

Functional genomics study in *Arabidopsis thaliana* of histidine biosynthesis

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Abstract

This study was aimed at analyzing the function of a gene thought to be required for histidine biosynthesis in the vascular plant *Arabidopsis thaliana*. A DNA insertion mutant of locus At2g36230 was isolated. Based on homology with the *Saccharomyces cerevisiae* His6 gene, At2g36230 was predicted to encode phosphoribosylformimino-5-aminoimidazole carboximide ribotide isomerase. Individual plants heterozygous for the *AtHis6::TDNA* insertion allele produced siliques in which approximately 25% of the seeds were aborted during early embryo development. The vegetative phenotype of the heterozygous plants was normal. When histidine was applied exogenously to the heterozygous plants the frequency of embryo-abortion was reduced to approximately 5%. The fact that the homozygous mutant seeds fail to grow while the maternal plant thrives suggests that histidine, unlike other amino acids, cannot be transported from the maternal plant to developing embryos.

Introduction

Arabidopsis thaliana was the first plant to have its genome sequenced and many of its genes have since been identified by modeling (Huala et al., 2001). Gene modeling is used to guide experiments leading to assignment of gene function. Currently a large effort is underway called the NSF 2010 project to identify the function of over 25,000 genes identified in the Arabidopsis genome. One project in the 2010 program is to isolate mutants in each of the annotated genes by identifying alleles carrying an inserted TDNA derived from *Agrobacterium tumefaciens* (Alonso et al., 2003). These mutants can be used to study gene function by assessing the phenotype of null mutations.

This study focused on a gene proposed to function in histidine biosynthesis. Very little is known about the functions of histidine or the biosynthesis pathway in plants (Miflin, 1980). The histidine pathway in plants is inferred primarily from the identification of genes from *Arabidopsis* that are homologous to either *Escherichia coli* or *Saccharomyces cerevisiae* genes for histidine biosynthesis (Jones and Fink, 1982; Winkler, 1996). Based on this analysis the common pathway for histidine biosynthesis in Arabidopsis and microorganisms is depicted in Fig 1.

Histidine is formed through a series of 10 reactions beginning with ATP and phosphoribosylpyrophosphate (PRPP). Histidine is unique among the proteogenic amino acids in that it is not derived directly from glycolysis or TCA cycle intermediates. Rather it is closely related to purine and pyrimidine metabolism. One of the initial substrates of histidine biosynthesis, PRPP is also the substrate for both purine and pyrimidine biosynthesis. The other initial substrate, ATP, is the end product of a branch of the pyrimidine pathway. Both PRPP and ATP are used in the first reaction catalyzed by

phosphoribosyltransferase. After the HIS7 (HisHF) step phosphoribosylformimino-5-aminoimidazole-carboxamide ribotide is cleaved releasing imidazole glycerolphosphate, which is converted in 4 more steps to histidine and 5-aminoimidazole-4-carboxamide ribotide, which is used to regenerate ATP. In bacteria the metabolic link between histidine and ATP synthesis was implicated by the finding that bacterial mutants before *hisHF* are either adenine auxotrophs or have severely reduced ATP pools (Winkler, 1996), suggesting that consumption of ATP for histidine synthesis without recycling of 5-aminoimidazole-4-carboxamide ribotide depletes the ATP pool. Whether such a link exists in plants has yet to be examined.

Several steps for histidine biosynthesis in *Arabidopsis* are encoded by single genes. This situation is valuable because mutation of any single gene is more likely to produce a phenotypic change than would be the case if the step were encoded instead by a family of genes with functional redundancy. The gene that was the focus of this study was At2g36230, which may encode phosphoribosylformimino-5-aminoimidazole carboxamide ribotide isomerase, a homolog of *hisA* in *E. coli* and *His6* in yeast. The cDNA derived from At2g36230 was previously cloned based on its ability to functionally complement an *E. coli hisA* mutant (Fugimori et al., 1998). Interestingly, the coding sequence of At2g36230 shows strong homology with *His6*, but none with *hisA*. Based on its homology with yeast *His6* At2g36230 is referred to hereafter as AtHis6.

