Histidine (His) is an essential ingredient for protein synthesis and is required by all living organisms. In higher plants, although there is considerable evidence that His is essential for plant growth and survival, there is very little information as to whether it plays any specific role in plant development. Here, we present evidence for such a role of this amino acid in root development in Arabidopsis (Arabidopsis thaliana) from the characterization of a novel Arabidopsis mutant, hpa1, which has a very short root system and carries a mutation in one of the two Arabidopsis histidinol-phosphate aminotransferase (HPA) genes, AtHPA1. We have established that AtHPA1 encodes a functional HPA and that its complete knockout is embryo lethal. Biochemical analysis shows that the mutation in hpa1 only resulted in a 30% reduction in free His content and had no significant impact on the total His content. It did not cause any known symptoms of His starvation. However, the mutant displayed a specific developmental defect in root meristem maintenance and was unable to sustain primary root growth 2 d after germination. We have demonstrated that the root meristem failure in the mutant is tightly linked to the reduction in free His content and could be rescued by either exogenous His supplementation or AtHPA1 overexpression. Our results therefore reveal an important role of His homeostasis in plant development.

His is one of the essential amino acids for protein synthesis and is required by all living organisms. Its biosynthetic pathway has been extensively characterized in bacteria and lower eukaryotes and consists of 10 enzymatic steps catalyzed respectively by (in the order of reactions) ATP-phosphoribosyltransferase, phosphoribosyl-ATP pyrophosphohydrolase (PRA-PH), phosphoribosyl-AMP cyclohydrolase (PRA-CH), N'-phosphoribosyl-(5''-phosphoribosyl)-4-imidazolecarboxamide isomerase (BBMII isomerase), Gln amidotransferase, cyclase, imidazoleglycerol-phosphate dehydratase (IGPD), histidinol-phosphate aminotransferase (HPA), histidinol-phosphate phosphatase, and histidinol dehydrogenase (which catalyzes the last two steps; Winkle, 1987; Carlomagno et al., 1988; Alifano et al., 1996). In Escherichia coli, these enzymes are encoded by eight genes (the PRA-PH and PRA-CH activities are encoded by a single gene) in a single operon (Carlomagno et al., 1988). However, in Saccharomyces cerevisiae, they are encoded by seven genes scattered throughout the genome. The difference in the number of genes between E. coli and S. cerevisiae is due to the multifunctional properties of two yeast proteins, HIS4 (with PRA-PH, PRA-CH, and histidinol-dehydrogenase activities) and HIS7 (with both Gln amidotransferase and cyclase activities; Mortimer et al., 1994; Alifano et al., 1996). Except for histidinol-phosphate phosphatase, genes encoding for all the other enzymes of the His biosynthetic pathway have been identified in plants (Noutoshi et al., 2005). In Arabidopsis (Arabidopsis thaliana), genomic sequence analysis reveals 10 putative genes in the His biosynthetic pathway (Noutoshi et al., 2005).

It has been well established that His biosynthesis is vital for the survival of plants. Physiological studies using specific inhibitors of the His biosynthetic pathway, such as triazole herbicides that inhibit the activities of IGPD, revealed that a complete blocking of His biosynthesis in plants was lethal (Guyer et al., 1995; Mori et al., 1995). A similar conclusion was reached from the characterization of a Nicotiana plumbaginifolia His auxotropic mutant, his1-1, which carried a mutation in the HPA encoding gene and was unable to survive without exogenous His supplement (El Malki and Jacobs, 2001), and an Arabidopsis mutant carrying a knock-out T-DNA insertion in the APG10 gene, which encodes a putative BBMII isomerase (Noutoshi et al., 2005). In addition to its role in protein synthesis, His is known to play some role in cross-pathway regulation (Guyer et al., 1995; Noutoshi et al., 2005). For example, in Arabidopsis, inhibiting His biosynthesis with a
triazole herbicide led to an enhanced expression of genes in other amino acid biosynthetic pathways and the accumulation of a number of amino acids (Guyer et al., 1995).

However, very little is known about whether His plays any specific role in plant development. This is partly due to the difficulty in experimentally separating the metabolic and regulatory functions of this essential amino acid and the lack of nonlethal (His) deficiency mutants. Our current understanding of the role of His in plants largely comes from studies of plants under severe His starvation, conditions that are usually lethal and can cause a wide range of nonspecific responses. As a result, there is little understanding about the role of this amino acid in plant growth and development under non- or mildly starved conditions.

