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Expression pattern of the *AHP* gene family from *Arabidopsis thaliana* and organ specific alternative splicing in the *AHP5* gene

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Abstract

Histidine-containing phosphotransmitters (AHPs) transfer a phosphoryl group from membrane receptors to effectors in the nucleus. Five *AHP* genes have been identified in *Arabidopsis*. Real-time reverse transcription polymerase chain reaction (RT-PCR) was employed to quantify contents of individual transcripts in *Arabidopsis* leaves, roots, stems, flowers and siliques. High organ specificity of gene expression was found in *AHP1*, *AHP2*, *AHP4* while expression of *AHP3* and *AHP5* appears more ubiquitous. We detected two *AHP5* specific PCR products (*AHP5* and *AHP5L*). Out of the five *AHP5* introns, the second one was retained unspliced in the longer product (*AHP5L*) while *AHP5* corresponded to a completely spliced *AHP5* mRNA. The ratio between alternately and completely spliced *AHP5* mRNAs was highest in flowers and lowest in siliques and stems. No evidence for alternative splicing in the remaining *AHP* transcripts was found. Two open reading frames were identified in AHP5L. The conserved part of the phosphotransfer domain remains unaffected in the longer one as the translation initiation at an intron ATG would result in a polypeptide coded by exons 3 to 6 and a short extension encoded by the intron part of the alternative open reading frame. However, the isoelectric point would shift by about 3 units towards neutral.

Additional key words: *Arabidopsis* two component systems, gene expression analysis, real time RT-PCR, signal transduction.

Introduction

Phosphotransfer from an activated histidine kinase receptor to a response regulator represents the basic molecular mechanism underlying signal transduction mediated by two-component systems of prokaryotic origin. In yeast and higher plants, but not in animals, a multistep two-component system of signal transduction has been reported (Urao *et al.* 2000). In the multistep two-component system, a histidine-containing phosphotransmitter (HPt) mediates phosphotransfer from an activated membrane histidine kinase receptor to a response regulator in the nucleus. In *Arabidopsis thaliana*, genes for 8 sensor histidine kinases (AHKs), 5 HPt proteins (AHPs) and 22 response regulators (ARRs) have been identified. A number of independent lines of evidence indicate that phosphorelay from AHK through AHP to ARR is involved in plant signalling networks (Hutchison and Kieber 2002, Hwang and Sheen 2001, Hwang *et al.* 2002). Recently, three members of the AHK family, namely WOL1/CRE1/AHK4, AHK2 and AHK3, were shown to be possible cytokinin receptors (Inoue *et al.* 2001, Yamada *et al.* 2001). The ethylene receptor family of *Arabidopsis* also consists of proteins of the AHK family (Sakai *et al.* 1998). A critical role for a putative sensor histidine kinase CKI1 in completion of megagametogenesis in *Arabidopsis* was demonstrated (Pischke *et al.* 2002, Hejátko *et al.* 2003). Another member of the AHK family, ATHK1, was implicated in osmosensing (Urao *et al.* 1999). Much less is known regarding the biological functions of AHPs.

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Abbreviations: AHP - histidine-containing phosphotransmitters; RT-PCR - reverse transcription polymerase chain reaction.
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AHPs are small proteins (14.5 - 18 kDa) with a predicted acidic isoeletric point (5.5 - 3.9). They consist of the typical phosphotransfer domain characterized by the conserved sequence X**H**QXKGSSXS, where the highlighted histidine residue represents the phosphoryl acceptor essential for phosphorelay. Their amino acid sequences are highly conserved, *e.g.*, the amino acid identity between AHP2 and AHP3 is 81 %, which might indicate a late evolutionary divergence in the AHP family. An additional protein highly homologous to the AHP family, AHP6, was found to contain an aspartate residue instead of the histidine in the phosphotransfer domain (Hwang *et al.* 2002). AHPs were shown to participate in phosphorelay with ARRs (Suzuki *et al.* 2000). Two-hybrid analysis has demonstrated that AHP2 is capable of interacting with ATHK1 and ARR10 (Imamura *et al.* 1999, Urao *et al.* 2000). Interestingly, the over-expression of AHP2 in transgenic *Arabidopsis* results in a hypersensitive cytokinin response in root and shoot elongation assays (Suzuki *et al.* 2002). ARRs fall into two classes based on structural criteria, the type-A and type-B response regulators (Imamura *et al.* 1999). Type-A ARRs consist of a receiver domain with only short N- and C-terminal extensions, and are transcriptionally induced to varying extents by nitrate and cytokinins independent of *de novo* protein synthesis, suggesting that some of type-A *ARRs* are cytokinin primary response genes (Brandstatter and Kieber 1998, D'Agostino *et al.* 2000). Type-B ARRs consist of an N-terminal receiver domain, and a GARP DNA binding domain, a presumed transactivation domain and nuclear localization signal (Riechmann *et al.* 2000, Hosoda *et al.* 2002). It was proven in yeast and *in planta* that ARR1 and ARR2 are nuclear localized transcription factors and that the transactivation activity is negatively regulated by the N-terminal receiver domain (Lohrmann *et al.* 1999, 2001, Sakai *et al.* 2000).

Independent lines of evidence indicate that several ARRs may be involved in hormone and light signalling in plants. Thus, ARR1 over-expressing plants display hypersensitive responses to exogenous cytokinins while a loss-of-function mutation in *ARR1* results in cytokinin hyposensitivity (Sakai *et al.* 2001). ARR4 was shown to be red light induced and to stabilize the active form of phytochrome B. This interaction might enable a crosstalk between red light-signalling and cytokinin-signalling pathways (Sweere *et al.* 2001). Involvement of ARRs in the regulation of plant development might be indicated by specific expression patterns, *e.g.*, *ARR21* is predominantly expressed in siliques (Horák *et al.* 2003). However, clarification of the *in planta* signalling pathways involving multi-step two-component system elements is currently the subject of intense research.

