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The *Arabidopsis* Histidine Phosphotransfer Proteins Are Redundant Positive Regulators of Cytokinin Signaling [W]

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Arabidopsis thaliana **histidine phosphotransfer proteins (AHPs) are similar to bacterial and yeast histidine phosphotransfer proteins (HPts), which act in multistep phosphorelay signaling pathways. A phosphorelay pathway is the current model for cytokinin signaling. To assess the role of AHPs in cytokinin signaling, we isolated T-DNA insertions in the five *AHP* genes that are predicted to encode functional HPts and constructed multiple insertion mutants, including an *ahp1,2,3,4,5* quintuple mutant. Single *ahp* mutants were indistinguishable from wild-type seedlings in cytokinin response assays. However, various higher-order mutants displayed reduced sensitivity to cytokinin in diverse cytokinin assays, indicating both a positive role for AHPs in cytokinin signaling and functional overlap among the AHPs. In contrast with the other four AHPs, AHP4 may play a negative role in some cytokinin responses. The quintuple *ahp* mutant showed various abnormalities in growth and development, including reduced fertility, increased seed size, reduced vascular development, and a shortened primary root. These data indicate that most of the AHPs are redundant, positive regulators of cytokinin signaling and affect multiple aspects of plant development.**

INTRODUCTION

Cytokinins are N^6-substituted adenine derivatives that have been implicated in many aspects of plant growth and development, including cell division, shoot initiation and development, vascular development, leaf senescence, deetiolation, and chloroplast differentiation (Mok and Mok, 1994, 2001; Sakakibara, 2006). A multistep phosphorelay model for cytokinin signaling has emerged that is similar to the two-component multistep pathways used by bacteria to sense and respond to environmental signals (Stock et al., 2000; West and Stock, 2001). A simple two-component system involves a His sensor kinase that perceives the signal and a response regulator that mediates the output of the pathway. The sensor His kinase autophosphorylates on a His residue in response to an input signal, and the phosphoryl group is transferred to a conserved Asp residue in the receiver domain of the response regulator. The activity of the response regulator is modulated by the phosphorylation state of its receiver domain. In a multistep phosphorelay pathway, additional His- and Asp-containing modules are present in one or more proteins. The phosphoryl group is transferred from a His in the receptor kinase to an Asp in the response regulator via His-Asp-His-Asp phosphotransfer reactions between alternating His and Asp residues (Perraud et al., 1999). In *Arabidopsis thaliana*, the cytokinin receptors CYTOKININ RESPONSE1 (CRE1; also known as WOODENLEG [WOL] or *ARABIDOPSIS* HISTIDINE KINASE4 [AHK4]) and its homologs AHK2 and AHK3 are similar to bacterial His sensor hybrid kinases that include a ligand binding, a His kinase, and a receiver domain in one protein (Inoue et al., 2001; Suzuki et al., 2001a; Ueguchi et al., 2001a; Yamada et al., 2001). The receptors are predicted to signal via the *Arabidopsis* histidine phosphotransfer proteins (AHPs) to the *Arabidopsis* response regulators (Hwang and Sheen, 2001; Hutchison and Kieber, 2002; Heyl and Schmülling, 2003; Kakimoto, 2003; Ferreira and Kieber, 2005).

The cytokinin receptors promote cytokinin responses: loss-of-function mutations of the cytokinin receptor *CRE1* result in reduced cytokinin sensitivity (Inoue et al., 2001; Franco-Zorrilla et al., 2002), double mutants show reduced response to cytokinin, and triple mutants in which all three cytokinin receptors are disrupted are cytokinin insensitive with small shoots, a short primary root, and reduced or no seed set (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). Cytokinins have been shown to bind to the CHASE domain of the AHKs, and cytokinin binding activates the His kinase function of the AHKs in yeast and bacterial systems (Inoue et al., 2001; Suzuki et al., 2001a; Ueguchi et al., 2001b; Yamada et al., 2001; Spíchal et al., 2004; Romanov et al., 2005).

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There are two primary classes of response regulators in *Arabidopsis* named type-A and type-B response regulators (ARRs). Type-B ARRs consist of a receiver domain and an extended C-terminal region that contains a DNA binding domain and a transcription activation domain (Imamura et al., 1999; Lohrmann et al., 1999; Sakai et al., 2000). A subset of type-B ARRs have been shown to act as positive regulators of cytokinin responses, and type-B ARRs are the direct upstream activators of the cytokinin-inducible type-A ARRs (Hwang and Sheen, 2001; Sakai et al., 2001; Mason et al., 2005). Type-A ARRs are transcriptionally upregulated in response to cytokinin and consist of a receiver domain and short C-terminal extensions (Brandstatter and Kieber, 1998; Taniguchi et al., 1998; Kiba et al., 1999; D'Agostino et al., 2000). In contrast with type-B ARRs, type-A ARRs are negative regulators of cytokinin signaling: loss-of-function alleles result in increased sensitivity to cytokinin (To et al., 2004). Genetic analysis has indicated that, in addition to their role in cytokinin signaling, a subset of type-A ARRs are involved in regulating circadian rhythm and shoot apical meristem function (Leibfried et al., 2005; Salomé et al., 2005).

A further component of cytokinin signaling is defined by the *CYTOKININ RESPONSE FACTOR* (*CRF*) genes, which encode transcription factors (Rashotte et al., 2006). Like the type-A ARRs, the CRFs are transcriptionally upregulated in response to cytokinin in a type-B ARR-dependent manner. Cytokinin also influences the subcellular location of the CRFs: they localize to the nucleus in response to cytokinin in an AHK- and AHP-dependent (but not ARR-dependent) manner. Insertion mutants of *CRF* genes result in reduced induction of cytokinin-inducible genes that overlap those whose induction is type-B ARR dependent. Mutation of the CRFs does not, however, reduce response to cytokinin in many cytokinin assays (Rashotte et al., 2006).

The AHPs are a family of six related proteins, including five (AHP1-AHP5) that contain the conserved amino acids required for function as a histidine phosphotransfer protein (HPt) and one (APHP1/AHP6) that is considered a pseudo-AHP, as it lacks the conserved His residue that is the target of phosphorylation (Suzuki et al., 2000; Mähönen et al., 2006b). Several lines of evidence suggest that the AHPs play a role in cytokinin signaling, mediating phosphotransfer between the cytokinin receptors and both the type-A and type-B response regulators. First, a combination of in vitro and heterologous complementation experiments have shown that AHPs can act as phosphorelay intermediates (Miyata et al., 1998; Suzuki et al., 1998, 2000). AHPs can be phosphorylated by AHKs, can phosphorylate both type-A and type-B ARRs, and can act in pathways that are responsive to cytokinin (Imamura et al., 1999; Suzuki et al., 2002; Mähönen et al., 2006a). In addition, AHP1, AHP2, and AHP4 accumulate in the nucleus in response to cytokinin treatment (Hwang and Sheen, 2001; Yamada et al., 2004), indicating that the AHPs respond to cytokinin in plant cells. To date, the most direct evidence that HPts mediate cytokinin signaling in plants has come from experiments using cultured periwinkle cells, in which cytokinin inducibility of a response regulator was reduced when a His phosphotransfer protein was silenced using RNA interference (Papon et al., 2004). Further evidence for a role for the AHPs has come from the observation that overexpression of AHP2 results in a slight increase in sensitivity to cytokinin in root elongation assays (Suzuki et al., 2002). By contrast, recent genetic analysis indicates that APHP1/AHP6, a predicted pseudo-AHP, acts as a negative regulator of the cytokinin response pathway (Mähönen et al., 2006b), most likely via a dominant negative mechanism.

We used a reverse genetic approach to investigate the role of AHP family members in cytokinin signaling. We isolated T-DNA insertions in the five canonical *AHP* genes and constructed various combinations of these mutations, including the *ahp1,2,3,4,5* quintuple mutant. Our results indicate that these genes have overlapping functions and that most act as positive regulators of cytokinin signaling. We also show that disruption of *AHP*s in *Arabidopsis* can alter root growth and vascular development, reduce seed set, and increase embryo and seed size.

RESULTS

Phylogenetic Analysis of Plant HPts

The *AHPs* are a family of six genes: *AHP1-AHP5* encode proteins that contain the conserved His residue that is required for phosphorylation, while the protein encoded by *APHP1/AHP6* does not (Suzuki et al., 2000). Phylogenetic analysis indicates that AHP2, AHP3, and AHP5 are more closely related to each other than to the other AHPs (Figure 1A). By contrast, AHP1 belongs to a distinct clade comprised of various dicot HPts. Interestingly, AHP4 groups with several rice (*Oryza sativa*) pseudo-HPts, while the *Arabidopsis* pseudo-HPt, AHP6, is not as closely related to these rice pseudo-Hpt genes. Monocot and dicot HPts cluster separately, with the exception of the rice pseudo-Hpt-AHP4 group, indicating that these gene families probably expanded independently in each lineage.

Isolation of T-DNA Insertions in the *Arabidopsis* HPt Loci

To study the function of the AHPs, we isolated lines harboring T-DNA insertions in *AHP1*, *AHP2*, *AHP4*, and *AHP5* by PCR screening and additional insertions in *AHP3* and *AHP5* using the SIGnAL T-DNA express website. Of the six insertion lines that we identified, four were in the Columbia (Col) ecotype (*ahp2*, *ahp3*, *ahp4*, and *ahp5-2*) and two were in the Wassilewskija (Ws) ecotype (*ahp1* and *ahp5-1*). The locations of the insertions were confirmed by DNA sequencing, and the effect of the T-DNA insertions on the expression of the *AHP* transcripts was examined by RT-PCR (Figure 1B). For *AHP1*, *AHP3*, *AHP4*, and both alleles of *AHP5*, no product was detected from RNA prepared from the mutants using primers flanking the T-DNA insertion site, indicating that full-length transcripts of the disrupted genes were not present in the insertion lines. However, some full-length *AHP2* transcript was detected, indicating that the T-DNA inserted in *AHP2* could be removed during RNA processing, and DNA sequence analysis indicated that the AHP2 transcript from the insertion line was correctly spliced. The expression of full-length AHP2 transcript in the *ahp1,2,3,4,5* mutant was, however, reduced to ~10% of that in wild-type plants (Figure 1C).