Here, we describe the characterization of a novel Arabidopsis mutant, hpa1, in the His biosynthetic pathway. This mutant carries a mutation in one of the two Arabidopsis HPA genes, AtHPA1, which encodes the enzyme catalyzing the transamination from L-Glu to 3-(imidazole-4-yl)-2-oxo-propyl phosphate to produce L-histidinol phosphate, the eighth step in the His biosynthetic pathway (Winkle, 1987). The mutant has a reduced level of free His content in comparison to the wild type. Interestingly, the mutant did not show any previously described symptoms of His starvation, such as lethality, a global increase in biosynthesis of other amino acids, or the albino and pale-green cotyledon and leaf phenotype of apg10, but it displayed a specific defect in root meristem maintenance. We demonstrated that the developmental defect of the mutant was tightly linked to the reduction in free His content and the mutation in AtHPA1 and could be rescued by either His supplementation or AtHPA1 overexpression. The results have therefore revealed a novel role of free His level in plant development.

RESULTS
Isolation and Morphological Characterization of hpa1

The hpa1 mutant was originally obtained for its extremely short root system (Fig. 1A). It has no obvious phenotype in the aerial parts and is fully fertile. Genetic analysis indicated that the short root phenotype was caused by a recessive mutation at a single locus (data not shown).

To establish the timing of the primary root growth defect, we monitored the primary root length in the wild-type and mutant seedlings within the first 10 d after germination (DAG; Fig. 1B). At 2 DAG, there was very little difference between the wild-type and mutant seedlings in primary root length, indicating that the root growth defect is probably not due to some developmental disorder during embryogenesis. However, a difference in root length occurred at 2 DAG and increased as the seedlings became older (Fig. 1B).

To characterize the growth defect at the cellular level, we examined a number of root tip-specific markers, including starch granule staining (root cap columella specific), DR5-β-glucuronidase (GUS; auxin accumulation marker normally highly expressed in the root cap columella cells and the quiescent center [QC]; Ulmasov et al., 1997), SCR-green fluorescent protein (GFP; expressed specifically in the QC and the endodermis; Sabatini et al., 1999), QC25 (QC specific; Van den Berg et al., 1995), J0631 (a root meristem boundary marker expressed in the epidermal cells of the mature part of the root but not in the meristematic region), and J0481 (expressed in the lateral root cap and root epidermal cells; http://www.plantsci.cam.ac.uk/Haseloff). These were examined in both mutant and wild-type seedlings at two different developmental stages, 3 DAG and 7 DAG. At 3 DAG, none of the markers displayed any obvious abnormal spatial pattern in the mutant root tips (Fig. 2, A, C, D, H, I, M, N, R, and S), indicating that, by this stage, the mutant roots had not yet developed any obvious cellular patterning defects. This observation is consistent with that obtained from the root growth analysis and indicates that the root growth phenotype in hpa1 does not result from developmental defects during embryogenesis. By 7 DAG, however, the mutant roots had developed a range of abnormal patterns in the meristematic region, ranging from a reduced meristematic zone (indicated by the closer proximity of the J0631-expressing region to the root tip; Fig. 2B), the reduced number of root cap columella cells expressing the correct cell-specific markers (such as DR5-GUS or starch granule staining; Fig. 2, E, F, T, and U), and the loss of J0481 expression in the lateral root cap (Fig. 2, O and P). Interestingly, the mutation had differential effects on two markers, QC25 and SCR-GFP, in the QC. It caused a reduction in QC25 expression (Fig. 2, J and
K), indicated by either a reduced level or a complete lack of GUS staining in most mutant roots (data not shown), but had no obvious effect on the expression of SCR-GFP in the QC (Fig. 2, Y and Z). The differential effects on the two markers indicate a partial loss of QC identity. The wide range of cellular patterning changes within the root meristematic region indicates that the mutant has some difficulties in maintaining proper cell patterning in the root meristems. The occurrence of root hairs and lateral roots at the proximity of the primary root tip region also indicates a defect in meristem maintenance (Fig. 2, A and B).