Alternative splicing enables eukaryotic cells to make more than one protein from a single gene, giving rise to protein isoform diversity. It can cause modification or deletion of protein activity, generate completely different protein activities, influence sub-cellular protein localization, modulate gene expression and affect mRNA stability or its translational efficiency. Over the past few years, the existence of alternative splicing and alternative translation initiation sites in plant genes has been encountered with increasing frequency (Haas *et al.* 2002). There are more than 27 000 *Arabidopsis* protein-coding genes and more than 1200 of them have alternatively spliced isoforms generating almost 3000 proteins (http://www.tigr.org/tdb/e2k1/ath1/altsplicing/splicing_variations.shtml). Similar to animals, most of them are proteins with regulatory function(s) or proteins involved in signalling (Kazan 2003). For example, isoenzymes of a chloroplast ascorbate oxidase are encoded by a single gene in higher plants, and their mRNAs are generated by alternative splicing of the two 3'-terminal exons. The shorter mRNA encodes a stromal isoenzyme while the inclusion of the last exon in the longer mRNA results in the hydrophobic membrane-binding domain of a thylakoid-localised isoenzyme (Ishikawa *et al.* 1997). Interestingly, alternative splicing in the ascorbate oxidase might be controlled by environmental stresses (Shigeoka *et al.* 2002). The truncated proteins encoded by the alternatively spliced mRNAs can regulate the content of the full-size protein, as found in the case of the *FCA* gene in *Arabidopsis* (Macknight *et al.* 1997, 2002). Another flower specific gene, *AG*-like MADS-box gene from *Crocus sativus*, generates two differentially spliced transcripts (Tsaftaris *et al.* 2005). Interestingly, alternative splicing in *WOL1* histidine kinase gene leading to three different cDNA species was described in *Arabidopsis* (Mähönen *et al.* 2000), however, its functional significance remains to be determined.

As part of our attempts to investigate the biological function(s) of AHPs, we have analyzed structure and contents of the individual transcripts encoded by the *AHP* gene family using RT-PCR and real-time RT-PCR, respectively. The results are discussed in the context of two-component signalling and alternative splicing in *Arabidopsis*.

Materials and methods

Plants and growth conditions: *Arabidopsis thaliana* (ecotype Columbia 0) seeds were grown on solid Murashige and Skoog (MS) medium supplemented with 1 % sucrose, adjusted to pH 5.7 before autoclaving and solidified by 1 % agar (*Duchefa*, Haarlem, The Netherlands) in plastic Petri plates (9 cm diameter). To synchronize germination, plates with seeds were kept in darkness at 4 ºC for 24 h, and subsequently transferred into a growth chamber *AR-36L Percival* (*Percival Scientific*, Perry, USA) and incubated at day/night temperature 21/19 ºC and 16-h photoperiod with a photosynthetic photon flux density of approximately

150 μmol m^{-2} s^{-1} provided by cool white fluorescent lamps. The seedlings were harvested after 8-d cultivation in the growth chamber. Plants were grown directly from seeds to maturity in soil in the growth chambers at the same conditions. After 8 weeks, the individual organs (roots, leaves, stems, flowers and siliques) were collected for analysis.

RNA isolation: Total RNA was extracted from the collected material 100 mg of fresh tissue using *Trizol Reagent* (*Invitrogen*, Paisley, UK) and following the manufacturer's recommended procedure. The residual DNA was removed by treating with RNase-free DNaseI (*Roche Applied Science*, Mannheim, Germany). Subsequently, DNaseI was inactivated at 70 ºC for 10 min and treated RNA was purified using ethanol precipitation with 3 M sodium acetate pH 5.2 and washing with 75 % ethanol. Isolated RNA was dissolved in an appropriate volume of RNase-free water and stored at -70 ºC.

Reverse transcription polymerase chain reaction: A two-step procedure was used for RT-PCR. In the reverse transcription step, oligoT primer RTP3 (CGT TCG ACG GTA CCT ACG TTT TTT TTT TTT TTT TT) was employed to prime the reverse transcription reaction using 5 μg of total RNA and Superscript II RNase H^- Reverse Transcriptase (*Invitrogen*) according to the recommended procedure (denaturation at 70 ºC for 10 min, cDNA synthesis at 42 ºC for 52 min, enzyme inactivation at 70 ºC for 15 min). The resulting cDNA served as a template in the PCR step. PCR was performed using a thermocycler (*Perkin-Elmer*, Wellesley, USA) programmed to the following temperatures and times: at 94 ºC for 15 - 45 s, 57 - 60 ºC for 30 - 45 s and 72 ºC for 30 - 90 s; 25 - 30 cycles. The reaction mixtures consisted of 1 U Taq-Purple polymerase and a provided buffer, final concentration of each dNTP was 1 mM, (*Top-Bio*, Prague, Czech Republic), primers (final concentration 1 μM), and other common components. As a control, PCR was performed for 25 cycles at 94 ºC for 45 s, 60 ºC for 45 s and 72 ºC for 90 s with primers ACT2u (5'TTC CTC AAT CTC ATC TTC TTC C3') and ACT2l (5'GAC CTG CCT CAT CAT ACT CG3') derived from 5' and 3' untranslated region, respectively, of the genomic sequence (At3g18780) of the *ACTIN2* gene. Thus, potential contamination by the genomic DNA would result in an additional PCR product of 1 760 bp. To allow for direct comparison of steady state contents of the *AHP* mRNAs in the organs analyzed, the amount of the individual cDNAs used in the subsequent PCR reactions was normalized to represent an equal actin RT-PCR band intensity. cDNA aliquots were used as templates in PCR reactions performed for 27 cycles of 94 ºC at 15 s, 60 ºC for 30 s and 72 ºC for 60 s except for *AHP4* requiring 30 cycles to yield a PCR product detectable by ethidium bromide staining in agarose gels. The primers were derived from 5' (upper, u) and 3' (lower, l) terminal exons of the individual *AHP* genes as follows: AHP1u = GCA AGA TTA CAC CAA ATC A; AHP1l = GCA CAA AGA AAG AAG TTC AC; AHP2u = AAA AAT CCT CTC CCA ATC TCC; AHP2l = AAT GGT TTC AAT TTT CTC GG; AHP3u = ATT GCT CAG TTA CAG AGA CGA; AHP3l = CAC TTG AGG GAT TCT ACC AC; AHP4u = AGC TCC AAG ATG ATG CAA ACC CTA A; AHP4l = GCT CGC CTG GAA ATA ATG TTC AAG C; AHP5u = CGT CGT TGC TCA GTT GC; AHP5l = CAT CGG TCT AAT TTA TAT CCA. To detect specifically the second intron in the alternatively spliced AHP5 mRNA, specific primers for second intron AHP5iu (5'TTG CTT ACT CTC TTA GGC TT3') and AHP5il (5'CCG CTC CCT AAT GTT TAT3') were used in a PCR performed for 35 cycles of 94 ºC for 25 s, 57 ºC for 25 s and 72 ºC for 30 s.