The T-DNA insertion sites in *AHP2*, *AHP4*, and *AHP5* are downstream of the sequence encoding the conserved His that is

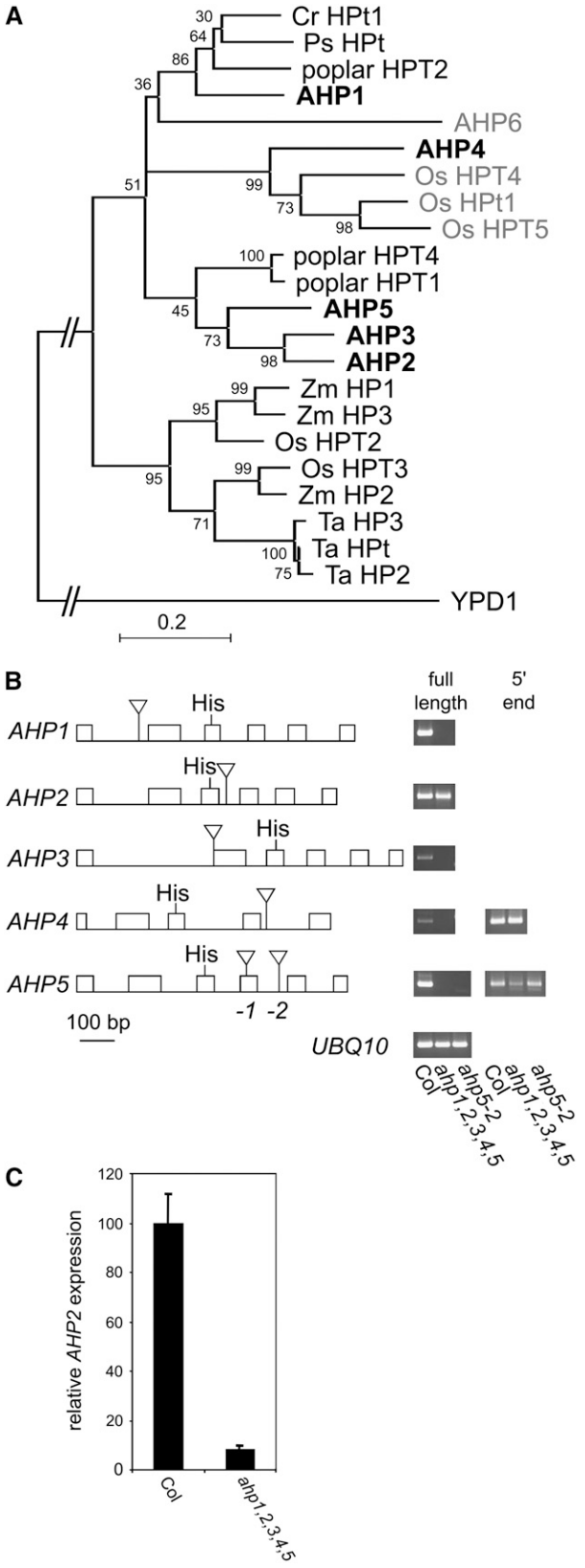

Figure 1. Characterization of *AHP* T-DNA Insertion Lines.

predicted to be phosphorylated during phosphorelay signaling. RT-PCR using primers 5′ to the T-DNA insertion site in *AHP4* and *AHP5* revealed that some partial transcript is present for each gene. It is, however, unknown whether the truncated proteins that they would encode are present in these mutant plants or if they encode functional HPts.

The *ahp5-1* allele (Ws) was used to make the *ahp1,5*, *ahp1,2,3,5*, and *ahp1,2,3,4,5* mutants, while the *ahp5-2* allele (Col) was used in all other *ahp* mutant combinations described here. The combination of Ws and Col alleles in some of the multiple mutants used in this study (see Methods for details on the combinations affected) could result in the segregation of genetic modifiers of the traits being examined. Therefore, the responses of these mutant combinations to cytokinin have to be interpreted with this in mind.

Primary Root Growth of *ahp* Mutants Is Less Sensitive to Cytokinin

To investigate the role of AHPs in cytokinin responsiveness, we measured root elongation of mutant and wild-type seedlings in the presence of various levels of exogenous cytokinin (Figure 2). Root elongation in wild-type seedlings is inhibited by increasing concentrations of the cytokinin benzyl adenine (BA) in the growth media, showing a sharp decrease in root elongation between 10 and 50 nM BA. Inhibition of root elongation in the single mutants was similar to that of wild-type plants. Root elongation of the double mutants *ahp1,2*, *ahp1,3*, *ahp2,4*, *ahp2,5-2*, *ahp3,4*, *ahp3,5-2*, *ahp4,5-2*, and *ahp1,5* and of the triple mutant *ahp2,3,4* was also similar to that of wild-type across the range of cytokinin concentrations examined, while *ahp2,3* was slightly less sensitive than the wild type to growth on 25 nM BA (Figures 2A to 2E). The *ahp1,2,3* triple mutant, however, was substantially less sensitive to cytokinin than the wild type and was less responsive to cytokinin over the whole range of BA concentrations used, indicating that AHP1, AHP2, and AHP3 affect the root elongation in response to cytokinin and have overlapping functions (Figure 2E). In addition, the roots of the *ahp1,2,3* mutants were longer than those of wild-type plants when grown on near-saturating levels of cytokinin (10 μM BA), indicating a reduced response to

(A) A phyologenetic tree of HPt amino acid sequences from plants. 1000 bootstrap replicates were used to construct the N-J tree, and bootstrap values as percentages are marked on the consensus tree. AHPs are highlighted in bold, and predicted pseudo-HPts are in gray.

(B) Position of T-DNA insertion sites in *AHP*s. Boxes represent exons, lines represent introns, His marks the position of the conserved His, and triangles represent T-DNA insertion sites. -1 and -2 mark the T-DNA insertion sites in *ahp5-1* and *ahp5-2*, respectively. Panels at the right present RT-PCR screening for presence of full-length transcripts (left-hand column) and 5′ truncated transcripts (right-hand column) in insertion mutants for the *AHP* gene shown at the left. *UBQ10* was amplified as a control. Bar = 100 bp.

(C) Real-time PCR quantification of full-length *AHP2* mRNA in shoots of Col and *ahp1,2,3,4,5* seedlings. *AHP2* expression relative to *TUB4* was plotted as a percentage of expression in Col. Error bars show SE ($n = 3$).

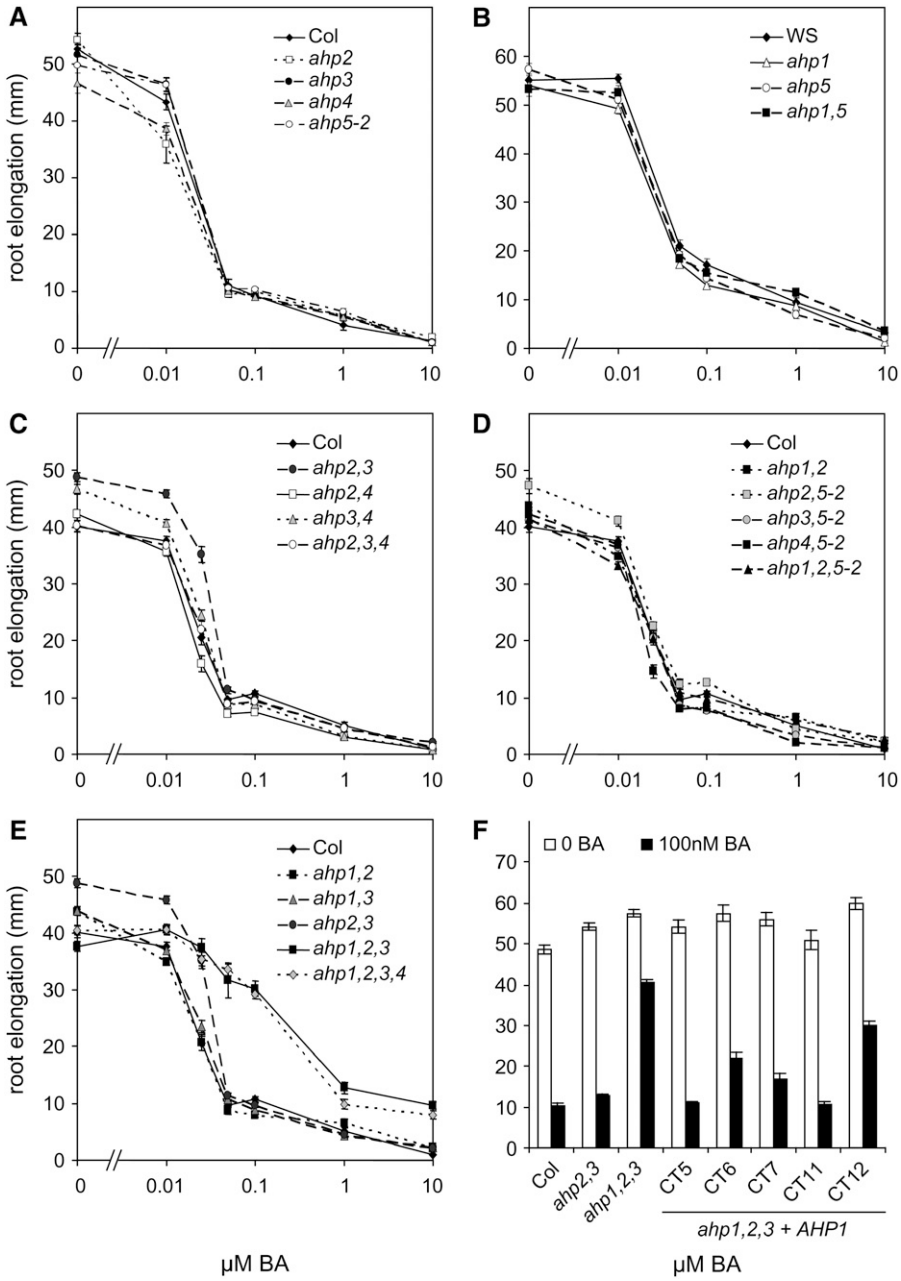

Figure 2. Primary Root Elongation in *ahp* Mutant Seedlings Is Less Sensitive to Inhibition by Cytokinin.