The hpa1 Mutant Carries a Mutation in an HPA Gene

A map-based strategy was used to clone the HPA1 gene. We first crossed hpa1 (in the Columbia [Col] background) to Landsberg erecta (Ler) wild type and selected progenies with the short root phenotype in the F2 population. Initial mapping analysis revealed a linkage between the short root phenotype and the simple sequence length polymorphism marker NG A249 on chromosome 5. We then developed a series of markers (M322, M326, M331, M339, and M35; for details, see Supplemental Table II) around NG A249 and carried out further mapping analyses. In 1,474 F2 mutant progenies, we observed 2, 1, and 0 recombination events between the hpa1 locus and M322, M331, and M326, respectively (Fig. 3A). We then cloned (by reverse transcription [RT]-PCR or genomic PCR) and sequenced all the coding regions for the predicted 22 genes between markers M322 and M331. Sequencing analysis revealed only one mutation (G-to-A base substitution), which is in the At5g10330 gene and results in an amino acid change from Ala-69 (GCT) to Thr-69 (ACT; Fig. 3B). At5g10330 encodes a putative HPA with 417 amino acids. The Ala residue affected by the hpa1 mutation is conserved in several organisms, including Arabidopsis, rice (Oryza sativa), and the alga Prototheca wickerhamii (Fig. 3B), but not in tobacco.
(Nicotiana tabacum; where a Ser residue occupies this position). In the Arabidopsis genome, there are two HPA genes, AtHPA1 (At5g10330) and AtHPA2 (At1g71920), and the positions of the two mutant alleles in AtHPA1. The numbers above or below the AtHPA1 scheme indicate the number of nucleotides in the exons or introns, respectively. The two genes share 99.1% identities in the transcribed regions at the genomic sequence level, but have very little sequence similarity in the 5' upstream regions except for a 187-bp fragment immediately adjacent to the transcribed regions. The T-DNA insertion in emb2196 and the point mutation in hpa1 are both in the second exon of AtHPA1.

The hpa1 Mutant Phenotypes Can Be Rescued by Either Constitutive Expression of the Wild-Type AtHPA1 or Exogenous His Supplementation

We carried out several experiments to check whether the root meristem defect of hpa1 is linked to the mutation in AtHPA1 and His biosynthesis. First, we expressed the AtHPA1 cDNA under the 35S promoter in hpa1 and found that root growth in the resultant transgenic lines (subsequently referred to as hpa1/35S-AtHPA1) was partially restored (to about 70% of that of the wild-type seedlings; Fig. 4A). We confirmed that this partial restoration in root growth is linked to the presence of the transgene (Fig. 4A, insert). Second, we found that hpa1 seedlings grown on media with added His no longer display the mutant phenotypes (Fig. 4B), including the root growth defect and the altered expression of cellular markers (Fig. 4, B, G, L, Q, and V), and that this rescuing effect of the exogenous His is concentration dependent (Supplemental Fig. 1). Within the concentration range (0.001–0.1 mM) tested, 0.01 mM His was optimal for the rescuing (the medium with 0.01 mM His is referred to as the His-containing medium hereafter).
results therefore establish a cause-effect relationship between the altered phenotypes in \textit{hpa1} and the mutation in \textit{AtHPA1}.

\textbf{AtHPA1 Encodes a Functional HPA Protein}

To establish whether \textit{AtHPA1} encodes a functional HPA protein, we transformed the \textit{E. coli} HPA (known as hisC in \textit{E. coli}) defective mutant \textit{UTH780} with the full-length \textit{AtHPA1} cDNA. Due to the lack of HPA activity, the \textit{UTH780} strain cannot grow on medium without His supplementation. However, the transformed \textit{UTH780} strain expressing the \textit{AtHPA1} cDNA grew on a minimal medium without His (Fig. 4, D and E). The ability of \textit{AtHPA1} to complement the \textit{E. coli} HPA defective mutant confirms that \textit{AtHPA1} encodes a functional HPA protein.

\textbf{The \textit{hpa1} Mutant Had a Reduced Level of Free His}

To establish the impact of the \textit{hpa1} mutation on His biosynthesis, we initially measured both the free and total His contents in 10-d-old \textit{hpa1} and Col wild-type seedlings. Although no significant difference was found between the mutant and the wild-type control in the total His content (in the hydrolyzed samples; Supplemental Table I), there was a clear reduction in free His content in the mutant compared with the wild-type control. The measurement also indicated that the reduction in concentration of His was similar...
in both shoots and roots (Table I). This observation prompted an extended measurement of the levels of 17 free amino acids (including His) in hpa1, hpa1/35S-AthHPA1, and Col wild-type seedlings (Table II). On the His-free medium, the concentration of free His in the mutant was about 30% lower than that in the wild-type seedlings throughout three different developmental time points (3, 7, and 21 DAG), indicating that the reduction is not age dependent. Importantly, the reduction in His content is tightly linked to the mutant phenotype, as the level of free His recovered in hpa1 seedlings when the mutant phenotypes (including the root growth defect and the altered expression of cell patterning markers) were rescued by either AthHPA1 constitutive expression or His supplementation (Table II). The close correlation between the mutant phenotypes and the reduced His level strongly indicates that the mutant phenotypes are linked to the reduction in free His content.