Methodology

An *Arabidopsis* line designated SALK_041416 isolated by the SIGnAL Project (Alonso et al., 2003) was obtained from the *Arabidopsis* Biological Resource Center (ABRC, Ohio State University), carrying a T-DNA insertion in exon two of AtHis6. Seeds from the line were germinated in Promix potting soil and the plants were grown in a growth chamber on a 24 hour diurnal cycle, 16 hours of light at 24°C and 8 hours of dark at 22°C. The light intensity was 100 $\mu\text{E m}^{-2} \text{s}^{-1}$. Genomic DNA was extracted from the leaves of 25 day old plants and Polymerase Chain Reaction (PCR) was carried out using DNA primers designed to amplify the T-DNA insertion region of the AtHis6. The primers were 041416RP, 5'-TGGTGGTCTCACTTGGTTCCAG-3'; 041416LP, 5'-CTTCCATCCTGCGCAACAAAA-3'; and LBB1, 5'-GCGTGGACCGCTTGCTGCAACT-3'. The PCR conditions were one cycle at 94°C for 5 minutes, 32 cycles at 94°C for 15 seconds, 59°C for 30 seconds and 72°C for 1 minute.

Prediction of signal sequences was performed using TargetP (<http://www.cbs.dtu.dk/services/TargetP/>). ClustalW was used to align protein sequences and MEGA was used to draw the phylogenetic tree.

The phenotype of heterozygous individuals was visually assessed. Embryo rescue was carried out by applying a 10 mM solution of histidine to the roots and leaves of heterozygous *AtHis6::TDNA* plants, and determining whether the frequency of aborted embryos was reduced.

Microscopic imaging of *Arabidopsis* embryos was carried out by removing immature seeds from a young silique, placing them on a microscope slide, and clearing the embryos with Hoyer's solution (25% w/v gum arabic, 333% w/v chloral hydrate, and 16% v/v glycerol) as described in (Meinke, 1994). The embryos were incubated overnight and then imaged with a compound microscope equipped with Nomarski optics and located at the Neurosciences Imaging Center at Rutgers University.

Results

Characteristics of AtHis6

The AtHis6 gene is composed of 7 exons producing a 1002 bp coding sequence. The gene model is presented in Figure 2 with exons shown in upper case black type and untranslated regions shown in lower case grey letters. The protein derived from AtHis6 is 304 amino acids with a predicted mass of 33364 Da. TargetP analysis predicted with nearly equal probability that the protein is localized either to chloroplasts or mitochondria. The sequence of AtHis6 protein is much more closely related with His6 from yeast than it is to *hisA* from *E. coli* or *Synechocystis* sp. (Fig 3). The evolutionary implications of the sequence homology is not clear, especially since cyanobacteria are thought to be the evolutionary ancestor of chloroplasts.

Mutation of AtHis6 results in embryo abortion

The PCR strategy for analysis of the AtHis6 mutant line is depicted in Figure 4. Primers RP and LP were designed to amplify an 890 bp segment from the wild type AtHis6 allele, whereas primers RP and LBb1 were designed to amplify a 440 bp segment of the *AtHis6::TDNA* allele. When both alleles are present, one on each homologous chromosome, both PCR products would be obtained, but if only the wild type allele or mutant allele were present only the 890 bp or 440 bp fragment would be obtained. Figure 4 also presents a gel electrophoresis analysis of 4 plants showing the heterozygous PCR pattern and 4 plants showing the wild type segregation pattern. Sequence analysis of the 440 bp PCR product confirmed that the TDNA is inserted in exon 2 at the position indicated in Fig 2.

Of 58 individuals derived from a heterozygous parent, 22 showed the wild type pattern and 36 showed the heterozygous pattern, whereas no individuals were identified showing the homozygous mutant pattern. In total, the results give a ratio of heterozygous:wild type of 1.6:1, close to that predicted for a homozygous lethal mutation, which would be predicted to give a ratio of 2:1.

The phenotype of AtHis6 heterozygotes was indistinguishable from the wild type. However, the heterozygous individuals produced a high percentage of aborted seeds, whereas the wild type segregants did not (Figure 5).