Real-time RT-PCR: To quantify the individual *AHP* mRNAs in *Arabidopsis* organs, a SYBR Green I real time RT-PCR assay was employed. PCR amplifications were performed in a 25 mm^3 reaction volume containing 0.2 fold SYBR Green I nucleic acid gel stain (*Molecular Probes*, Carlsbad, USA), 0.5 μM each of upper and lower primers 1 mM each dNTP, 5 mm^3 of cDNA (see above), 1 U Taq DNA polymerase 1.1 and the provided buffer at the recommended concentration (*Top-Bio*) using the *Rotorgene 3000* (*Corbett Research*, Mortlake, Australia). Gene specific primer pairs are listed in Table 1. Actin cDNA fragments amplified with ACTfwd and ACTrev (corresponding to *ACTIN2* and *ACTIN8* genes, An *et al.* 1996, Szyroki *et al.* 2001) were used to normalize the steady-state mRNA levels. The general PCR conditions were as follows: denaturation at 94 ºC for 30 s, annealing at 55 ºC for 30 s and extension at 72 ºC for 30 s, fluorescence reading at 82 ºC for 15 s; 35 cycles. The general procedure was optimized for each set of primers to prevent non-specific PCR products and primer dimer formation (Table 1). A melting curve analysis was performed immediately after PCR by monitoring the fluorescence as the temperature was increasing slowly from 50 to 90 ºC. An aliquot of the PCR product was run on a 2 % agarose gel to confirm that each primer pair amplified product(s) of the expected molecular mass. The identity of the PCR products was further verified by DNA sequencing. The threshold cycle was defined as the cycle at which a statistically significant increase in the fluorescence value above the threshold value was first detected. The individual gel-purified PCR fragments were used to generate calibration curves. To enable statistical analysis, two fully independent real time RT-PCR experiments (starting with seedling cultivation) were performed and each real time PCR sample was run in triplicate.

Statistical analysis: The mean of the three replicates and the standard deviations were calculated for copy numbers of the individual *AHP* and *ACTIN* (*ACT*) transcripts. The abundance of the *AHP* transcripts is presented as an *AHPn*/*ACT* copy number ratio. The standard deviation of the ratio was calculated as the square root of the sum of the individual standard deviations. Two independent real

time RT-PCR experiments were evaluated using the Student's *t*-test to assess the statistical significance of differences in the *AHPn*/*ACT* copy number ratios in the individual organs investigated.

DNA sequencing: To identify unequivocally the unspliced intron in the alternatively spliced *AHP5* mRNA and to confirm the identities of the RT-PCR products corresponding to the individual *AHP* mRNAs, the RT-PCR products were separated on a 2 % agarose gel, DNA isolated using *Qiaquick Gel Extraction Kit*, (*Qiagen*, Hilden, Germany) and sequenced using the upper primers (except AHP5rtl primer) on an ABI 310 (*Perkin Elmer*) automatic sequencer.

Sequence analyses were performed using Biology Workbench (http://workbench.sdsc.edu/). The programs *BLASTN*, *SIXFRAME* and *CLUSTALW* were used to analyze the nucleotide sequences. Protein sequence analysis was performed using programs *CLUSTALW*, *PI*, *RPSBLAST* and *AASTATS*.

Table 1. Oligonucleotides used in real-time RT-PCR analysis. Modifications of the general real-time RT-PCR protocol employed for the particular primer pairs to overcome non-specific PCR products and primer dimer formation are shown. AHP5i corresponds to the alternatively spliced AHP5 transcript.

Gene/transcript	Primer	Sequence	Modification
AHP1	AHP1rtu	5'TAGGAGCACAGAGAGTTAAGA3'	30 cycles
At3g21510	AHP1rtl	same as AHP1l	
AHP2	AHP2rtu	same as AHP2u	30 cycles
At3g29350	AHP2rtl	5'CTTTGTCTTTAACGCCTTGTA3'	
AHP3	AHP3rtu	5'AGCTGCAAGATGAATGTAGTC3'	30 cycles
At5g39340	AHP3rtl	same as AHP3l	
AHP4	AHP4rtu	5'TGTTGAAGAAGTTTCCGCATTA3'	
At3g16360	AHP4rtl	5'AAGCATCCTTCCGCATTT3'	
AHP5	AHP5rtu	5'CTCTCTATTCTTCGACGACTG3'	annealing at 55 ºC
At1g03430	AHP5rtl	5'GTTCTGAACATCGCAACAT3'	
AHP5i	AHP5irtu	5'CTTTGCTTACTTTAGGCTT3'	fluoresc. reading at 78 ºC
	AHP5irtl	5'TCCACCTGTAAACACC3'	
ACT2, ACT8	ACTfwd	5´GGTGATGGTGTGTCT3´	annealing at 54 ºC, 30 cycles
At3g18780, At1g49240	ACTrev	5´ACTGAGCACAATGTTAC3´	fluoresc. reading at 85 ºC