We also noticed changes in other amino acids in the mutant. For example, the levels of free Asp, Lys, Arg, and Glu were also reduced in the mutant. The reduction of Asp and Lys occurred across the three different developmental time points examined, while that of Arg and Glu occurred only at the two early time points (Table II). However, the reduction of Glu, Lys, and Arg in the mutant did not correlate with the mutant phenotypes, as it persisted in hpa1 seedlings no longer displaying the mutant phenotypes, such as hpa1/35S-AthHPA1 seedlings or hpa1 seedlings grown on the His-containing medium. Consistent with this conclusion, supplementation of these amino acids (Glu, Lys, and Arg) in the growth medium did not rescue the root growth defect of hpa1 seedlings (Supplemental Fig. 2). The reduced level of Asp appeared to be correlated with the mutant phenotype (i.e. it was reduced in the mutant seedlings and restored to the wild-type level when the mutation was either complemented by constitutive AthHPA1 expression or rescued by a supplementation of His in the medium). However, as in the case of Glu, Lys, and Arg, addition of Asp to the growth medium also failed to rescue the mutant phenotypes (Supplemental Fig. 2), indicating that the change in this amino acid is not linked to the mutant phenotype. In fact, the addition of a combination of the above-mentioned four amino acids to the medium also failed to rescue the mutant phenotype (Supplemental Fig. 2).

His Dependency of hpa1 Roots Is Age Related

It has been recently reported that in Arabidopsis a proper balance of His is particularly important at early developmental stages (Noutoshi et al., 2005). To investigate whether the dependency of hpa1 roots on His is also age related, we grew hpa1 seedlings first on the His-containing medium (with 0.01 mM His) and then transferred them to the His-free medium at different dates (1, 4, 7, and 15 DAG). The growth of the primary roots in the subsequent 5 d following the transfer from the His-containing to the His-free medium was measured (Fig. 4C). Seedlings transferred after 1 d on the His-containing medium had similar root growth as those grown on the His-free medium from the start. When the seedlings were transferred between 4 and 7 DAG, their primary root growth was about one-half that of wild-type seedlings. When the seedlings were transferred after 15 d on the His-containing medium, the growth rate of the mutant primary roots was very similar to that of the wild-type roots. In fact, hpa1 plants that stayed on the His-containing medium for the first 15 d maintained similar root growth to the wild-type plants after several weeks on the His-free medium (Supplemental Fig. 3). These observations indicate that younger hpa1 seedlings are more dependent on His addition than older ones for maintaining root growth.

Expression of the HPA Transcripts in the Root Meristem Is Not Lower Than in Other Parts of the Plant

One possible explanation of the specific impact of the hpa1 mutation on roots is that root tips may have a lower expression of the HPA genes than other parts of the plants and are therefore more sensitive to defects in HPA activity. To check whether this is true, we examined the level of the HPA transcripts (due to the high sequence identity of the two HPA genes, we were unable to design gene-specific markers) in several different organs/tissues and also in different regions of roots in the wild-type plants. The results showed that the level of the HPA transcripts in the root tip region was not lower than that in other parts of roots or the aboveground organs/tissues (Fig. 5), indicating that the special sensitivity of the root meristem to the hpa1 mutation is unlikely to be due to a lower expression of the HPA transcripts.

Complete Knockout of AthHPA1 Is Embryo Lethal

The moderate impact of the hpa1 mutation on His content raises a question about the relative contribution

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**Figure 5.** Expression of HPA transcripts in different organs/tissues and different parts of roots. Leaves or basal or middle or tip segments of roots (see “Materials and Methods” for details) were from 5-DAG seedlings, and the stems, flowers, and siliques were from 4-week-old plants. The analyses were carried out using real-time RT-PCR. The value is the relative expression fold of HPA between samples; the lowest expression of the siliques sample was set as a basis (unit 1) for the fold calculation.
of AtHPA1 in His biosynthesis and the impact of the hpa1 mutation on the function of the protein. We noticed, on the National Center for Biotechnology Information Web site, the existence of an uncharacterized T-DNA insertional mutant, emb2196 (embryo defective 2196), in AtHPA1 in the Meinke’s embryo defective mutant collection (http://www.seedgenes.org/). According to the description on the Web site, the homozygous emb2196 mutant is embryo lethal and carries a mutation in the second exon about 90 bp downstream of the ATG start codon (Fig. 3C). To confirm that the insertion in emb2196 was indeed in AtHPA1, we carried out PCR amplification with (HPA) gene-specific forward primers against the 5’ upstream regions of the two HPA genes and a reverse primer specifically against the T-DNA insert. Amplification of a band of the correct size was obtained only when the AtHPA1-specific forward primer was used (Fig. 6C; in combination with the T-DNA-specific reverse primer). When the AtHPA2-specific primer was used (Fig. 6C), we did not obtain any amplification. This result confirms that the insertion in emb2196 is in AtHPA1. We then screened a segregating plant population from a heterozygous emb2196 plant (homozygous mutant plants could not be obtained due to embryo lethality) using the AtHPA1-specific forward and the T-DNA-specific reverse primers and confirmed that the T-DNA insertion cosegregates with the embryo-lethal phenotype (Fig. 6D). The results therefore demonstrate that AtHPA1 makes a vital contribution to HPA activity and His biosynthesis in Arabidopsis and also indicate that the mutated HPA protein in hpa1 is not functionally inactive and must have some residual activities that are sufficient to maintain the vitality of the hpa1 embryo/plants.