When seeds of several heterozygotes were counted, approximately $25\pm 6\%$ of the seeds were aborted (Table 1). Assuming that the TDNA allele of AtHis6 is recessive and leads to embryo abortion, one would expect it to segregate at a 3:1 ratio.

***AtHis6::TDNA* mutants are auxotrophic for histidine**

To test whether embryo abortion is due to histidine auxotrophy, heterozygous *AtHis6* plants were fed 10 mM histidine by applying the solution on the surface of leaves and onto the soil. This treatment reduced the frequency of embryo abortion to below 5% (Table 1).

A Chi squared test for this change yielded a $P=1.85$ with one degree of freedom indicating that histidine was able to rescue homozygous *AtHis6::TDNA* embryos. However, when plants were fed 1 mM histidine, the seeds were not rescued. The seeds that were aborted in 10 mM histidine-fed plants appeared larger than in the plants that were not fed histidine.

***AtHis6::TDNA* mutants abort at the pre-globular stage of embryo development**

Mutant embryos were observed microscopically and none that were observed progressed beyond the pre-globular stage of development (Figure 6). Mutant and wild type sibling embryos are contrasted in figure 6A (mutant) and B (wild-type). In addition, many mutant embryos showed an abnormal pattern of cell division in which some suspensor cells were observed to divide longitudinally to the axis of the suspensor (see cells in figure 6C). Normally, the division of suspensor cells occurs perpendicularly to the axis of the suspensor.

Discussion

The absence of growing homozygous *AtHis6::TDNA* plants in the population and the 3:1 segregation of alive to dead seeds in the siliques of heterozygous *AtHis6* plants suggest that *AtHis6* is an essential gene for *Arabidopsis* embryo development and that the *TDNA* allele of this gene is recessive to wild-type. Rescue of these seeds by histidine feeding suggests that *AtHis6* functions in the histidine biosynthesis pathway.

While addition of histidine to an *AtHis6* heterozygous plant rescues most of the embryos, there are still ~5% that aborted, nearly 80% more than in WT. These seeds aborted at a later stage of development than in the non-histidine-fed plants. Therefore, we hypothesize that a certain histidine concentration is important for embryo development, which cannot be efficiently supplied by application of histidine to the surfaces of the plant. This hypothesis is supported by the necessary increase in histidine concentration from 1 mM to 10 mM for embryo rescue.

In total the results show that a heterozygous *AtHis6* individual is able to synthesize sufficient histidine to support its own growth, yet is unable to supply adequate levels of histidine to support embryo development of its own *AtHis6::TDNA* progeny. There are several possible explanations. First, it is possible that a barrier exists that prevents transport of histidine from parent to progeny. This would imply that embryos are entirely autotrophic for histidine, an idea that runs counter to the common presumption that embryos are reliant on the parent plant for transportable metabolites such as amino acids (Bewely et al., 2000). The idea that embryos are autotrophic for histidine also is not supported by the finding that histidine transport proteins exist that are expressed in embryonic tissues (Chen and Bush, 1997; Frommer et al., 1994; 1995). Since the function of plant histidine transporters was not studied within a plant system, there is a possibility that they do not actually transport histidine within plants. Another possible explanation for the embryo lethality of *AtHis6* mutants is that the heterozygous parent may produce

sufficient histidine to support its own growth, but not enough additional histidine to support its progeny. Perhaps histidine is not produced in sufficient amounts in the specific tissue that are used to supply the embryos. A third possibility is that a block in the middle of the histidine pathway either causes the accumulation of a toxic intermediate that kills embryos, or that the mutation blocks recycling of adenine for ATP synthesis without blocking the usage of ATP at the first step in the pathway. The third hypothesis could be consistent with the complementation of the embryo lethal phenotype by histidine feeding because histidine biosynthesis is catalyzed by ATP phosphoribosyltransferase an enzyme that is known to be feedback-regulated by histidine in plants (Miflin, 1980). Therefore, histidine feeding could block ATP phosphoribosyltransferase preventing accumulation of the hypothetical toxic intermediate or depletion of ATP.