Results

RT-PCR analysis of *AHP* transcripts: As an initial step in the investigation of biological functions of the *AHP* gene family, we analyzed expression patterns of the individual *AHP*s using RT-PCR. Total RNA was isolated from individual organs and developmental stages including roots, leaves, stems, flowers and siliques harvested from 8-week-old plants, and 8-d-old seedlings. Gene specific pairs of primers were designed in the first and last exons to cover most of the transcribed regions (Fig. 1*A*). Striking differences were observed in expression patterns of the individual *AHP*s under conditions when PCR amplification of reverse-transcribed cDNA displayed comparable levels of actin signal in all samples (Fig. 2*A*). The highest level of *AHP1* transcript was detected in roots. The transcript was also clearly detected in flowers, siliques and seedlings, while only barely detectable levels of *AHP1* transcript were found in stems. The highest contents of *AHP2* transcript were observed in leaves and flowers, and somewhat lower contents were found in roots, stems, siliques and seedlings. *AHP3* transcript was present in comparable contents in all organs examined. *AHP4* transcript was most abundant in leaves, clearly detectable in flowers, stems and siliques, and below detection limit in roots. The contents of *AHP5* transcript were present comparable in all organs examined except for lower levels in siliques. In addition to the product of the expected size of approximately 440 bp, a longer product appeared in the position corresponding to approximately 580 bp when PCR amplification of reverse-transcribed cDNA was performed with primers (AHP5u and AHP5l) specific for *AHP5* transcript (Fig. 2*A*). Southern blot analysis (data not shown) revealed a strong and specific hybridization of both PCR products to an *AHP5* probe, therefore, the products were designated AHP5 (440 bp) and AHP5L (580 bp).

Alternative splicing in *AHP5*: Detection of the two distinct yet specific RT-PCR products indicated the presence of alternatively spliced *AHP5* transcripts. To confirm that the two products corresponded to a completely and an alternatively spliced *AHP5* transcript, and to identify the unspliced intron, AHP5 and AHP5L

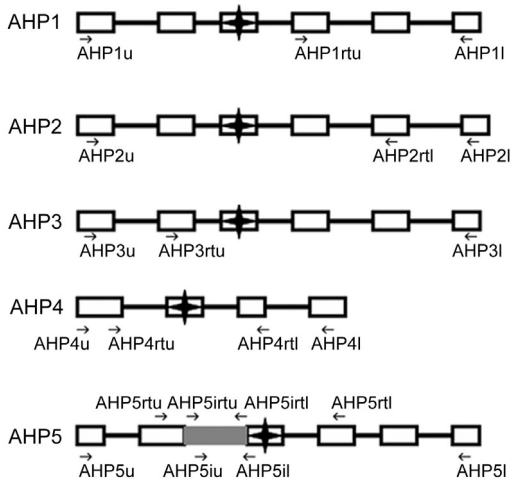

Fig. 1. Exon-intron structure in the *AHP* genes. Exons are shown as *white boxes*, lines between the boxes represent introns. The *grey box* represents exon 2 of *AHP5* gene. *Asterisks* depict positions encoding the invariant histidine residue of the phosphotransfer domain. The size of the individual open reading frames (ORFs) is 465, 471, 468, 384 and 474 bp for *AHP1*, *AHP2*, *AHP3*, *AHP4* and *AHP 5*, respectively. Positions of the individual primers employed in RT-PCR and real-time RT-PCR experiments are marked by *arrows*.

obtained from leaf RNA were sequenced using primer AHP5u (and AHP5rtl – data not shown). Sequence analysis revealed that in AHP5L the second intron of 144 bp (intron 2 starting at positions 201 bp, possessing GT-AG consensus) remained unspliced, while AHP5 corresponds to a completely spliced *AHP5* transcript (Fig. 3). The intron size was in good agreement with the