Root elongation of wild-type and single *ahp* mutant insertion lines **(A)**, *ahp* mutant lines in the Ws ecotype **(B)**, and double, triple, and quadruple *ahp* mutant combinations (**[C]** to **[E]**). Root elongation of wild-type, *ahp2,3*, *ahp1,2,3*, and complemented *ahp1,2,3* lines carrying a wild-type *AHP1* construct **(F)**. Each experiment was repeated at least twice with consistent results. Error bars show SE; $n > 9$ for **(A)** to **(E)** and $n > 7$ for **(F)**.

cytokinin in addition to the reduced sensitivity (Figure 2E). The response of the *ahp1,2,3,4* mutant was not significantly different from that of the *ahp1,2,3* mutant, which, along with the other mutant combinations analyzed, indicates that AHP4 does not play a substantial role in this response.

To confirm that the altered cytokinin responses were the result of disruption of the AHPs, a wild-type *AHP1* cDNA under the control of the 2-kb *AHP1* upstream region was introduced into the *ahp1,2,3* triple mutant. Homozygous T3 progeny were assayed for sensitivity to cytokinin using the root elongation assay. Cytokinin sensitivity was increased in five lines out of a total of 14 independent transformed lines examined, indicating that decreased sensitivity to cytokinin was the result of disruption of the AHP function (Figure 2F).

Lateral Root Formation in *ahp* Mutant Seedlings Is Less Sensitive to Cytokinin

Addition of exogenous cytokinin inhibits lateral root formation in *Arabidopsis* (To et al., 2004; Li et al., 2006). To investigate the role of AHPs in this response, the number of lateral roots of seedlings grown in the presence of various levels of exogenous cytokinin was counted (Figure 3). The number of lateral roots in wild-type seedlings was reduced for plants grown on increasing concentrations of BA. A similar response to growth on cytokinin was seen in most of the genotypes tested. However, the triple mutant *ahp1,2,3* showed reduced sensitivity of lateral root formation to cytokinin. In general, inclusion of *ahp4* to various mutation combinations slightly increased sensitivity to cytokinin, suggesting that AHP4 acts as a weak negative regulator in this cytokinin response.

Chlorophyll Levels in *ahp* Mutant Seedlings Are Less Sensitive to Cytokinin

Shoot size and the chlorophyll content in the shoot are reduced in *Arabidopsis* seedlings grown on exogenous cytokinin (To et al., 2004; Mason et al., 2005; Figure 4). In the absence of cytokinin, the cotyledons and shoots of *ahp* mutants were similar to those of wild-type plants. When grown on exogenous BA, wild-type *Arabidopsis* plants developed smaller shoots, leaf development was delayed, and the shoots became yellow (Figure 4A). This response was reduced in the *ahp1,2,3* mutant but not in the *ahp* mutant combinations *ahp1,2*, *ahp1,3*, and *ahp2,3* (Figures 4A and 4B). The shoots of *ahp1,2,3* plants were smaller with growth on exogenous BA but developed more visible leaves and remained greener than those of wild-type plants grown in the presence of cytokinin, indicating that AHP function is important for response to cytokinin in the shoot as well as the root.

Altered Hypocotyl Elongation Response to Cytokinin in *ahp* Mutant Seedlings

To further investigate the effect of *ahp* mutations on the response of aerial parts of the plant to cytokinin, we measured the effect of cytokinin on hypocotyl elongation of plants grown in low-light

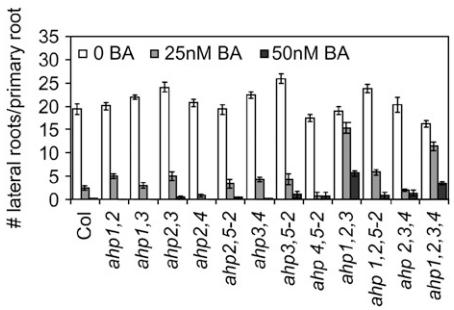

Figure 3. Lateral Root Formation by *ahp* Mutant Seedlings Is Less Sensitive to Inhibition by Cytokinin.

The average number of visible lateral roots from the primary root 9 d after imbibition is shown for each genotype. Experiments were repeated at least twice with similar results. Error bars show SE ($n > 10$).

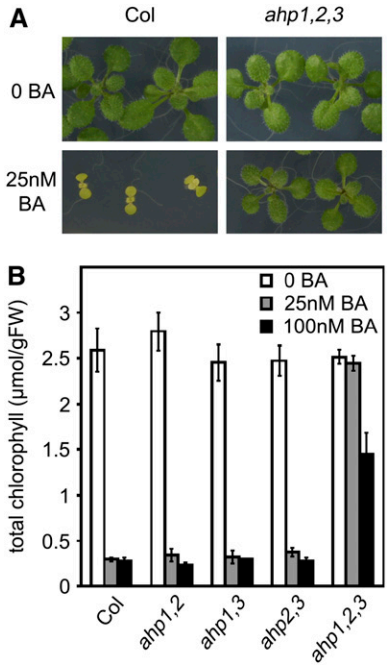

Figure 4. Chlorophyll Content of *ahp* Mutant Shoots Is Less Sensitive to Cytokinin.

(A) Shoots of 14-d-old wild-type and *ahp1,2,3* seedlings grown on plates supplemented with 0 and 25 nM BA.

(B) Quantification of shoot chlorophyll content of seedlings 14 d after imbibition after growth on plates supplemented with the marked concentrations of BA. Three samples of three to five seedlings were assayed for each genotype on each concentration of BA. The experiment was repeated three times with similar results. Error bars show SE. FW, fresh weight.

conditions and in the dark. Hypocotyl elongation is inhibited in wild-type plants grown in the presence of exogenous cytokinin in the light (Figure 5A). In most *ahp* mutant plants, hypocotyl elongation was also inhibited by growth on cytokinin. The mutants *ahp1,2,3*, *ahp1,2,3,4*, *ahp2,3,5-2*, and *ahp2,3,4,5-2*, however, showed reduced inhibition of hypocotyl elongation in response to cytokinin, consistent with reduced sensitivity to the hormone. The greatest difference from the wild-type response was seen in the *ahp1,2,3,4,5* mutant, which actually showed an increase in hypocotyl elongation in the presence of cytokinin (Figure 5A). The *ahp1,2,3,5* mutant also showed an increase in hypocotyl elongation when grown on plates supplemented with 100 nM BA but a similar hypocotyl length when grown on plates with either no supplemental BA or 10 µM BA. The increased hypocotyl elongation observed in response to cytokinin in *ahp1,2,3,5* and *ahp1,2,3,4,5* could reflect suboptimal cytokinin function in these mutants in the absence of exogenous BA. This suggests that the *ahp1,2,3,4,5* mutant has the most reduced cytokinin function in this assay and that this defect could be partially suppressed by exogenous cytokinin via the residual cytokinin signaling in this mutant.

Hypocotyl elongation of wild-type plants in the dark is also inhibited in the presence of cytokinin (Vogel et al., 1998). To

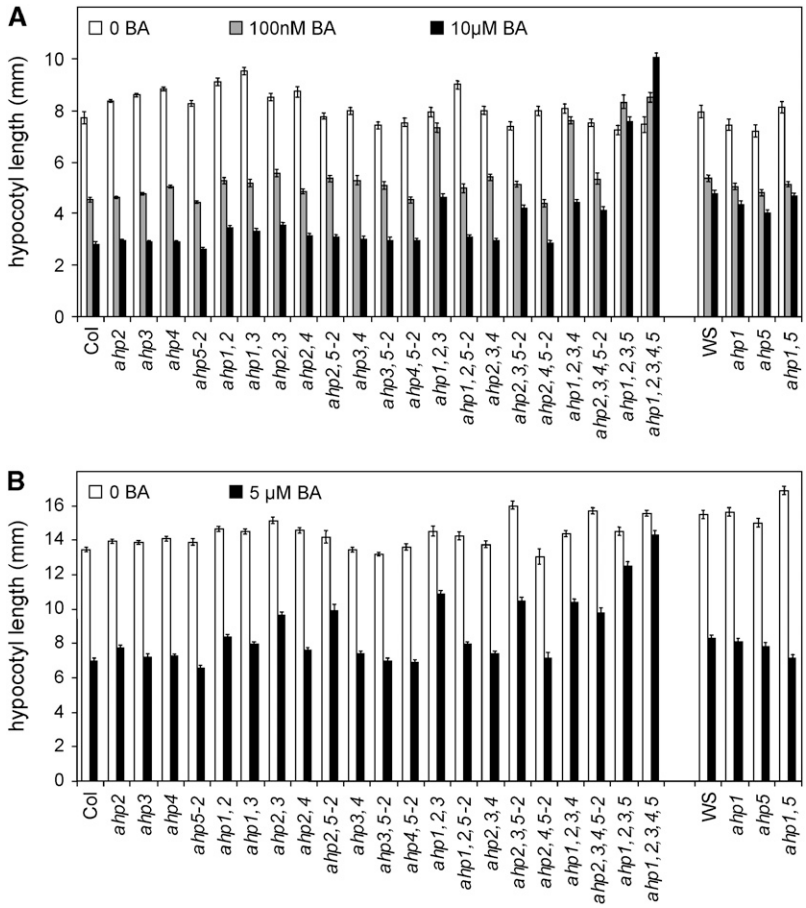

Figure 5. Inhibition of Hypocotyl Elongation in the Presence of Cytokinin Is Reduced in *ahp* Mutants.