The phenotype of a histidine mutant of *Arabidopsis* is the first example suggesting that an amino acid cannot be supplied to embryos by the maternal plant. Since this idea runs counter to the common presumption that embryos are reliant on the parent plant for transportable metabolites such as amino acids, the present results indicate that more research is needed to characterize the relationship between parental and embryonic tissues with respect to amino acid nutrition.

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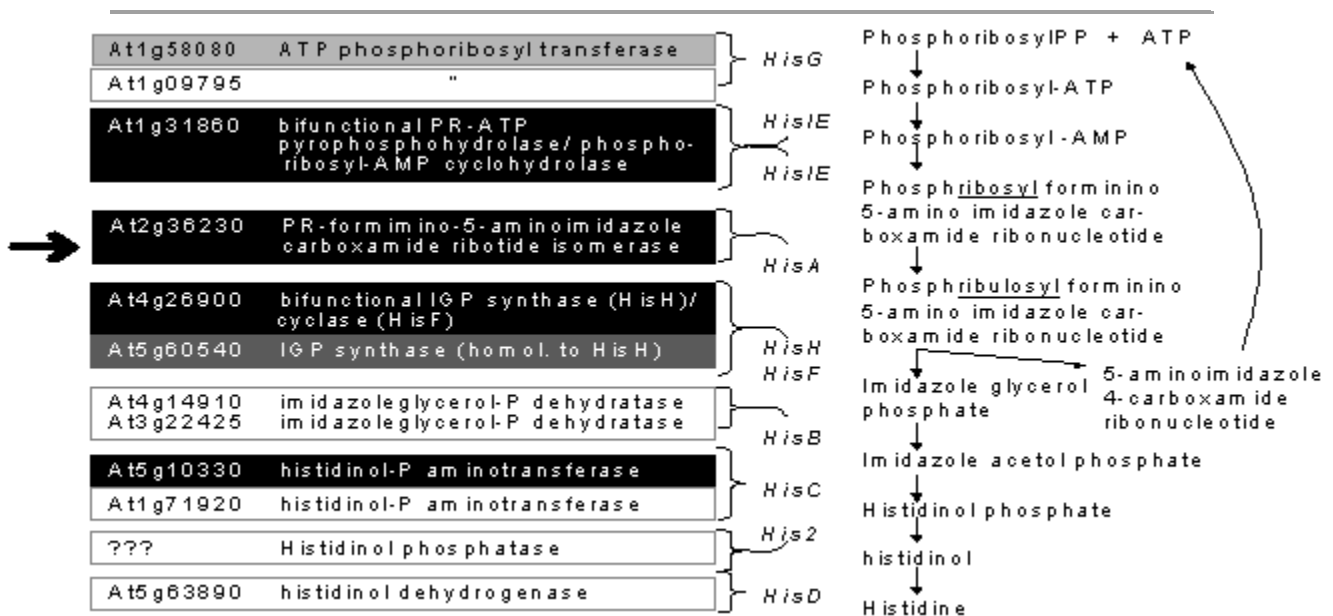


Figure 1: Supposed histidine biosynthesis pathway in *Arabidopsis*. The genes identified for each step in *Arabidopsis* are indicated on the left. The single gene encoding *AtHis6* (*hisA*) is indicated by the arrow on the left.

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1   ataaaccgat atctaaaccc tagaatatct agaaccagat acgtcactgg tgcttactga
61  ttacagtttc agttccaaag ATGAGAACTT TGAGCTCCCA ATTATACTCC AATGGTGGTC
121 TCACCTGGTT CCAGAAGAAG AATCAGAGCT CTCTGTTCAT CAAGCATCTC AGAGTCTCTA
181 AACCATCGAG GGTTCAGCTC ATATCTGCTG TTCAATTCCG TCCGTGCATC GATATTCATA
241 AGgtatgaaa tcaatttcac cgattagaaa atcctcactg gctgttgagt gcagtgaatt
301 catttatgga tctatctctg tgtgatgtga ttttcgatag GGAAAAGTGA AACAGATAGT