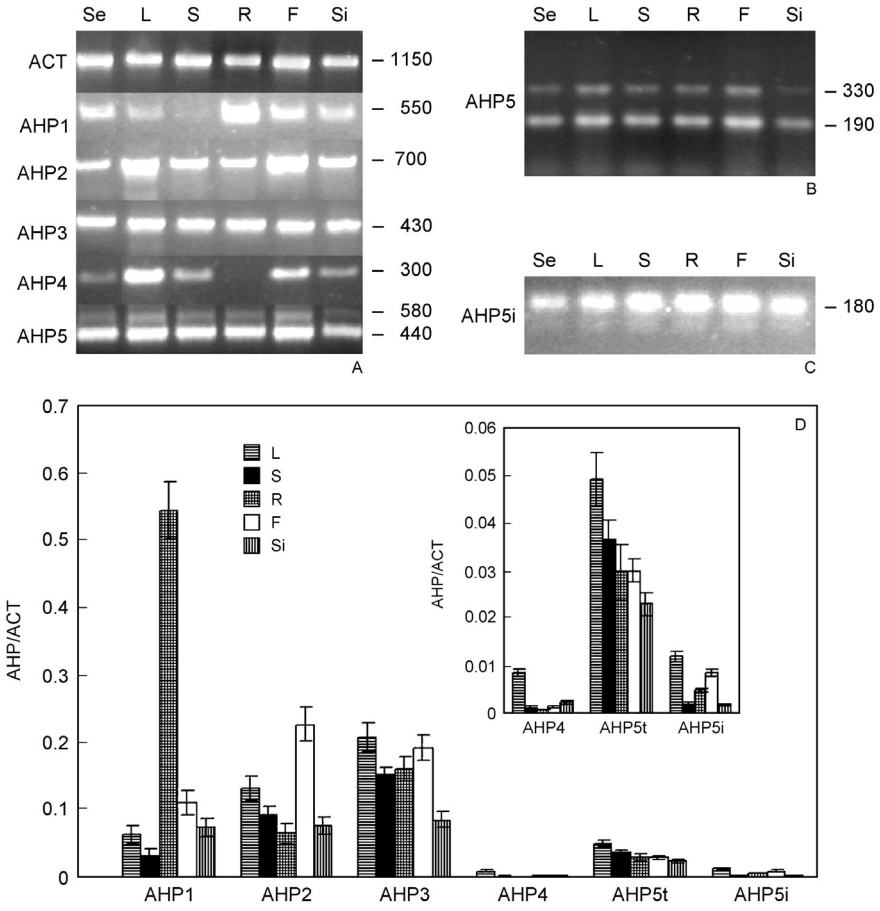

Fig. 2. Organ specific expression and alternative splicing in *AHP* gene family. *A* - RT-PCR-based expression profile of *AHP* genes in the individual *Arabidopsis* organs. Presented is a comparison of *AHP1*, *AHP2*, *AHP3*, *AHP4* and *AHP5* amplification achieved by 27, 27, 27, 30 and 27 PCR cycles, respectively, with amplification of the *ACTIN* housekeeping genes (25 PCR cycles). Using individual gene specific primer pairs, every RT-PCR reaction resulted in a single, gene specific fragment (based on match of predicted and observed size of the fragment upon sequencing) except for *AHP5*. *B* - RT-PCR analysis with a primer pair (AHP5rtu and AHP5rtl) designed for real-time RT-PCR quantification of properly and alternatively spliced *AHP5* transcripts (30 cycles). *C* - RT-PCR analysis with a primer pair (AHP5irtu and AHP5irtl) designed for real-time RT-PCR quantification of alternatively spliced *AHP5* transcript only (35 cycles). *D* - Real-time RT-PCR based quantification of organ specificity of expression in *AHP* gene family. Copy number of the individual transcripts was determined using gene specific primers listed in Table 1. Transcript abundance is expressed as a ratio *AHP/ACT*. Total RNA was isolated from 8-d-old seedlings (Se), and the individual organs of 8-week old plants: leaves (L), stems (S), roots (R), flowers (F) and siliques (Si).

AHP5

TATCAAC*AGGGATTTCTGGATAATCAGTTCTCAGAGTTGAGAAAGTTGCAAGATGAAGGAACCCCTGATT
TTGTAGCTGAAGTTGTCTCTCTATTCTTCGACGACTGTTCCAAGCTTATTAATACCATGTCTATATCCCT*
GGAGCGGCCAGATAATGTGGATTTCAAACAGGTGGATTCAGGTGTTCATCAACTCAAGGGTAGTAGCTCC*
AGTGTCGGTGCAAGGAGGGTGAAAAATGTGTGCATATCTTTCAAGGAATGTTGCGATGTTCAGAACCGTG
AAGGGT*GTCTAAGGTGTTTACAGCAGGTGGATTATGAATATAAGATGTTAAAGACTAAACTTCAGGATCT
CTTTAATT*TAGAGAAA

AHP5L

TATCAAC*AGGGATTTCTGGATAATCAGTTCTCAGAGTTGAGAAAGTTGCAAGATGAAGGAACCCCTGATT
TTGTAGCTGAAGTTGTCTCTCTATTCTTCGACGACTGTTCCAAGCTTATTAATACCATGTCTATATCCCT
GTAAGCTTTGCTTACTCTCTTAGGCTTCTCTTTTTTATCTAACATGGGACTTTGTCTCTAATCCTCCTTC
ATATTCATTTTTGAGGGTGTAAATTAGCTTCATATATTCATGACAGCATTTTATGAGTTTATTATAAACA
TTAGGGAGCGGCCAGATAATGTGGATTTCAAACAGGTGGATTCAGGTGTTCATCAACTCAAGGGTAGTAG
CTCC*AGTGTCGGTGCAAGGAGGGTGAAAAATGTGTGCATATCTTTCAAGGAATGTTGCGATGTTCAGAAC
CGTGAAGGGT*GTCTAAGGTGTTTACAGCAGGTGGATTATGAATATAAGATGTTAAAGACTAAACTTCAGG
ATCTCTTTAATT*TAGAGAAA

Fig. 3. Intron 2 is retained in the alternatively spliced *AHP5* transcript. AHP5 and AHP5L fragments resulting from PCR amplification using AHP5u/AHP5l primer pair were subjected to sequence analysis to identify the unspliced intron. DNA sequencing with AHP5u primer revealed absence of introns in AHP5 fragment while complete intron 2 sequence (shown in *bold italics*) was found in AHP5L. *Asterisks* mark positions from which introns 1 (70 bp), 2 (201 bp), 3 (270 bp), 4 (367 bp) and 5 (417 bp) are removed in the fully spliced *AHP5* transcript. Except for intron 2 in AHP5L no intron sequence was found in either AHP5L or AHP5 confirming that intron 2 sequence detected in AHP5L is not due to contaminating genomic DNA.

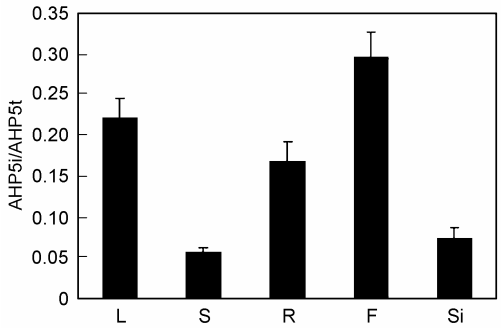

Fig. 4. Extent of alternative splicing in *AHP5* transcript is organ-dependent. The extent of alternative splicing in individual organs was investigated by a real-time RT-PCR assay. Copy number of both fully and alternatively spliced *AHP5* transcripts (AHP5t) was determined using primers AHP5rtu and AHP5rtl while primers AHP5irtu and AHP5irtl were used to assay copy number of alternatively spliced *AHP5* transcript (AHP5i) only. The results are expressed as a ratio of alternatively spliced transcript (AHP5i) to total *AHP5* transcript (AHP5t). Total RNA was isolated from the individual organs of 8-week old plants: leaves (L), stems (S), roots (R), flowers (F) and siliques (Si).

size difference deduced from gel mobility of AHP5 and AHP5L (Fig. 2*A*). Absence of the remaining introns in AHP5L proved that AHP5L cannot originate from genomic DNA contaminations in the RNA preparations.