(A) Graph shows mean normalized hypocotyl lengths of seedlings grown for 4 d in low light on plates supplemented with the marked concentrations of BA.

(B) Graph of mean normalized hypocotyl lengths of seedlings grown for 7 d in the dark on plates supplemented with 0 or 5 μM BA. Each experiment was repeated at least twice with consistent results. Error bars show SE ($n > 17$).

investigate the role of AHP function in this cytokinin response, we measured hypocotyl elongation of seedlings grown in the dark in the presence and absence of cytokinin (Figure 5B). As was seen for wild-type seedlings, hypocotyls of *ahp* mutants were shorter in the presence of cytokinin, but a reduced response was observed in *ahp2,3,5-2*, *ahp2,3,4,5-2*, *ahp1,2,3*, *ahp1,2,3,4*, *ahp1,2,3,5*, and *ahp1,2,3,4,5*, with the *ahp1,2,3,4,5* mutant showing the most diminished response. These data indicate that all of the AHPs can contribute to the response of hypocotyl elongation to cytokinin and that cytokinin signaling is not completely eliminated in *ahp1,2,3,4,5*.

Higher-Order *ahp* Mutants Have Altered Primary Root Development

The morphology of the AHP single mutants and most of the higher-order *ahp* mutants that we examined appeared superficially similar to that of wild-type plants. However, a clear difference in primary root growth could be seen in the *ahp2,3,5-2* triple mutant and also in *ahp* mutant plants that included *ahp2*, *ahp3*, and *ahp5* mutations (*ahp1,2,3,5*, *ahp2,3,4,5-2*, and *ahp1,2,3,4,5*) compared with wild-type plants. These mutant plants produced a very short, narrow primary root (Figures 6 and 7; see Supplemental Figure 2 online). Xylem development in the narrow primary root of *ahp2,3,5-2* plants was less extensive than in the larger primary root of wild-type plants (Figures 6B and 6C). The phenotype of *ahp1,2,3,4,5* was studied in more detail because this mutant represented the greatest disruption of *AHP* expression.

We examined primary root growth in wild-type and *ahp1,2,3,4,5* mutant seedlings over time. The primary root of the quintuple *ahp* mutant elongated relatively normally for the first few days after imbibition and then dramatically slowed growth at approximately day 3 (Figure 7B). The short primary root phenotype of the *ahp1,2,3,4,5* mutant was rescued by introduction of a genomic copy of *AHP5* into the *ahp1,2,3,4,5* mutant, indicating that the short primary root in *ahp1,2,3,4,5* is the result of reduced AHP function (Figure 7A).

The cessation of root growth in *ahp1,2,3,4,5* may result from loss of root meristem function. To test this, we examined the expression of a mitotic marker, *cyc1At:GUS*, in the *ahp1,2,3,4,5*

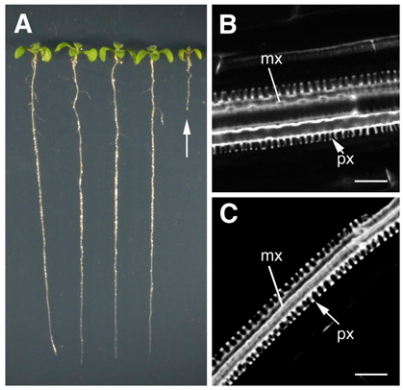

Figure 6. Primary Root Phenotype of *ahp2,3,5*.

(A) Seven-day-old seedlings, from left to right: Col, *ahp2,3*, *ahp2,5-2*, *ahp3,5-2*, and *ahp2,3,5-2* showing the short primary root of *ahp2,3,5-2*. **(B)** and **(C)** Protoxylem (px) and metaxylem (mx) are present in a mature Col root **(B)** and in a mature *ahp2,3,5-2* root **(C)**. Bars = 10 μm.

quintuple mutant background. This cyclin:GUS reporter construct includes the *cyc1At* cyclin destruction box (CDB), which degrades the CDB–β-glucuronidase (GUS) protein at the end of mitosis; this reporter has been found to be an excellent marker for cell division (Donnelly et al., 1999). In wild-type plants, GUS staining in the meristem region of the young root increased from day 1 to day 7 after imbibition (Figure 7C). In *ahp1,2,3,4,5* plants, GUS expression for the first day following germination was close to that of the wild type but then declined sharply (Figure 7D). This corresponds with the cessation of root growth and indicates that cell division is no longer occurring in the root meristem of older primary roots in the *ahp1,2,3,4,5* mutant.

Vascular Development Is Reduced in Primary Roots of *ahp1,2,3,4,5*

Like the *ahp1,2,3,4,5* mutant, *wol* alleles of the *AHK4*/*CRE1*/*WOL* cytokinin receptor have a short root (Scheres et al., 1995; Mähönen et al., 2000; de Leon et al., 2004; Kuroha et al., 2006). Another striking aspect of the *wol* mutant phenotype is loss of phloem and metaxylem development in the root (Scheres et al., 1995; Caño-Delgado et al., 2000; de Leon et al., 2004; Mähönen et al., 2006b). In wild-type *Arabidopsis* roots, xylem is present as a band of vessels with protoxylem cells nearer the cortex and more central metaxylem cells. On either side of the xylem are the phloem poles, which consist of sieve elements and companion cells (Mähönen et al., 2006b). The *ahp1,2,3,4,*5 mutant primary root had a reduced vascular cylinder compared with the wild type; xylem, but no phloem vessels, were visible in transverse sections of *ahp1,2,3,4,5* primary roots. Protoxylem vessels with annular wall thickening, but not metaxylem vessels, which have more reticulate wall thickening, were seen in the primary root in the *ahp1,2,3,4,5* quintuple mutant (Figures 7F and 7H). The protoxylem that was visible in the primary root of *ahp1,2,3,4,5* formed a central cylinder of vessels that displayed radial symmetry, in contrast with the clear asymmetry seen in the wild-type vasculature.

The observation of metaxylem in the primary root of *ahp2,3,5-2* mutants (Figure 6C), but not *ahp1,2,3,4,5* mutants (Figures 7F and 7H), is likely to be the result of residual cytokinin signaling through AHP1 and/or AHP4 in *ahp2,3,5-2*. However, the *ahp2,3,5-2* and *ahp1,2,3,4,5* mutants were constructed using different *ahp5* alleles; furthermore, the *ahp5-1* allele that was included in the *ahp1,2,3,4,5* mutant was isolated in a Ws background. Therefore, the difference in xylem development between the two mutants could also reflect an effect of the distinct *ahp5* allele that was used or an ecotype effect on vascular development. Nevertheless, these observations clearly indicate that AHP function is important for vascular development in the primary root.

Adventitious Root Development in the *ahp1,2,3,4,5* Mutant

While cell division in the meristem of the primary root declined during the first week of growth and the root did not reach more than ~5 mm long, many *ahp1,2,3,4,5* mutant plants developed adventitious roots that grew longer than the primary root, suggesting that meristem activity is more robust in the adventitious roots (Figure 8A). Therefore, we examined cell division in the meristems of the adventitious roots using the *cyc1At:GUS* reporter construct. Wild-type plants did not produce adventitious roots when germinated and grown under our normal long-day growth conditions. To induce adventitious root initiation in wild-type seedlings, plants were grown in the dark for 3 d before transfer to long-day growth conditions. The adventitious roots produced by wild-type plants showed a large region of GUS staining in the meristems (Figure 8B), consistent with a high level of cell division. GUS staining from the *cyc1At:GUS* reporter in the adventitious root systems of *ahp1,2,3,4,5* seedlings was variable, but in both short and longer adventitious roots was frequently more extensive than that observed in the primary root (Figures 8A and 8C). This indicates that in contrast with the primary root, the meristems of adventitious roots of *ahp1,2,3,4,5* seedlings can have high levels of cell division, consistent with their more robust growth.

Extensive development of both protoxylem and metaxylem vessels and radial asymmetry could be seen in adventitious roots of *ahp1,2,3,4,5* plants, in contrast with the lack of metaxylem and apparent radial symmetry in the primary root (Figures 8D and 8E).