DISCUSSION

The hpa1 Mutant Is a Novel Plant His-Deficient Mutant and Is Defective in Root Meristem Maintenance

In this article, we describe the characterization of a novel Arabidopsis mutant in the His biosynthesis pathway, and we establish that the mutant carries a mutation in one of the two HPA genes (Fig. 3B). To our knowledge, hpa1 is the first plant mutant with reduced His content. Another Arabidopsis mutant of the His biosynthetic pathway, apg10, has recently been characterized (Noutoshi et al., 2005). This mutant carries a mutation in a putative BBMII isomerase-encoding gene (only one copy in Arabidopsis) and displays an albino and pale-green cotyledon and true leaf phenotype at young seedling stages. As in hpa1, the mutant phenotypes in apg10 can be rescued by exogenously supplied His. Surprisingly, apg10 seedlings were found to contain 6 times more free His than the wild-type counterpart. It was suggested by the authors that the increased His level in apg10 was probably the result of a cross-pathway compensatory mechanism (Noutoshi et al., 2005). Interestingly, such a cross-pathway compensatory mechanism, if it exists at all, did not overcome the impact of the hpa1 mutation on His biosynthesis, let alone cause the several fold increase in His content as observed in apg10.

At the morphological level, the hpa1 mutant clearly has difficulties in sustaining root growth (Fig. 1). Our results show that the root growth defect is associated with a wide range of alterations in cellular patterning in the root meristematic region (Fig. 2). Because some of the alterations occurred in markers that are known to be linked to meristem functions or maintenance, such as the auxin maximum and QC identity, it would suggest that the root growth defect in the mutant is linked to some impairments in the mechanisms involved in root meristem maintenance.

The hpa1 Seedlings Do Not Show Any Symptoms Previously Associated with Severe His Starvation

It has been well established that His is essential for plant survival and that a complete blocking of His biosynthesis is lethal (Guyer et al., 1995; El Malki and Jacobs, 2001). Our confirmation that the embryo-lethal phenotype in emb2196 is caused by a T-DNA insertion in the AtHPA1 gene also reinforces this view. The nonlethal nature of hpa1 plants, together with the amino acid measurement, indicates that hpa1 does not suffer from a significant His starvation. Such interpretation is supported by several other observations. First, the hpa1 mutant plants had a similar total His concentration to the wild-type controls.
Table I. Free His concentration (mg/100g FW) in shoot and root of 10-DAG Arabidopsis plants grown on basic medium