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TDNA insertion site for SALK_041416

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361 TGGATCCACA CTTCGTGACT TGAAGAGGA TGGTT▼CAGTT CTCGTTACGA
      ATTTTGAATC
421 GGATAAGTCT GCGGAAGAGT ATGCAAAGAT GTACAAAGAA GATGGGCTTA CGGGTGGTCA
481 TGTGATAATG CTTGGAGCAG ACCCTTTAAG CCAAGCTGCA GCTATTGGAG CATTACATGC
541 TTATCCTGgt atgtgttttag ttgaatgtca catggtgttt gttttatatt gtttgaattt
601 gaaatgtaaa gttttgatgt gttatatatg atacaagatg atgaattcta gGCGGTTTGC
661 AAGTTGGAGG TGGAAATCAAT TCTGAGAATT GCATGAGTTA TATTGAGGAG GGAGCAAGCC
721 ATGTCATTGT CACTTCGgta tagctgtcat cttagctttag tgaatatgta atcatagtaa
781 gtatagctga tgataattat gttttcgttt tgtatttcag TATGTATTTA ACAATGGAAA
841 GATTGATCTT GAAAAGACTCA AAGATATTGT GAGCATTGTT GGGAAACAAA GACTGATATT
901 GGACCTTAGC TGCAGAAAAG AGgtgattct ctgactagtt tcatcttcag tttaatgtta
961 ttggtcatga aaagaagatc ggcatttgag gtatttataat ggttattttg ttgctcagGA
1011 TGGAAGATAC GCAATTGTTA CTGATAGATG GCAGAAGTTC AGCGACGTC A TCCTTGACGA
1081 GAAATCATTG GAGTTTCTTG GAGGATTTTC AGATGAGTTT CTGGTGCATG GCGTAGACGT
1141 TGAAGGAAAAG AAgtaaagctt taaatatctc aacgctgtga ttttagtgct ttagagtaaa
1201 ttcgttttgc attgaaaata cagtatttag tcagtatgta ctgatcagtg gtgttaacac
1261 ttcttctctc aaagGCTAGG GATCGATGAA GAGCTTGTAG CATTGCTTGG CAACTACTCT
1321 CCGgtgagta ataacatgca gtttcttatg attcggaaact aatcctatgg acttaacgat
1381 aattaacaga acttcactct attcaaaatg cttgtgatgt tagtgaacc ggaattggat
1441 tagatgtagt cgttataact cacttttagac atagagataa gtcgattgc aaacttgttc
1501 atttgagctt gatcttaaat gttttttgac cttgacagAT TCCAGTAACT TATGCGGGAG
1561 GTGTGACGGT AATGGATGAT GTAGAGAGGA TCAAGGACGC AGGAAAAGGA CGTGTAGATG
1621 TAACAGTGGG AAGTGCCTTA GACATTTTCG GGGGAAATCT TCCATACAAA GACGTCGTCTG
1681 CTTGGCATCA CAAGCAGCAC TCACTCCATT GAatctgcca ttttcacctt tgctcatctt

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Figure 2: Structure of the AtHis6 gene and position of the Agrobacterium TDNA Insertion.

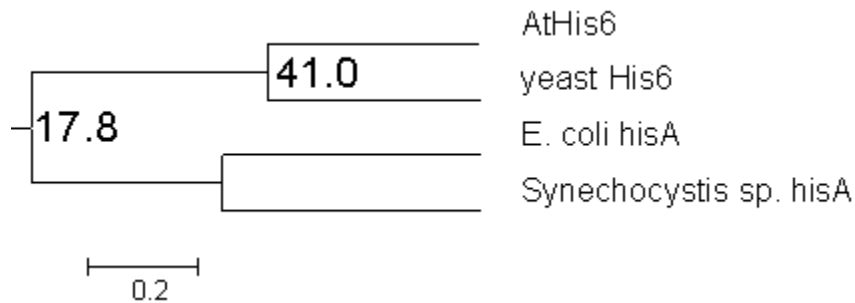


Figure 3: Alignment of His6 (*hisA*) protein sequences. The alignment was created with ClustalW and the tree was drawn with MEGA. The percent identity is given at the branches of the tree. The GenBank accession numbers for the sequences are AtHis6, NP_181165; yeast, NP_012244; E. coli, NP_310852; Synechocystis sp., NP_442856.