Adult Shoot Phenotype of the *ahp1,2,3,4,5* Mutant

The *ahp1,2,3,4,5* seedlings germinated and produced the first two leaves at the same time as wild-type Col plants. Continued shoot development depended on adventitious root development from the mid to upper hypocotyl. A proportion of the plants survived to maturity, producing a rosette, bolting, flowering, and setting seed. Flowering time was variable and delayed, but the leaf number at flowering was similar to that of the wild type, indicating that flowering was not delayed developmentally (total leaf number ± SE: 15.9 leaves ± 0.4 for the wild type and 14.2 ± 0.4 for *ahp1,2,3,4,5*). The size of the rosette of the *ahp1,2,3,4,5* plants was generally smaller than that of wild-type plants but was

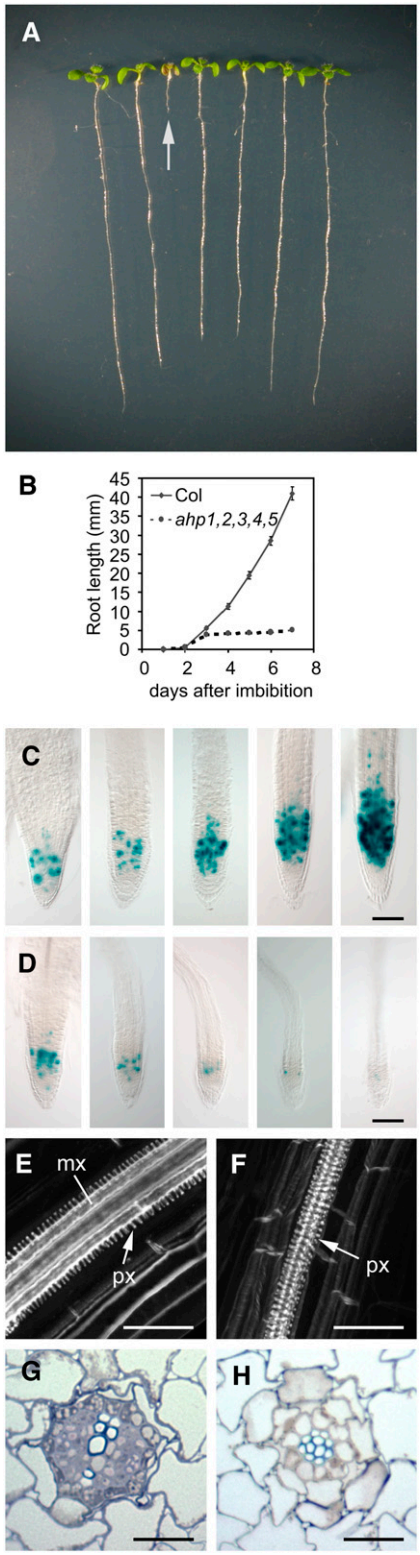

Figure 7. *ahp1,2,3,4,5* Has a Short Primary Root with Altered Vascular Development.

(A) Seven-day-old seedlings of (from left to right) wild-type, *ahp1,2,3,4*, *ahp1,2,3,4,5*, and individuals from four independent *ahp1,2,3,4,5* lines complemented with a wild-type *AHP5* construct. The arrow indicates the *ahp1,2,3,4,5* primary root.

(B) Growth of wild-type and *ahp1,2,3,4,5* primary roots from 1 to 7 d after imbibition.

(C) and **(D)** *CycAt:GUS* expression in wild-type **(C)** and *ahp1,2,3,4,5* **(D)** primary roots. Roots 1, 2, 3, 5, and 7 d after germination are shown from left to right.

(E) and **(F)** Confocal laser scanning microscopy (CLSM) visualization of protoxylem (px) and metaxylem (mx) in the wild-type primary root **(E)** and protoxylem **(F)** but no metaxylem in the primary root of *ahp1,2,3,4,5*.

(G) and **(H)** Transverse sections of 7-d-old wild-type **(G)** and *ahp1,2,3,4,5* **(H)** primary roots stained with toluidine blue.

Bars = 100 μm in **(C)** and **(D)** and 20 μm in **(E)** to **(H)**.

sensitive to growth conditions and was variable within the population and in the progeny of plants of all sizes. The variation in size generally correlated with the extent of adventitious root formation (data not shown).

Silique and Seed Development of the *ahp1,2,3,4,5* Quintuple Mutant

The siliques of the *ahp1,2,3,4,5* quintuple mutant were shorter than those of wild-type plants, and fewer seed were produced per silique (seed number in well-filled siliques ± SD: 54.2 ± 7.2 for the wild type and 9.8 ± 4.6 for *ahp1,2,3,4,5*). While developing seeds in wild-type siliques appeared very similar to one another, development of the seed in *ahp1,2,3,4,5* siliques appeared uneven, and seed abortion was often observed (Figure 9A). The seed that was produced by the quintuple mutant was larger than that from wild-type plants: seed length was ~20% larger in *ahp1,2,3,4,5* seed than in wild-type seed. The increase in seed size correlated with an increase in the size of the mutant embryos (Figures 9B to 9E). The altered seed growth seen in *ahp1,2,3,4,5* was rescued in plants harboring a transgenic copy of wild-type *AHP5*, indicating that this was the result of disrupted AHP function in *ahp1,2,3,4,5* (Figure 9E). A similar alteration in seed growth was seen in the *ahp2,3,5-2* mutant (Figure 9E), indicating that AHP2, AHP3, and AHP5 together are particularly important for seed development.

ahp Mutations Affect the Cytokinin Primary Response

The type-A response regulators are cytokinin primary response genes (D'Agostino et al., 2000; Rashotte et al., 2003). To determine whether the phenotypes of the *ahp* mutants were associated with reduced cytokinin primary signal transduction, we tested the effect of the mutations in the *ahp1,2,3,4,5* mutant on the induction of type-A ARR genes *ARR5*, *ARR8*, and *ARR9*. Ten-day-old light-grown seedlings were treated with a range of concentrations of cytokinin up to 10 μM BA, and expression of the type-A *ARR*s was visualized by RNA gel blot analysis (Figure 10).

In wild-type plants, type-A *ARR* expression was induced in response to cytokinin treatment, as has been shown previously (D'Agostino et al., 2000; To et al., 2004). *ARR* transcript levels were lower in the *ahp1,2,3,4,5* mutant than in wild-type plants in

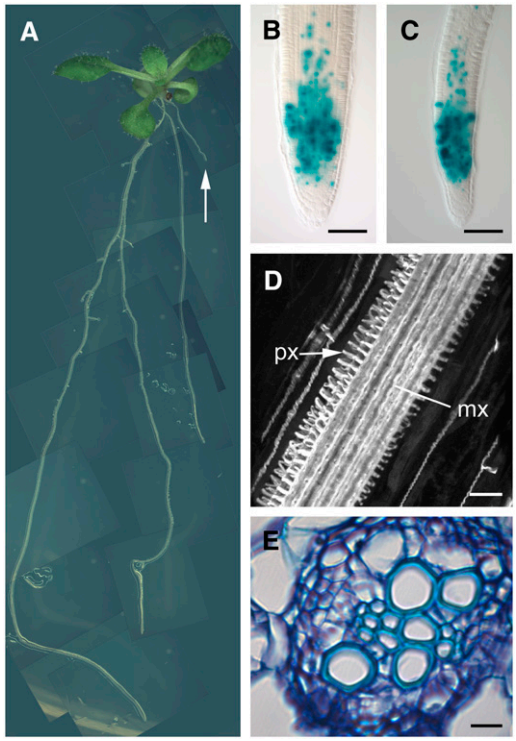

Figure 8. Phenotype of Adventitious Roots of *ahp1,2,3,4,5*.

(A) An *ahp1,2,3,4,5* mutant plant with a short primary root (arrow) and long adventitious roots.
(B) and **(C)** Wild-type **(B)** and *ahp1,2,3,4,5* **(C)** adventitious roots showing extensive *CycAt1:GUS* expression.
(D) CLSM visualization of protoxylem (px) and metaxylem (mx) in an *ahp1,2,3,4,5* adventitious root.
(E) Transverse section of an *ahp1,2,3,4,5* adventitious root stained with toluidine blue.
Bars = 100 μm in **(B)** and **(C)** and 10 μm in **(D)** and **(E)**.

the absence of exogenous cytokinin. There was a detectable increase in the steady state levels of type-A *ARR* transcripts in *ahp1,2,3,4,5* seedlings after cytokinin treatment, but their expression remained much lower than that observed in wild-type seedlings treated with cytokinin (Figure 10). The reduced expression of type-A ARRs in *ahp1,2,3,4,5* is consistent with reduced flux though the primary cytokinin signal transduction pathway in this mutant.

DISCUSSION

We have described the characterization of plants disrupted in the five genes encoding predicted functional HPts in *Arabidopsis*. Based on their response to cytokinin in a variety of assays, our analysis demonstrates that multiple AHPs act redundantly as positive regulators of cytokinin signaling. Furthermore, the phenotype of plants carrying various combinations of *ahp* mutations indicates that AHP function is required for normal development of the embryo and the adult plant and provides evidence that cytokinin signaling from the cytokinin receptors via the AHPs is important for these processes.

AHPs Act Redundantly Primarily as Positive Regulators of Cytokinin Signaling

Single *ahp* mutants did not display altered cytokinin sensitivity or any obvious effect on growth and development. Some double and higher-order *ahp* mutants showed increasing resistance to cytokinin in various assays for cytokinin sensitivity of the shoot and the root, indicating functional overlap between members of the family and that these AHPs act as positive regulators of cytokinin signaling. These observations provide evidence that AHP1, AHP2, AHP3, and AHP5 each act as a positive regulator of cytokinin signaling and that they act partially redundantly. This is consistent with the high degree of sequence similarity among the AHPs and their overlapping patterns of expression (Suzuki et al., 2000; Tanaka et al., 2004). We do discern a function for AHP1 in various cytokinin response assays in the shoot despite evidence from previous studies that indicated that *AHP1* transcripts were not expressed at a detectable level in aerial parts of the plant.

The role of AHP4 in cytokinin signaling appears to be minor in the responses that were analyzed, which is consistent with transcriptome analyses indicating that *AHP4* transcript levels are much lower than those of the other four AHPs (Zimmermann et al., 2004). The effect of the *ahp4* mutation is dependent on the response examined and the genetic background. In some

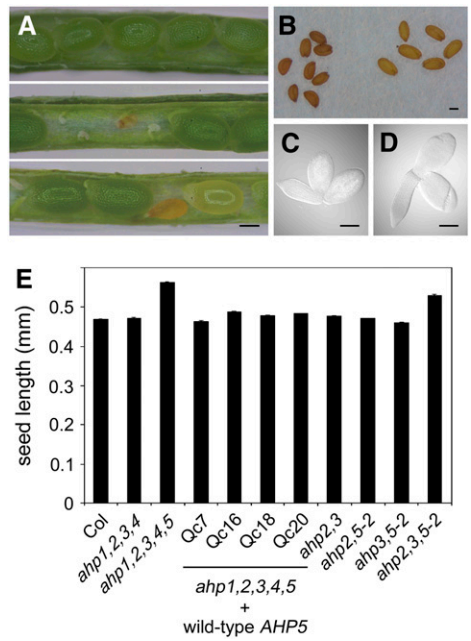

Figure 9. *ahp1,2,3,4,5* Affects Seed Development.