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>3-d Wild Type</th>
<th>3-d hpa1</th>
<th>7-d Wild Type</th>
<th>7-d hpa1</th>
<th>7-d hpa1/35S-AtHPA1</th>
<th>7-d hpa1 on His</th>
<th>21-d Wild Type</th>
<th>21-d hpa1</th>
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<tbody>
<tr>
<td>Asp</td>
<td>35.84 ± 0.14</td>
<td>34.91 ± 0.03*</td>
<td>37.44 ± 0.46</td>
<td>35.34 ± 0.31*</td>
<td>35.62 ± 0.23</td>
<td>36.24 ± 0.49</td>
<td>33.47 ± 0.46</td>
<td>31.00 ± 0.98*</td>
</tr>
<tr>
<td>Thr</td>
<td>16.89 ± 0.19</td>
<td>15.2 ± 0.05*</td>
<td>16.45 ± 0.54</td>
<td>15.57 ± 0.35</td>
<td>15.47 ± 0.42</td>
<td>16.32 ± 0.48</td>
<td>16.81 ± 0.78</td>
<td>17.11 ± 0.02</td>
</tr>
<tr>
<td>Ser</td>
<td>7.22 ± 0.14</td>
<td>8.04 ± 0.08*</td>
<td>7.79 ± 0.02</td>
<td>8.24 ± 0.06*</td>
<td>7.98 ± 0.04</td>
<td>8.07 ± 0.06</td>
<td>6.78 ± 0.33</td>
<td>8.11 ± 0.14*</td>
</tr>
<tr>
<td>Glu</td>
<td>66.30 ± 0.42</td>
<td>58.75 ± 0.43*</td>
<td>66.32 ± 1.08</td>
<td>58.24 ± 0.91*</td>
<td>58.97 ± 0.11*</td>
<td>58.79 ± 0.13*</td>
<td>55.90 ± 1.70</td>
<td>51.99 ± 0.10</td>
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<tr>
<td>Gly</td>
<td>4.75 ± 0.23</td>
<td>4.65 ± 0.01</td>
<td>4.27 ± 0.28</td>
<td>4.30 ± 0.29</td>
<td>4.23 ± 0.03</td>
<td>4.12 ± 0.08</td>
<td>4.74 ± 0.38</td>
<td>4.07 ± 0.11</td>
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<td>Ala</td>
<td>18.97 ± 0.20</td>
<td>18.02 ± 0.03</td>
<td>19.63 ± 0.38</td>
<td>17.72 ± 0.73</td>
<td>19.03 ± 0.02</td>
<td>18.42 ± 0.24</td>
<td>18.79 ± 0.14</td>
<td>17.35 ± 0.02*</td>
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<tr>
<td>Cys</td>
<td>1.69 ± 0.02</td>
<td>1.52 ± 0.01</td>
<td>1.68 ± 0.08</td>
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<td>1.56 ± 0.08</td>
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<td>1.61 ± 0.05</td>
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<td>Val</td>
<td>7.03 ± 0.10</td>
<td>6.31 ± 0.01</td>
<td>6.64 ± 0.34</td>
<td>5.85 ± 0.16</td>
<td>5.96 ± 0.06</td>
<td>6.18 ± 0.05</td>
<td>6.12 ± 0.18</td>
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<td>Met</td>
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<td>1.15 ± 0.09</td>
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<td>1.01 ± 0.01</td>
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<td>0.90 ± 0.04</td>
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<tr>
<td>Ile</td>
<td>2.35 ± 0.12</td>
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<td>Tyr</td>
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<td>3.77 ± 0.19</td>
<td>3.89 ± 0.11</td>
<td>3.70 ± 0.30</td>
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<td>Phe</td>
<td>6.96 ± 0.09</td>
<td>7.37 ± 0.04</td>
<td>7.57 ± 0.12</td>
<td>7.46 ± 0.1</td>
<td>7.21 ± 0.10</td>
<td>7.37 ± 0.01</td>
<td>8.28 ± 0.06</td>
<td>8.14 ± 0.01</td>
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<td>Lys</td>
<td>3.36 ± 0.08</td>
<td>2.75 ± 0.05*</td>
<td>3.53 ± 0.08</td>
<td>2.95 ± 0.08*</td>
<td>3.06 ± 0.13</td>
<td>2.89 ± 0.15*</td>
<td>3.05 ± 0.02</td>
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</tr>
<tr>
<td>His</td>
<td>1.15 ± 0.01</td>
<td>0.77 ± 0.03*</td>
<td>1.11 ± 0.03</td>
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<td>1.02 ± 0.04</td>
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<td>Arg</td>
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<td>Pro</td>
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<td>15.50 ± 0.55</td>
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<td>16.21 ± 0.29</td>
<td>15.32 ± 0.12</td>
<td>16.77 ± 0.17*</td>
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</table>

Values are the mean ± SD of three independent samples. *, Values significantly (P < 0.01) different from the control of the same age by t test.

Table II. Free amino acid concentration (mg/100g FW) in hpa1, hpa1/35S-AtHPA1, and wild-type plants

Values are the mean and standard deviation of three independent samples. *, Values significantly (P < 0.01) different from the control of the same age by t test.

( Supplemental Table I), indicating that the mutant does not suffer from a serious His shortage. Considering that free His makes up only about 2% of the total His concentration of plants (Tables I and II; Supplemental Table I), it is not surprising that the observed change in free His has no significant impact on the overall His supply and does not cause any obvious starvation response. Second, the mutant does not show any significant growth retardation in the aerial parts. This is in sharp contrast with previous observations in the tobacco His auxotrophic his1 mutant (El Malki and Jacobs, 2001) or in plants treated with herbicides that specifically block His biosynthesis (Guyer et al., 1995). In both cases (the tobacco his1 mutant and herbicide-treated plants), His starvation caused an overall growth inhibition (Guyer et al., 1995; El Malki and Jacobs, 2001). The necessity of His supply for plant survival is also demonstrated by the lethal phenotypes of the knockout mutants of the APG10 (Noutoshi et al., 2005) and AtHPA1 (Fig. 6) genes. If hpa1 suffers from His starvation, we would expect a more global impact on growth rather than the observed localized specific effect. Third, it has been reported that His starvation usually leads to an increased accumulation of other amino acids. For example, Guyer et al. (1995) observed that Arabidopsis plants had enhanced levels of many amino acids, including Ala, Asp, Glu, Phe, Pro, Thr, Try, Tyr, and Val, following a treatment with the herbicide IRL1803, which specifically inhibits the activity of IGPD, the enzyme catalyzing the seventh step of the His biosynthetic pathway (Guyer et al., 1995). However, in hpa1 there was no such global increase in amino acid accumulation. In fact, quite a few amino acids were actually reduced in amount (Asp, Glu, Thr, and Val; Table II). Since hpa1 did not display any of the previously known symptoms of His starvation, it would seem that mutant phenotypes are an unlikely part of a general (His) starvation response. The sharp contrast between the relatively normal and healthy hpa1 plants and the lethal phenotype of the knockout mutants in both AtHPA1 and APG10 genes also supports such a view.