Quantitative expression analysis in *AHP* gene family and organ specificity of *AHP5* alternative splicing: We developed a real-time RT-PCR assay to reliably quantify the expression of the *AHP* gene family in individual organs. Primer pairs (Table 1, Fig. 1) were designed carefully to discriminate between the individual highly homologous *AHP* genes, and fully and alternatively spliced *AHP5* transcripts. Identity of the PCR products was verified by DNA sequencing and checked routinely by melting curve analysis immediately following real-time RT-PCR. *ACTIN* transcripts were used to normalize the copy number of the *AHP* PCR products. The results (Fig. 2*D*) represent mean values obtained from two sets of independently cultivated plants. We could detect transcripts, though at highly varying levels, of all five *AHP* genes in every organ analyzed, including roots, rosette leaves, stems, flowers and siliques harvested from 8-week-old plants. The highest expression was found in *AHP1-3* genes, steady state levels of total *AHP5* transcripts (see below) varied between 2.3 and 4.9 %, and those of *AHP4* transcript were below 1 % of actin control.

The highest degree of organ specificity was found in *AHP1* gene expression – steady state level of *AHP1* transcript reached almost 55 % of the actin control in roots, while only 11.0, 7.4, 6.3, and 3.1 % were found in flowers, siliques, leaves, and stems, respectively. *AHP2* transcript accumulated preferentially in flowers (over 20 % of the actin control). Abundance of *AHP3* transcript was close to 20 % of the actin control in all the organs examined except siliques. Steady state level of *AHP4* transcript was approximately 5-fold higher in leaves compared to other organs investigated.

Two sets of specific primers (AHP5rtu/l and AHP5irtu/l, Table 1) were used to discriminate between the steady state levels of the fully and alternatively spliced *AHP5* transcripts. The primer pair AHP5rtu/l amplifies both fully and alternatively spliced AHP5 transcripts (Fig. 2*B*). Thus, the real-time RT-PCR signal obtained using this primer pair corresponds to the copy number of both *AHP5* transcripts – AHP5t. The primer pair AHP5irtu/l is designed to selectively amplify intron 2 (Figs. 1, 2*C*), thus, the resulting real-time RT-PCR signal represents the copy number of the alternatively spliced *AHP5* transcript – AHP5i alone. AHP5t was

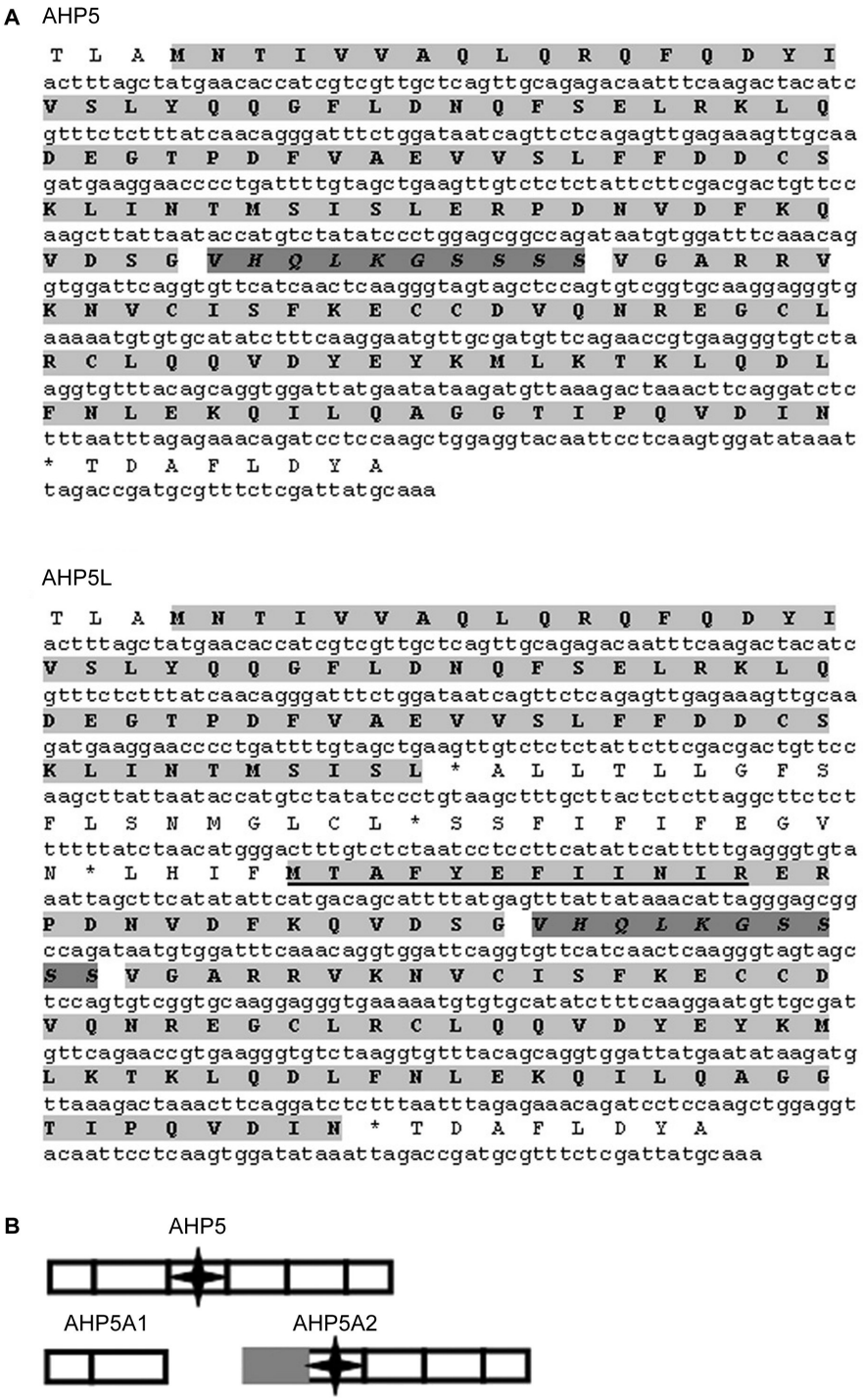

Fig. 5. The alternatively spliced *AHP5* transcript encodes two putative proteins. *A* - Fully spliced *AHP5* transcript encodes a single polypeptide chain of 158 amino acid residues (*highlighted in grey*) comprising the conserved sequence motif XHQXKGSSXS of a phosphotransfer domain (*in italics* and *highlighted in dark grey*). The second intron retained in the alternatively spliced *AHP5L* transcript introduces a stop codon that causes premature translation termination following the last codon of the second exon which might result in a truncated protein AHP5A1. An intron-borne ATG might serve as a translation re-initiation point of the second truncated protein, AHP5A2, consisting of 12 amino acid residues encoded by intron 2 (*underlined*) and a polypeptide encoded by exons 4 - 6 which includes the complete conserved sequence motif of the phosphotransfer domain (*italics*, *dark grey*). *B* - Schematic representation of the standard AHP5, and the truncated AHP5A1 and AHP5A2 proteins. *White boxes* correspond to the individual exons, *gray box* represents peptide sequence encoded by intron 2, and *asterisk* depicts the position of the invariant histidine residue of the phosphotransfer domain.