(A) Even seed development in wild-type silique (top panel) and seed abortion in *ahp1,2,3,4,5* siliques (bottom panels).
(B) *ahp1,2,3,4,5* seed on the right is larger than that of the wild type on the left.
(C) and **(D)** Size comparison of wild-type **(C)** and *ahp1,2,3,4,5* **(D)** embryos.
(E) Mean seed length for the genotypes shown. Error bars show SE ($n > 300$).
Bars = 200 μm.

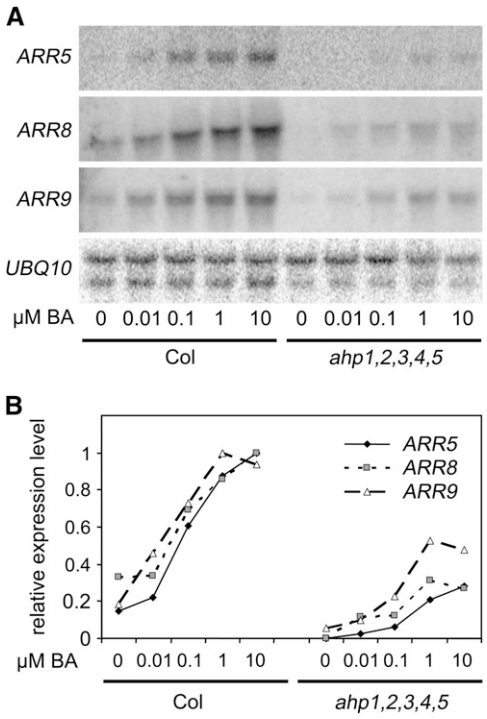

Figure 10. *ahp* Mutations Affect the Cytokinin Primary Response.

(A) RNA gel blot analysis of type-A ARR gene expression in 10-d-old seedlings treated with BA.

(B) Quantification of ARR gene expression normalized to UBQ10 expression and to the maximum signal observed for that ARR gene after induction with cytokinin.

cytokinin response assays, such as lateral root formation, inclusion of the *ahp4* mutation in some multiple *ahp* mutant combinations appeared to slightly increase cytokinin sensitivity, which, in combination with the sequence similarity of AHP4 to the rice pseudo-HPts (Figure 1A), suggests that AHP4 may act as a negative regulator of the cytokinin response pathway. However, in other cytokinin response assays, such as hypocotyl elongation, AHP4 appears to play no role or a slightly positive role in some mutant combinations, suggesting that the role of AHP4 is complex.

The genetic redundancy and overlapping function displayed by the AHPs is also seen in other cytokinin signaling elements, including the cytokinin sensor kinase receptors (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006), the type-A ARRs (To et al., 2004), which are negative regulators of the pathway, the type-B ARRs (Mason et al., 2005), and the recently identified CRFs (Rashotte et al., 2006). In addition, evidence for antagonism between different paralogs has been presented for both the type-A and the type-B ARRs (To et al., 2004; Mason et al., 2005). Thus, each step of the cytokinin signaling pathway is encoded by a partially redundant, multigene family. This extensive redundancy and antagonism among paralogs may serve to fine-tune cytokinin signaling in response to environmental variations, during development, and in distinct tissues of the plant.

Mutation of *AHPs* Affects Induction of Cytokinin Primary Response Genes

The phenotypes of the *ahp* mutants are likely to be the result of reduced phosphotransfer signaling in the cytokinin primary signal transduction pathway. AHPs are predicted to act as the middle component of the phosphorelay pathway from the cytokinin receptors to the ARRs based on several lines of evidence. Studies using yeast two-hybrid assays have demonstrated direct interaction of the AHPs with the cytokinin sensor kinase receptors and with both the type-A and type-B ARRs (Imamura et al., 1999; Urao et al., 2000; Suzuki et al., 2001b). Further, cytokinin-responsive phosphotransfer from the AHKs to the AHPs and phosphotransfer from the AHPs to the type-A ARRs and type-B ARRs have been demonstrated in vitro, and cytokinin-responsive phosphorelays have been constructed in bacteria and in yeast (Imamura et al., 1999, 2001; Suzuki et al., 2001b, 2002; Mähönen et al., 2006a). This, coupled with the phosphorelay paradigm and our genetic analysis, suggests that the AHPs act in cytokinin signaling in *Arabidopsis* plants as direct downstream targets of the cytokinin receptors and that the ARRs are direct downstream targets of the AHPs. Reduced expression of cytokinin primary response genes has been found in higher-order mutant combinations of the cytokinin receptors and the type-B ARRs (Higuchi et al., 2004; Nishimura et al., 2004; Mason et al., 2005). Consistent with the AHPs functioning with these two-component elements as part of the primary cytokinin signaling pathway, disruption of the AHPs in the *ahp1,2,3,4,5* mutant compromises the expression of multiple type-A primary response genes both in the absence and in the presence of exogenous cytokinin.

AHPs Play a Role in Various Developmental Processes

Growth of seedlings on exogenous cytokinin revealed a role for *AHP1*, *AHP2*, and *AHP3* in the response of root elongation, lateral root formation, and shoot chlorophyll content to cytokinin and a positive role for *AHP1*, *AHP2*, *AHP3*, and *AHP5* in the response of hypocotyl elongation to cytokinin.

Reduced AHP function in the *ahp2,3,5-2* and *ahp1,2,3,4,5* mutants resulted in a short primary root, reduced vascular development, particularly in the *ahp1,2,3,4,5* mutant, and reduced fertility, but larger embryos, larger seeds, and enhanced adventitious root development. This indicates that *AHP2*, *AHP3*, and *AHP5* are particularly important and that *AHP1* and/or *AHP4* may play a minor role in these processes, which have been associated previously with reduced cytokinin function (Werner et al., 2003; Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). The defect in vascular development in the *ahp1,2,3,4,5* mutant, which is similar to that seen in *ahk* triple cytokinin receptor mutants and *CRE1 wol* mutants (Scheres et al., 1995; Mähönen et al., 2000; de Leon et al., 2004; Kuroha et al., 2006), was associated with a defect in the normal asymmetry observed in the vascular cylinder of wild-type roots. These observations provide intriguing clues for a role of these genes, and by inference cytokinin, in regulating polarity during development. Alternatively, the lack of xylem polarity in the primary root of the *ahp1,2,3,4,5* mutant may be a secondary consequence of the reduced number of vascular cell files available for development

of other vascular cell types later in development and the differentiation of all of these cell files as protoxylem (Scheres et al., 1995; Mähönen et al., 2006b).

Comparison with Other Mutants That Affect Cytokinin Function

The reduced sensitivity of some higher-order *ahp* mutants to cytokinin is consistent with the reduced cytokinin sensitivity observed with some higher-order mutant combinations of the cytokinin receptors and type-B ARRs (Higuchi et al., 2004; Nishimura et al., 2004; Mason et al., 2005) and with the model that these signaling elements act together in a phosphorelay that mediates the response to cytokinin. The more severe root growth phenotypes found in higher-order *ahp* mutants containing disruptions of *AHP2*, *AHP3*, and *AHP5* (e.g., *ahp1,2,3,4,5*) are similar to those of the cytokinin receptor triple mutant and *wol* alleles of *CRE1*, which have a short primary root (Scheres et al., 1995; Mähönen et al., 2000; de Leon et al., 2004; Higuchi et al., 2004; Nishimura et al., 2004; Kuroha et al., 2006). The reduced seed set and increased seed size observed in the *ahp* mutants is similar to some triple combinations of cytokinin receptor mutants and to transgenic plants that have lower levels of endogenous cytokinin as a result of overexpression of the cytokinin-degrading enzyme cytokinin oxidase (Werner et al., 2003; Riefler et al., 2006). In each of these plants, root development and seed set and/or seed size are affected by reduction in cytokinin action. Thus, our data provide evidence, in combination with the work of other labs, that the cytokinin signaling that is needed for normal development is at least in part mediated by the AHPs.

The *ahp2,3,5-2* and *ahp1,2,3,4,5* mutants produced short primary roots that grew for a few days after germination before cell division in the primary root meristem diminished and growth arrested. Adventitious roots were initiated from the hypocotyl and grew longer than the primary root, consistent with a more robust meristem activity as revealed by *cyc1At:GUS* staining. Similarly, more robust adventitious root growth is seen in the cytokinin receptor mutant *ahk2,3,4* (Kuroha et al., 2006). This suggests that reduced cytokinin signaling in *ahp1,2,3,4,5* inhibits root meristem function and/or maintenance and that primary and adventitious root development has different requirements for this signal. It is possible that the more severe effect on the primary root meristem may not simply reflect a direct role of the AHPs in regulating cell proliferation in the meristem but may also be a secondary consequence of the altered vascular development that occurs in the mutant primary root. The reduced vascular development in the *ahp1,2,3,4,5* and *ahp2,3,5-2* mutants could itself be the result of defects in cell division that result from reduced cytokinin action: *ahk2,3,4* and *CRE1 wol* mutants also have short roots with fewer vascular cell files resulting from reduced periclinal cell divisions in the procambium (Scheres et al., 1995; Mähönen et al., 2000; Nishimura et al., 2004).