The Phenotype of hpa1 Is Caused by a Moderate Reduction in Free His Concentration and Reveals an Important Role of His Homeostasis in Plants

Our results show that hpa1 seedlings had reduced free His content across different stages of seedling development and that this reduction in free His concentration is tightly linked with the mutant phenotypes. The reduction occurred when the mutant displayed the altered phenotypes and disappeared when the mutant phenotypes were either complemented by AtHPA1 overexpression or were rescued by exogenous His supplementation. Interestingly, hpa1 seedlings grown on the His-containing medium also maintained their free His at a similar level as that in the wild type, suggesting that there is an endogenous mechanism to keep His concentration at a certain level.

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Coincidentally, of the different amino acids, the concentration of free His is the lowest in Arabidopsis plants and is maintained at a constant level in both roots and shoots (Table I) and throughout different developmental stages (Table II). Such tight control of free His content may reflect the importance of maintaining this amino acid at the appropriate level.

**A Regulatory Role for His Homeostasis?**

Interestingly, both *apg10* and *hpa1* show some degree of age-related His dependency. In *apg10*, the mutant phenotype only occurred at the early seedling stage (Noutoshi et al., 2005). Similarly, the dependency of *hpa1* on exogenous His supply also appears to be age dependent. Young seedlings are more dependent on exogenous His supply than the older ones for maintaining root growth (Fig. 4C; Supplemental Fig. 3). Since free His content did not vary significantly in both *hpa1* and wild-type seedlings across different developmental stages, the observed difference in His dependency between different aged *hpa1* seedlings is more likely due to differential sensitivities to His.

**His Dependency in Arabidopsis Is Age Dependent**

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**A Regulatory Role for His Homeostasis?**

Our results have established a link between His homeostasis and root meristem maintenance and illustrates the importance of maintaining the right His content in plants. Interestingly, observations made on the *apg10* mutant also support a role for His homeostasis in plant development as it was reported that the albino and pale-green cotyledon and leaf phenotype of the mutant was linked to an increase in His content (Noutoshi et al., 2005). Although the two mutations (*hpa1* and *apg10*) have different impacts on His concentration and development (which may reflect the differential roles of His homeostasis at different levels), they both reveal a function of His homeostasis. The next challenge is to establish how such regulation takes place.

As discussed above, the root meristem failure in *hpa1* is unlikely to be part of a global His starvation response. Theoretically, there are at least two possible explanations as to why the root meristem fails in the mutant. The first one is that the root meristem may suffer from a localized His shortage. Our current experimental data do not support this argument. For example, the level of free His and also the impact of the *hpa1* mutation on the His content seem very constant and uniform within the plant and throughout different developmental stages, indicating that either the biosynthetic capacity is very evenly balanced within a plant and across developmental stages or that some balancing mechanisms may exist to ensure a uniform distribution of this amino acid. In either scenario, a localized starvation could not be established easily. In addition, the lethal phenotype of *emb2196* indicates that *AHPA1* is vital for the survival of the whole plant, and our expressional analysis indicated that it is expressed fairly constitutively in different parts of wild-type plants; it is therefore difficult to imagine how the mutation in *AHPA1* could only have a very localized effect on His supply in the root meristem but not in other parts of the mutant plants. Nevertheless, it is not possible to completely rule out the possibility of a localized His starvation in the *hpa1* root meristems without a direct His measurement.

An alternative explanation of the cause of the root meristem failure in *hpa1* is that there are different sensitivities among the different parts, organs, tissues, or cells of plants and that root meristems are particularly sensitive to changes in His homeostasis, probably within a certain range. Such specific high sensitivity of the root meristem could lead to a localized developmental response, such as the root meristem failure. There is some evidence that differential sensivities to His homeostasis in plants do indeed occur at different developmental stages. For example, in both the *apg10* and *hpa1* mutants, His dependency changes at different ages, although the level of His content appears constant. Further investigation is needed to establish the true mechanisms of the root meristem defect in *hpa1*, and this may open the door to His signaling mechanisms in plants.