comparable in stems, roots and flowers, while significantly higher levels were encountered in leaves, and the lowest levels were found in siliques. However, this pattern was not matched by the alternatively spliced *AHP5* transcript indicating organ specificity of alternative splicing in the *AHP5* gene (Fig. 2*D*). To visualize clearly the organ specificity of the *AHP5* alternative splicing, the results were expressed as AHP5i/AHP5t ratios (Fig. 4). No significant differences in values obtained for the individual organs were found using *t*-test analysis of two independent experiments. Thus, the quantitative results confirmed reliably that ratio between the alternatively and fully spliced products differs significantly in individual organs. The representation of the alternatively spliced *AHP5* transcript was found to be the highest in flowers and the lowest in siliques and stems. No dramatic differences were found in the representation of the alternatively spliced *AHP5* transcript when leaves and roots were compared.

***In silico* analysis of the ORFs resulting from *AHP5* alternative splicing:** The normally spliced *AHP5* transcript encodes a polypetide chain consisting of 158 amino acid residues (Fig. 5*B*) with a predicted molecular mass of 17.9 kDa and a predicted isoelectric point of 4.8 (as calculated using Biology WorkBench). The alternatively spliced *AHP5* transcript could encode two distinct putative proteins. The first one would be translated starting from the translation initiation ATG codon of the *AHP5* open reading frame (ORF), and terminate at a stop codon represented by the first triplet of the unspliced intron 2. The resulting protein, AHP5A1, would consist of 68 amino acid residues in a polypetide chain with a predicted molecular mass of 7.7 kDa and an isoelectric point of 3.9 (Fig. 5*B*). The second one might be translated from an ATG found at position 109 in the unspliced intron 2, and would terminate at the regular stop codon of the *AHP5* ORF (Fig. 5*A*). Thus, the second putative protein, AHP5A2, would consist of 103 amino acid residues in a polypetide chain with a predicted molecular mass of 11.7 kDa and an isoelectric point of 7.7 chain that would include most of the standard phosphotransfer domain and the intact conserved sequence motif, XHQXKGSSXS (Fig. 5*B*).

Discussion

We analyzed the expression patterns of the individual members of a gene family coding for AHPs, histidine-containing phosphotransmitters, in *Arabidopsis*. Using real-time RT-PCR, transcripts of all five *AHP* genes could be reliably detected and quantified in roots, rosette leaves, stems, flowers and siliques. Various degrees of organ specificity were encountered in the gene family with the highest one observed for the *AHP1* gene. Surprisingly, two specific RT-PCR products were detected for *AHP5* transcripts. Sequencing of the RT-PCR products revealed that alternative splicing of the second intron of *AHP5* gene gives rise to the two products. Interestingly, the fraction of alternatively spliced *AHP5* transcripts apparently differs in the individual organs of *Arabidopsis*. A longer open reading frame identified in the alternatively spliced *AHP5* transcript might encode a protein containing the conserved sequence motif, XHQXKGSSXS, crucial for AHP mediated phospho-relay, however, lacking the N-terminal part of the native AHP5 and possessing an isoelectric point more neutral compared to the acidic pI of native AHP5.

Based on expression analysis using real-time RT-PCR, three groups with respect to overall transcript abundance become apparent in the *AHP* gene family. *AHP1-3* transcripts represent the group with the highest transcript abundance ranging typically between 6 and 23 % of the *ACTIN* transcript level, and reaching a high of 54 %. The abundance of the *AHP5* and *AHP4* transcript falls within 2 - 5 % and 0.08 - 0.9 % of the *ACTIN* transcript level, respectively. Regarding organ specificity, two groups can be defined. Steady state levels of *AHP3* and *AHP5* transcripts were fairly similar in all organs investigated. Taken together with a high degree of sequence similarity, functional redundancy might be anticipated for *AHP3* and *AHP5*. In contrast, *AHP1*, *AHP2* and *AHP4* expression appears highly organ specific, and to some extent complementary. For example, *AHP1* is expressed predominantly in roots with the transcript level approaching 55 % of the *ACTIN* transcript level while *AHP4* transcript reached only 0.08 % in roots. Previously, the expression profile of the *AHP* gene family was investigated using Northern blot analysis (Miyata *et al.* 1998, Tanaka *et al.* 2004). The Northern blot analysis detected *AHP2*, *AHP3* and *AHP5* transcripts in all organs analyzed except for an apparent absence of *AHP3* transcript in siliques that might be due to low amount and/or quality of RNA isolated from siliques. High degree of organ specificity, in contrast, was found in *AHP1* and *AHP4* expression. Thus, our results partly confirm and, more importantly, further extend those obtained previously by Northern blot analysis, and thereby represent the first rigorous quantitative data on organ specific expression of the *AHP* gene family. These data would be useful, among others, when a particular signal transduction chain is being elucidated. Coordinate expression among members of a single signal transduction cascade is critical for its functionality. Thus, new members of the chain, putatively identified based, *e.g.*, on sequence comparison, can be assigned a role in a given pathway based on their coordinate expression with other known upstream and downstream members of the chain. Interestingly, expression profile of *AHP1* gene apparently correlates with that of the cytokinin receptor gene *AHK4/CRE1/WOL*, whose expression is highest in roots and low in rosette leaves (Higuchi *et al.* 2004).