While the size of the rosette of the *ahp1,2,3,4,5* mutant plants was generally smaller than that of wild-type plants, the *ahp1,2,3,4,5* mutant did not reveal a strong direct effect of AHP function on rosette size. Rosette size was variable within the population and correlated with the extent of adventitious root formation, which was also variable within the population. A similar effect of adventitious rooting on shoot development has been described for plants carrying *wol* mutant alleles of the cytokinin receptor CRE1 (Scheres et al., 1995; Kuroha et al., 2006). In view of the strongly reduced shoot size of *ahk2,3* and *ahk2,3,4* cytokinin receptor mutants and plants with reduced endogenous cytokinin levels (Werner et al., 2003; Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006), these observations suggest that the residual cytokinin signaling in *ahp1,2,3,4,5* can be sufficient to generate near-normal-size rosettes. Alternatively, cytokinin signaling from the cytokinin receptors may not occur solely through the AHPs.

The decrease in seed set and increase in seed size in *ahp1,2,3,4,5* plants compared with wild-type plants indicates that AHP action is important for proper seed development. Increased seed size has been linked to decreased cytokinin signaling by the observations that plants with reduced endogenous cytokinin produce larger seed than wild-type plants, as do some *ahk2,3,4* cytokinin receptor mutants (Werner et al., 2003; Riefler et al., 2006). The increase in seed size in *ahp1,2,3,4,5* mutants was slightly less than that seen in *ahk2,3,4* mutants in which some seed is set (~20% increase in seed length for *ahp1,2,3,4,5* compared with ~30% increase for *ahk2,3,4*; Figure 9; Riefler et al., 2006).

Thus, the developmental effects of disrupting all five *AHP* genes are generally less severe than those observed for the triple cytokinin receptor mutants. In particular, the shoot and reproductive phenotypes of the *ahp1,2,3,4,5* mutant are less severe than those of *ahk2,3,4* mutants, which have very small rosettes and, if they flower, produce few flowers and little or no seed (Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). In addition, some induction of type-A ARR genes is seen in the *ahp1,2,3,4,5* mutant in contrast with the lack of induction seen in the *ahk2,3,4* mutant (Higuchi et al., 2004; Nishimura et al., 2004). The most likely reason for these differences in phenotype is residual *AHP2* function resulting from the precise splicing and concomitant removal of the T-DNA insertion from the AHP2 transcript that occurs with low efficiency in plants carrying this *ahp2* allele. It is also possible that the *ahp4* and *ahp5* alleles that we used produce truncated proteins that are partially functional, which could contribute to the residual cytokinin responsiveness in the quintuple *ahp1,2,3,4,5* mutant. Alternatively, a noncanonical AHP could mediate cytokinin signaling, or the cytokinin sensor kinases could signal directly to the ARRs or through other signaling elements.

In sum, the genetic analysis presented here provides strong in vivo evidence that AHP1, AHP2, AHP3, and AHP5 act primarily as positive regulators of cytokinin signaling, affecting induction of cytokinin primary response genes, sensitivity to exogenous cytokinin, and developmental processes that have been linked to cytokinin. Like other components of the cytokinin signal transduction pathway, the AHP family members act redundantly with overlapping function in these cytokinin responses. Taken together with results from other studies, this analysis adds support for the model in which the AHPs act in the primary cytokinin signal transduction pathway to shuttle phosphate from the AHK receptors to the ARR proteins. It also provides evidence that AHPs mediate signaling from the AHKs not only to achieve correct expression of known cytokinin primary response genes, but also to downstream developmental processes, such as primary root and seed development, that have been linked previously to cytokinin action.

Given that each element of the cytokinin signal transduction pathway is encoded by a multigene family and that more than one family member has been linked to each cytokinin response, it remains to be determined if there is any specificity in the interactions among these various signaling components. Whether there are other outputs from the AHK receptors, and/or other inputs that regulate the ARR proteins, and whether these inputs and/or outputs are mediated by the AHPs await future investigation.

METHODS

Isolation of *AHP* Mutants

In all, 80,000 *Arabidopsis thaliana* lines from the Salk T-DNA collection in the Col ecotype were screened for T-DNA insertions in the *AHP*s by PCR, as described previously (Alonso et al., 2003). Insertions in *AHP2* and *AHP4* were identified from this screen. Insertions in *AHP1* and *AHP5* were identified in the Ws ecotype in screens using the *Arabidopsis* knockout facility. Gene-specific primers used were as follows: AHP1sense, 5′-CAGAGAATATGGATTTGGTTCAGAAG-3′; AHP1anti, 5′-ATGTGGGATTTAGATGTGGATTTAGAAAC-3′; AHP2sense, 5′-CTTGGGATTGGCTATTTCCAGAAATCCAA-3′; AHP2anti, 5′-CAATGGTTTCAATTTTCTCGGATGAGATC-3′; AHP3sense, 5′-TCATGAGGTCAAGTTGATGAGAGTATATG-3′; AHP3anti, 5′-TTTGTATTTGACAGTGAGACTGCGTTGAC-3′; AHP4sense, 5′-TGATCATTTGATAACACCTACCATTTGTG-3′; AHP4anti, 5′-CCACTCTTTTCTGTATTCCGTTATTAGAG-3′; AHP5sense, 5′-ATTTTTCCTGTTTTGTAACTGTGGACGAT-3′ or 5′-GCTAGCAACTTCCCTATCTATAAAATCA-3′; and AHP5anti, 5′-TCCTTTCTCAATCTATTGTCACAATCATG-3′ or 5′-AACTACAGAGGAGTTTCGGTTTCATTTGT-3′. T-DNA primers used were JMLB1 (5′-GGCAATCAGCTGTTGCCCGTCTCACTGGTG-3′) for insertions in Col and JL202 (5′-CATTTTATAATAACGCTGCGGACATCTAC-3′) for insertions in Ws. Additional insertions in *AHP3* and *AHP5* in the Col ecotype (SALK_041384 and SALK_079857) were identified at the SIGnAL T-DNA express website (http://signal.salk.edu/cgi-bin/tdnaexpress). The locations of the T-DNAs in the insertion lines were confirmed by sequencing.

The *ahp1* and *ahp5* alleles in the Ws ecotype, designated *ahp1-1* and *ahp5-1*, were crossed to give the *ahp1,5* double mutant in a Ws background. The *ahp1* mutant isolated in the Ws ecotype was crossed to Col twice before being crossed to single mutants isolated in the Col ecotype. The single mutants were then crossed to generate the double mutants *ahp1,2*, *ahp1,3*, *ahp2,3*, *ahp2,4*, *ahp2,5-2*, *ahp3,4*, *ahp3,5-2*, and *ahp4,5-2*. Double mutants were crossed to generate *ahp1,2,3* and *ahp2,3,4*. The *ahp5-1* allele was used to make *ahp1,2,3,5* and the *ahp1,2,3,4,5* quintuple mutant: *ahp5-1* was crossed to *ahp4* and then the double mutant was crossed to *ahp1,2*. An F2 plant homozygous for insertions in *ahp1* and *ahp5*, heterozygous for the insertion in *ahp4*, was crossed to *ahp2,3*. F1 plants heterozygous for insertions in all five loci were identified by PCR, and the quintuple mutant *ahp1,2,3,4,*5 was identified by PCR in the F4 generation. The *ahp5-2* allele was used to construct the other double and triple mutants described here.

Phylogenetic Analysis of AHP Amino Acid Sequences

For AHP1, AHP2, AHP3, and AHP5, the full-length AHP amino acid sequences associated with the Arabidopsis Genome Initiative numbers stated below were used, while for AHP4, the amino acid sequence BAB01275.1 was used because this was the sequence encoded by the AHP4 cDNA that we amplified. Other plant HPt sequences were identified by BLAST searches of the Uniprot database. Rice (*Oryza sativa*) amino acid sequences were identified from the complete genome sequence available through The Institute for Genomic Research using BLAST and HMMER-based searches with a Hidden Markov Model of the Hpt domain (S.H. Shiu, J.J. Kieber, and G.E. Schaller, unpublished results). The sequences were aligned using ClustalW (Thompson et al., 1994) within the MEGA3 software package (see Supplemental Figure 1 online). The consensus N-J tree was calculated using MEGA3 (Kumar et al., 2004). Branches were marked with bootstrap values for 1000 repetitions as percentages.

Growth Conditions for Adult Plants and Seedlings

For growth on soil, seeds were sown directly on soil or on wet filter paper and cold-treated for 3 d before transfer to growth conditions. Plants were grown at 23°C in ~75-μE light under long-day conditions (16 h light/8 h dark).

For growth on plates, seeds were surface-sterilized and cold treated at 4°C for 3 to 9 d in the dark. Seedlings were grown either on vertical plates containing 1× Murashige and Skoog (MS) salts, 1% sucrose, and 0.6% phytagel (Sigma-Aldrich) or on horizontal plates containing 1× MS salts, 1% sucrose, and 0.8% Phytablend. Plates were incubated at 23°C in ~100-μE constant light (for root elongation and lateral root formation assays and for measurement of shoot chlorophyll content), in ~75-μE constant light (for analysis of gene expression) or long days (for other phenotypic analyses and GUS staining), and in ~5-μE constant light or darkness (for hypocotyl elongation assays).

Complementation Analyses

For complementation with *AHP1*, the wild-type cDNA was amplified and cloned downstream of the 2-kb promoter region of *AHP1*. The resulting promoter-cDNA construct was inserted into the pCAMBIA1390 binary vector. For complementation with *AHP5*, an *AHP5* genomic fragment that included 2-kb upstream sequences of *AHP5* was amplified by PCR and inserted into pENTR-D (Invitrogen). The AHP5 genomic clone was transferred into pGWB4 (T. Nakagawa, Simane University, Japan), creating an AHP5-GFP construct. *ahp1,2,3* plants were transformed with the *AHP1* construct, and *ahp1,2,3,4,5*/+ plants were transformed with the *AHP5* construct by floral dip (Clough and Bent, 1998). Transformants were selected on MS plates supplemented with 50 μg/mL hygromycin and 50 μg/mL carbenicillin. For complementation of *ahp1,2,3*, T1 hygromycin-resistant lines were selected, and homozygous T3 progeny from 12 independent lines were examined in seedling cytokinin response assays as described above. For complementation of *ahp1,2,3,4,5*, plants homozygous for insertions in *AHP1*, *AHP2*, *AHP3*, and *AHP4* and heterozygous for insertion in *AHP5* were transformed with a genomic copy of *AHP5*. T1 lines were selected, plants with an insertion in *AHP5* were identified by PCR, and T2 lines homozygous for the T-DNA insertion in *AHP5* and heterozygous for the transgene were analyzed. Results were confirmed with T3 lines.