**MATERIALS AND METHODS**

**Marker Lines**

The DR5-GUS, SCR-GFP, and QC25 marker lines were kindly provided by Ben Scheres (Utrecht University, Netherlands). The enhancer trap lines J0631 and J0481 (generated by Jim Haseloff; http://www.plantsci.cam.ac.uk/~ Haseloff) were obtained through the Nottingham stock center. The markers were introduced into *hpa1* by crossing, and morphological examinations were carried out in the F3 generation.

**Plant Material and Growth Conditions**

Wild-type and mutant seeds were surface sterilized with 10% commercial bleach in 100% ethanol for 10 min, followed by several washes with 100% ethanol. The seeds were then dried in a laminar flowhood for 20 to 30 min. All the media used in this study for seed germination and seedling growth were based on a basic medium containing the following ingredients: 200 μM KCl; 80 μM MgSO4; 40 μM CaCl2; 44 μM NaH2PO4; 2.3 μM MnSO4; 1 mM KNO3; 0.18 μM KI; 2 μM H3BO3; 0.28 μM ZnSO4; 4 μM CuSO4; 4 μM Na2MoO4; 4 μM CoCl2; 4 μM
Fe-EDTA; 0.5 g L⁻¹ MES, pH 5.7, 1% agar-agar; and 0.5% Suc. For the His-containing medium, His is added before autoclaving. Plates were sealed with paraffilm and placed vertically (to allow root growth along the surface of agar) in a growth cabinet (Percival Scientific), with a 15-h-light/9-h-dark cycle, 300 μmol m⁻² s⁻¹ photon flux density, and at a constant temperature of 20°C. For the 21-d-old seedlings used for amino acid measurement, seedlings were ground in liquid nitrogen. Free amino acids were extracted from leaves of 4-week-old plants grown on soil in a growth room at 22°C; and leaves, root tips (the first 0.4 cm from the root tip), the middle (the middle section between 0.4-cm root tip and 1-cm basal root segment), and basal root sections (1-cm root from the stem junction) from 5-DAG seedlings. First-strand cDNA was synthesized from total RNA using a commercial cDNA synthesis kit (Invitrogen) and stored at −20°C before use. The primers used in RT-PCR analyses were as follows: PLA forward 5'-TGATCATGAGATCGGTTTAGAT-3', PLA reverse 5'-TTTTGCGGAAATTTGAA-3', ACTIN forward 5'- CCTCT-ATGGCGTGTCGTA-3', and ACTIN reverse 5'- CCTAGAGAAACGG- GAGT-3'. Quantitative real-time PCR was performed with an ABI Prism 7000 sequence detection system (Applied Biosystems) using the SYBR green master mix (Applied Biosystems). Each 20-μL reaction contained 2× SYBR green master mix, 2 μL of cDNA, and 0.1 μL of forward and reverse primers (20 μM). The PCR conditions were set as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 15 s, 58°C for 20 s, and 72°C for 30 s. Fluorescence data were collected during the 72°C step and were analyzed with Sequence Detector version 1.7 software (Applied Biosystems). The quantity of cDNA was calculated from the threshold cycle by interpolation from the standard curve. The amounts of cDNA template in each sample were normalized using the amplification of ACTIN primers.

Characterization of emb2196

To establish which of the HPA genes the insertion in emb2196 was located in, we used the following gene-specific forward primers, 5'-TCCCAATTAGGCTTACCTGCT-3' (At5g10300 specific) and 5'-AAACTGTAGTTAG-TATCCTGG-3' (At1g71920 specific), together with a T-DNA-specific reverse primer 5'-TCCCCAAATACCAATACATTACACT-3', and carried out PCR amplification using genomic DNA from a heterozygous emb2196 plant. The analysis confirmed that the insertion was in ATHPA1. To establish whether the embryo-lethal phenotype cosegregated with the T-DNA insertion, we first obtained a segregating population from a heterozygous (emb2196/EMB2196) plant and assessed the individuals of the population for the production of lethal embryos at the flowering stage to establish whether they were wild type or heterozygous with regard to the embryo-lethal allele. We then extracted genomic DNA from selected wild-type and heterozygous plants and carried out genome-typing analysis using the ATHPA1-specific forward and T-DNA-specific reverse primers to check whether the embryo-lethal phenotype cosegregated with the T-DNA insert.

Free Amino Acid Content Measurement

For measuring amino acid contents, samples (600 mg in fresh weight) of the wild-type and hpa1 seedlings were ground in liquid nitrogen. Free amino acids were extracted with 80% ethanol for 30 min. The extract was filtered into an evaporating dish, concentrated by evaporation at 60°C, and resuspended in 5 mL of citric acid and 1 mL of sulfosalicylic acid. The solution was filtered through Whatman 3MM filter paper and centrifuged at 10,000g for 10 min. The supernatant was analyzed by a Hitachi amino acid analyzer (835-50) for amino acid contents.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number AF1127255.

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LITERATURE CITED