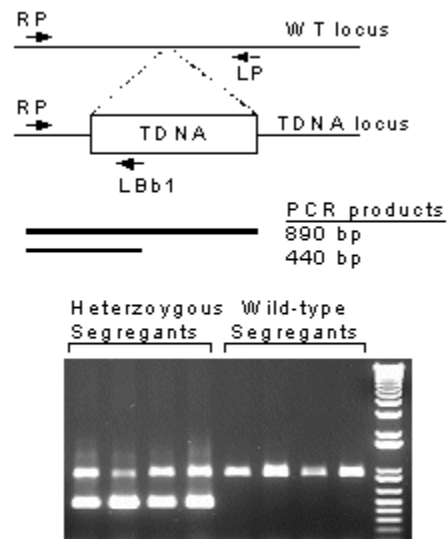


Figure 4: PCR Amplification of Wild Type and TDNA Insertion of His6 Loci. Top: Primers designed to amplify WT and TDNA insertion loci in His6, respectively. Bottom: Amplicons produced by the above primer scheme on an electrophoresis gel.



Figure 5: Aborted seeds in siliques from *AtHis6::TDNA* heterozygotes. A) A silique from a *AtHis6::TDNA* heterozygote (top) is compared with a silique from a wild type segregant (bottom). The arrows indicate several aborted seeds. B) a close-up image of an aborted seed (arrow).

Table 1: Histidine-Feeding Complementation of Aborted Seed Phenotype

Plant # 2	8	9	10	18**	20**	23	27	
Genotype via PCR	WT	WT	Het	Het	Het	Het	Het	Het
% seed abortion <1	<1	23.7	31.3	4.5	5.4	22.6	22.8	

**Histidine fed heterozygotes. All others were treated with water in place of the histidine solution. Wild Type plants were monitored as controls.

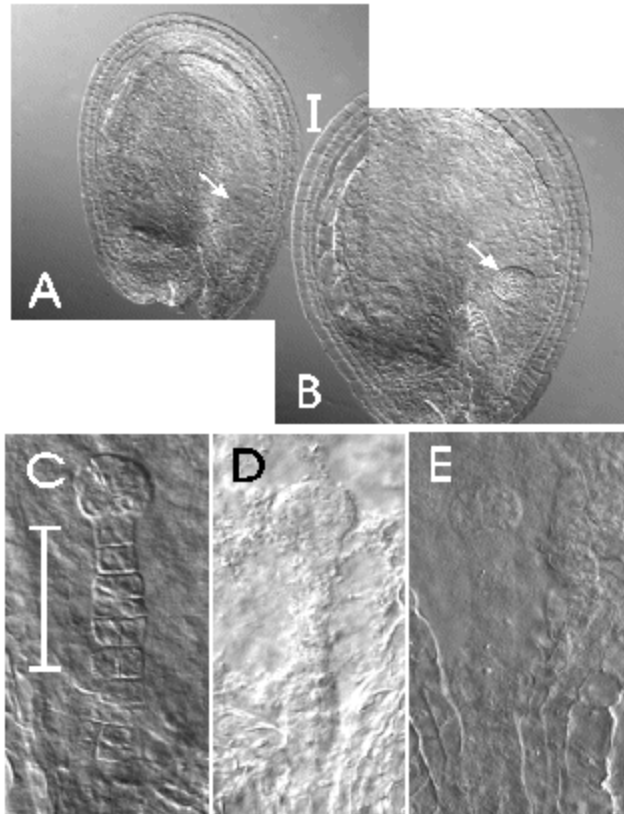


Figure 6: Microscopic observations of aborted *AtHis6::TDNA* embryos. An aborted embryo (A) compared with a normal embryo (B) at the late globular stage of development. C,D,E: Different examples of embryo abortion in the *AtHis6::TDNA* mutant.

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