RNA Expression Analysis

AHP RNA expression in the T-DNA insertion lines was assayed by RT-PCR. RNA was extracted from seedlings grown on vertical plates for 10 d under constant light. cDNA was generated using Superscript III RT (Invitrogen). *AHP* cDNA was amplified by 35 cycles of PCR using a 5′ primer at the ATG and 3′ primers either upstream of the insertion site or at the stop codon of the AHP open reading frame. Primers used were as follows: cAHP1s, 5′-TTCTCGAGGCCATGGATTTGGTTCAGAAGCAG-3′; cAHP1a, 5′-GTGGATCCGAAACAACAATGGACAGAGATG-3′; cAHP2s, 5′-TTCTCGAGGCCATGGACGCTCTCATTGCTCAG-3′; cAHP2a, 5′-TAGGATCCTAAAGAAAGAAAGTCATCATC-3′; cAHP3s, 5′-TTCTCGAGGCCATGGACACACTCATTG-3′; cAHP3a, 5′-TTGGATCCATCTTGCTGTTCTGTTGTC-3′; cAHP4s, 5′-CACCATGCAGAGGCAAGTGGCACTC-3′; cAHP4RTLa, 5′-CTATCCAGCCGATTGAAATCAAATGATC-3′; cAHP4a,

5′-ATGGATCCATAATTGATTATTGGCTC-3′; cAHP5s, 5′-TACTCGAGATGAACACCATCGTCGTTGCTC-3′; cAHP5HAa, 5′-CTACCCTTGAGTTGAGCAACACCTGAATC-3′; and cAHP5a, 5′-CAGGATCCATCGGTCTAATTTATATCCAC-3′. UBQ10sF1 (5′-GATCTTTGCCGGAAAACAATTGGAG-3′) and UBQ10R1 (5′-CGACTTGTCATTAGAAAGAAAGAGATAACAGG-3′) primers were used as controls.

Real-time PCR analysis was used to quantify residual full-length *AHP2* RNA expression. RNA was prepared from three independent samples of shoots of 10-d-old seedlings using an RNeasy kit (Qiagen) according to the manufacturer's instructions. cDNA was prepared as above. PCR was performed with two sample replicates on a light cycler using SYBR green technology for product detection and the amplification program 94°C for 1 min, 35 cycles of 94°C for 15 s, 60°C for 15 s, and 72°C for 15 s. The primers used were as follows: AHP2RTs1, 5′-CTACAAGATGATGGAAGTCCTG-3′; AHP2RTa1, 5′-CACCAACACTTGAGCTACTAC-3′; TUB4s, 5′-AGAGGTTGACGAGCAAGATGA-3′; and TUB4a, 5′-ACCAATGAAAGTAGACGCCA-3′. Threshold cycle values were generated by subtracting blanks and the baseline average over cycles 1 to 10 with the threshold set at 10× SD over the cycle range for each sample. Fold change in expression of *AHP2* relative to *TUB4* was calculated between samples.

For analysis of cytokinin-induced gene expression, 10-d-old seedlings were treated with the described concentrations of BA, or DMSO vehicle control, in liquid 1× MS and 1% sucrose. Samples were taken after 45 min, and RNA was prepared using TRI reagent (Molecular Research Center) according to the manufacturer's instructions, separated on a 1.2% agarose, formaldehyde gel, and transferred to nylon membrane (GeneScreen; NEN Life Science Products). Hybridizations, with full-length cDNAs of the appropriate cytokinin primary response gene as probes, were performed using Rapid hyb buffer (Amersham Biosciences) according to the manufacturer's instructions.

Seedling Cytokinin Response Assays

For primary root elongation and lateral root formation assays, *Arabidopsis* seeds were grown on vertical plates containing the appropriate concentration of BA in 0.1% DMSO for 10 d. Root lengths at day 3 and day 8 were marked on the plates. The plates were photographed at 9 d, and root growth between days 3 and 8 was measured using ImageJ (National Institutes of Health). The number of lateral roots that were visible on day 9 was counted with the aid of a dissecting microscope.

For determination of chlorophyll content, shoots of 2-week-old seedlings grown on the stated concentration of BA were harvested, and chlorophyll was extracted with methanol. Chlorophyll content was determined spectrophotometrically and normalized to fresh weight as described previously (Porra et al., 1989).

For hypocotyl elongation assays, seeds were grown on horizontal plates containing the appropriate concentration of BA for the stated number of days. Seedlings were then knocked over, photographed, and measured using ImageJ software as above. Wild-type Col plants were grown on each plate. The ratio of hypocotyl lengths of seedlings of each genotype on each plate to the Col hypocotyl length on that plate was calculated. The mean hypocotyl lengths of seedlings of each genotype on each concentration of BA were plotted normalized to the mean Col hypocotyl length on that concentration of BA.

Histology and Microscopy

For GUS staining, sample tissues were treated with ice-cold 90% acetone for 20 min, rinsed three times with sodium phosphate buffer, and then incubated with reaction buffer (1 mM X-glucuronic acid, 100 mM sodium phosphate, pH 7.0, 0.1% Triton X-100, 0.5 mM each potassium ferri- and ferrocyanide, and 10 mM EDTA) overnight at room temperature. The tissues were destained in 70% ethanol, rehydrated in water for 10 min, mounted in chloral hydrate, and examined immediately.

For sectioning, seedlings were embedded in either Spurr's resin or paraplast. For embedding in Spurr's resin, seedlings were fixed by submersion in 2% paraformaldehyde and 1% glutaraldehyde in 50 mM sodium phosphate buffer, pH 7.4, washed three times in sodium phosphate buffer, and postfixed with 1% OsO_4 in 50 mM sodium phosphate buffer, pH 7.4. Samples were then washed three times in deionized water and dehydrated in an ethanol series before transfer to propylene oxide and infiltration with 1:1 propylene oxide:Spurr's resin overnight. Samples were embedded in Spurr's resin in flat molds at 70°C for 24 h and then cut into 1-μM-thick sections. Sections were stained with 1% toluidine blue in 1% sodium borate for 30 s at 60°C. For embedding in paraplast, seedlings were fixed overnight at 4°C in 4% paraformaldehyde in 100 mM sodium phosphate buffer, pH 6.8, washed three times in sodium phosphate buffer, dehydrated in an ethanol series, stained with 0.1% eosinY overnight, and washed with 100% ethanol. Samples were then transferred to citrisolv through an ethanol/citrisolv series and infiltrated with 1:1 citrisolv:paraplast overnight at 50 to 55°C. Six further 100% paraplast changes were performed before samples were embedded in flat molds. Samples were cut into 5-μM-thick sections. Sections were stained with 0.01% toluidine blue.

To examine embryo size, embryos were dissected out of seeds in the last silique before the silique in which the seed coats of all the seeds was brown. Embryos were fixed in 1:3 acetic acid:ethanol for 25 min, rehydrated in water for 10 min, mounted in chloral hydrate, and examined immediately.

Samples were viewed and images were recorded using either a Nikon Eclipse 800 microscope with SPOT camera and software or a Nikon Optiphot2 microscope with a micropublisher 3.3 RTV camera and QImaging software.

To examine seed size, seeds from five plants for each genotype were scanned at 1200 dpi using a flatbed scanner. More than 300 seeds of each genotype were measured using ImageJ software as above.

Vascular staining with basic fuchsin was performed as described (Mähönen et al., 2000). Seedlings were mounted in 50% glycerol, and CLSM images were made using a Zeiss LSM510 confocal microscope with a HeNe laser (543 excitation; long-pass emission filter LP560). Z-sections were collected and projected together to give a composite image of the xylem vessels.

Accession Numbers

Sequence data from this article can be found in the GenBank/EMBL data libraries under the following accession numbers: AHP1, At3g21510; AHP2, At3g29350; AHP3, At5g39340; AHP4, At3g16360 and BAB01275.1; AHP5, At1g03430; TUB4, At5g01010; and UBQ10, At4g05320. Wheat (*Triticum aestivum*) sequences: Ta HPt, AY342358; Ta HP2, BK005644; and Ta HP3, BK005645. Maize (*Zea mays*) sequences: Zm HP1, AB024293; Zm HP2, AB024292; and Zm HP3, AB089191. Pea (*Pisum sativum*) sequence: Ps HPt, AJ831475. Poplar (*Populus x canadensis*) sequences: hpt1, AJ841793; hpt2, AJ841794; hpt3, AJ841795; and hpt4, AJ841796. Madagascar periwinkle (*Catharanthus roseus*) sequence: Cr HPt1, AF346308. Rice sequences: Os *Hpt2*, Os08g44350; Os *Hpt*3, Os09g39400; Os *Hpt*1, Os01g54050; Os *Hpt*4, Os05g09410; and Os *Hpt*5, Os05g44570. Yeast (*Saccharomyces cerevisiae*) sequence YPD1, U62016. pCAMBIA1390 binary vector, AF234307.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Alignment of HPt Amino Acid Sequences from Plants.

Supplemental Figure 2. *ahp* Mutant Combinations That Include *ahp2*, *ahp3*, and *ahp5* Have a Short Root.

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