Protein-protein interactions between AHP1 and AHK4/ CRE1/WOL were shown in yeasts (Grefen and Harter 2004 and references therein). *AHP3* and *AHP5* expression profiles follow that of the cytokinin receptor gene *AHK2* which is expressed to about the same degree in leaves and roots (Higuchi *et al.* 2004).

Recently, involvement of AHP and ARR in ethylene signal transduction has been proposed (Hass *et al.* 2004). Within the ethylene receptor family, the most pronounced tissue specific expression was found in *ETR2* with a marked preference for flowers and leaves (Sakai *et al.* 1998) that corresponds to *AHP2* expression pattern. The apparent major players in ethylene perception, ETR1 and ERS1, were found in all *Arabidopsis* tissues investigated (see Grefen and Harter 2004) precluding any prediction regarding the putative interacting AHP. Recently, expression patterns of Type-B response regulators were analyzed by RT-PCR and β-glucuronidase fusion constructs in *Arabidopsis* (Mason *et al.* 2004). RT-PCR analysis revealed expression, although at different levels, of all members of subfamily I of the type-B ARRs thus resembling the general expression pattern in *AHP* family. Broad expression patterns were found in *ARR1, 2, 10, 11* and *12*. *ARR14* and *ARR18* demonstrated greater specificities of expression but could be detected in all tissues. Expression of members of subfamilies II and III were more restricted than members of subfamily I, being found predominantly in the flowers and/or siliques. No complete match could be identified over every organ analyzed for any particular *ARR* and *AHP* gene. Thus, a histochemical analysis of the *AHP* family using β-glucuronidase fusions is expected to bring a resolution sufficient for predicting putative *AHP* and *ARR* partners.

Interestingly, based on the Northern blot analysis, alternative splicing and/or alternative polyadenylation in *AHP4* was proposed to explain variation of the apparent size of *AHP4* transcript(s) in individual organs (Tanaka *et al.* 2004). Our RT-PCR results exclude alternative splicing as a possible mechanism responsible for the putative multiple forms of *AHP4* transcripts.

Standard maturation of transcripts corresponding to the *AHP* gene family in *Arabidopsis* requires five and three introns to be spliced correctly in AHP1 - AHP4 (Fig. 1*A*). The presented RT-PCR data prove standard splicing in *AHP1* - *AHP4* transcripts in all organs examined. Surprisingly, the AHP5L PCR product corresponding to an alternatively spliced *AHP5* transcript was found to accompany the major PCR product, AHP5, that corresponds to the fully spliced *AHP5* transcript. DNA sequencing of AHP5L revealed that intron 2 was not spliced from the *AHP5* transcript. The alternatively spliced *AHP5* transcript has remained unnoticed previously, probably due to the low resolution and sensitivity of Northern blot analysis of the *AHP* gene family (Tanaka *et al.* 2004). Here, the alternatively spliced *AHP5* transcript was detected in all organs examined, however, the representation of the alternatively spliced *AHP5* transcript in total *AHP5* transcripts (inferred from the *AHP5i*:*AHP5t* ratio) differed in the individual organs with the highest representation found in flowers and the lowest in stems and siliques. Organ dependent preferential enrichment of the alternatively spliced *AHP5* transcript might suggest its biological function, however, the current data do not allow us to draw any firm conclusions on its nature. Among the genes involved in the multistep two-component systems of *Arabidopsis*, alternative splicing has been identified only in *WOL1/CRE1/AHK4* histidine kinase gene (Mähönen *et al.* 2000) though its functional significance remains to be clarified. Among genes affecting cytokinin metabolism, alternative splicing was found in the orchid gene coding for cytokinin oxidase, DSCKX1. Apparently three transcripts are derived from this gene, however, their functional significance also remains to be determined (Yang *et al.* 2003).

A stop codon in intron 2 is expected to abort translation of a polypeptide chain initiated at the translation initiation ATG of the *AHP5* gene leading to a truncated protein without the conserved residue required for phosphorelay. However, an alternative open reading frame was identified that starts with an ATG in the intron 2 (at position 109 bp) and terminates at the standard stop codon of *AHP5* gene. Though lacking the N-terminal part, the corresponding polypeptide, AHP5A2, would cover most of the phosphotransfer domain including the conserved histidine residue within the intact sequence motif, XHQXKGSSXS, that is pivotal in AHP mediated phosphorelay. However, in AHP5A2, the conserved sequence motif resides in a protein with a predicted isoelectric point of 7.7 that is dramatically different to the value (4.8) calculated for the standard AHP5. The dramatic shift in the protein charge might influence both the catalytic properties and protein-protein interactions of the phosphotransmitter domain resulting in a novel function that might include competition with the standard AHP5 protein. Thus, AHP5A2 might be able to accept phosporyl group but unable to transfer it to a downstream ARR due to a dramatic difference in charge. Alternatively, difference in charge might result in strong binding of AHP5A2 to a downstream ARR thus preventing phosphorelay from the standard AHP5. Interestingly, a similar pattern of protein splitting into two distinct putative polypetide chains was observed in the *FCA* gene. The *FCA* floral promotion gene contains 20 introns, and alternative splicing of intron 3 and 13 produces four different transcripts, designated α, β, γ, and δ (Macknight *et al.* 1997). Transcript α, in which all of the introns are accurately spliced and removed, encodes the full-length protein, including two RNA binding motifs and a WW domain that is known to play a role in protein-protein interactions. Transcript α is alternatively spliced to retain intron 3, which introduces an early translation termination codon and includes a second open reading frame. The first open reading frame would encode a putative protein containing little more than the N terminus and lacking the RNA binding and WW domains. The second open reading frame starts at an ATG within the coding region of the second RNA

binding domain and terminates at the standard termination codon thus including only the WW domain. However, functional significance, if any, of the two putative proteins remains unknown (Macknight *et al.* 2002). Thus, the reported results represent a starting point for the elucidation of the functional significance of alternative splicing in the *AHP5* gene.

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