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Functional Annotation, Transcriptional Characterization and Enzymatic Contributions of Essential Amino Acid Biosynthesis-Related Genes

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FUNCTIONAL ANNOTATION, TRANSCRIPTIONAL CHARACTERIZATION, AND ENZYMATIC CONTRIBUTIONS OF ESSENTIAL AMINO ACID BIOSYNTHESIS-RELATED GENES

by

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In plants, essential amino acid biosynthesis predominantly or exclusively occurs in the plastid. The plastid in the heterokont alga *Nannochloropsis oceanica* is surrounded by four membranes, which add great complexity to intracellular trafficking and communication. *N. oceanica* genes in essential amino acid biosynthesis were functionally annotated. The biosynthesis pathways resemble the pathways in *Arabidopsis thaliana*, but the gene content seems to be simpler in *N. oceanica*.

In addition, two *A. thaliana* mutants with loss-of-function mutations in the *aspartate kinase-homoserine dehydrogenase 2* (*AK-HSDH2*) gene were characterized. These *ak-hsdh2* mutants demonstrate unexpected accumulation of aspartate-derived amino acids (ADAAs), particularly threonine, in leaves. Microarray analysis supports that other ADAA biosynthesis-related genes are not up- or down-regulated in the mutants. The AK and HSDH activities were measured in mutant and wild-type plants. AK-HSDH2 appears to be an important contributor to the overall HSDH activity, but not a major contributor to the overall AK activity. The changes in the amino acid content over a range of leaf developmental stages were examined using mutants with loss-of-function mutations in *AK-HSDH2* and/or another *AK* or *HSDH* gene. The accumulation of ADAAs in the mutants is a dynamic process.

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CHAPTER I

BIOSYNTHESIS OF ESSENTIAL AMINO ACIDS IN *NANNOCHLOROPSIS OCEANICA*

Introduction

Overview of essential amino acids

Amino acids are building blocks of proteins and precursors of many secondary metabolites that are important to the growth, development, and reproduction of living organisms (Coruzzi and Last, 2000). Most bacteria, archaea, fungi, algae, and higher plants are capable of *de novo* amino acid biosynthesis. Unlike these organisms, humans and other animals cannot synthesize aspartate (Asp)-derived amino acids (Lys, lysine; Met, methionine; and Thr, threonine), aromatic amino acids (Phe, phenylalanine; Tyr, tyrosine; and Trp, tryptophan), and branched-chain amino acids (Ile, isoleucine; Leu, leucine; and Val, valine) themselves. Therefore, these nine amino acids are considered "essential" to human and animal nutrition. In plants, Asp-derived, aromatic, and branched-chain amino acids are predominantly or exclusively synthesized in the plastid (Jander and Joshi, 2009; Binder, 2010; Tzin and Galili, 2010).

Unique features of heterokont Nannochloropsis oceanica

As a heterokont, *Nannochloropsis* is considered to be the product of an ancient secondary endosymbiosis event: the engulfing of eukaryotic red alga by a heterotrophic eukaryote (McFadden, 2001). The plastid in *Nannochloropsis* is therefore surrounded by four membranes (Murakami and Hashimoto, 2009), which add great complexity to the trafficking and communication between the plastid and other subcellular compartments.

This prompted us to manually annotate *N. oceanica* genes involved in essential amino acid biosynthesis.

Methods

The biosynthetic pathways for Asp-derived, aromatic, and branched-chain amino acids in *N. oceanica* were manually annotated by adding predicted functions to a list of *N. oceanica* putative genes. Most of the *Arabidopsis thaliana* enzymes in these pathways are known. The protein sequence for each *A. thaliana* enzyme was obtained using the gene locus through The Arabidopsis Information Resource (TAIR, http://arabidopsis.org/), an online compilation of molecular and genetic data. This protein sequence was used to scan the *N. oceanica* genome via a BLAST (Basic Local Alignment Search Tool) search (https://benning-linux.bch.msu.edu/blast/blast_cs. html). The chromosomal location of close matches was recorded and used to find the *N. oceanica* cDNA sequence (https://benning-linux.bch.msu.edu/cgi-bin/gb2/gbrowse/Nanno/). The cDNA was translated (http://www.expasy.org/) and checked for protein domains (http://pfam.sanger.ac.uk/) to confirm expected function. Lastly, as a validation step, the protein sequence for the *N. oceanica* gene was used in a "reverse" BLAST analysis to search for homologous *A. thaliana* genes (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Results

Annotation of Asp-derived amino acid biosynthesis-related genes

Like most bacteria, archaea, fungi, higher plants, and other algae, *N. oceanica* synthesizes Lys, Met, and Thr from Asp (Jander and Joshi, 2009). There are two branch-

point metabolites in the pathway: Asp semialdehyde and homoserine 4-phosphate (Figure 1.1). Asp semialdehyde is the substrate for branch-point enzymes dihydrodipicolinate synthase (DHDPS) and homoserine dehydrogenase (HSDH). These two enzymes control the flux between the Lys branch and the Met and Thr branch. Homoserine 4-phosphate is the substrate for branch-point enzymes cystathionine gamma synthase (CGS) and Thr synthase (TS). These two enzymes control the metabolic flow between the Met branch and the Thr branch. In *A. thaliana*, there are three mono-functional Asp kinases (AKs), one mono-functional HSDH, and two dual-functional AK-HSDHs (Figure 1.1).

Based on BLAST similarity, PFAM domain support, and reverse BLAST analysis, we predict that *N. oceanica* has one AK, one HSDH, and one AK-HSDH (Figure 1.1 and Appendix A). One interesting difference between *N. oceanica* and *A. thaliana* is that *N. oceanica* has two cobalamin-independent Met synthases (MS) and one cobalamin-dependent Met synthase (MetH); *A. thaliana* has three cobalamin-independent Met synthases, but no cobalamin-dependent Met synthase (Figure 1.1 and Appendix A).

It should be noted that in the *N. oceanica* genome sequence database, the putative *dihydrodipicolinate reductase* (*DHDPR*) and *diaminopimelate epimerase* (*DAPE*) genes, and the two *Thr synthase* (*TS*) genes appear to be partial (Figure 1.1 and Appendix A). The *DHDPR* and *DAPE* genes and one *TS* gene are missing the 5'-ends of the coding regions; the other *TS* gene does not have the 3'-end of the coding region (Appendix A). In *N. oceanica*, there are five putative genes encoding CGS or cystathionine beta lyase (CBL) (Figure 1.1 and Appendix A). Overall, *N. oceanica* appears to have all the genes for Asp-derived amino acid biosynthesis.

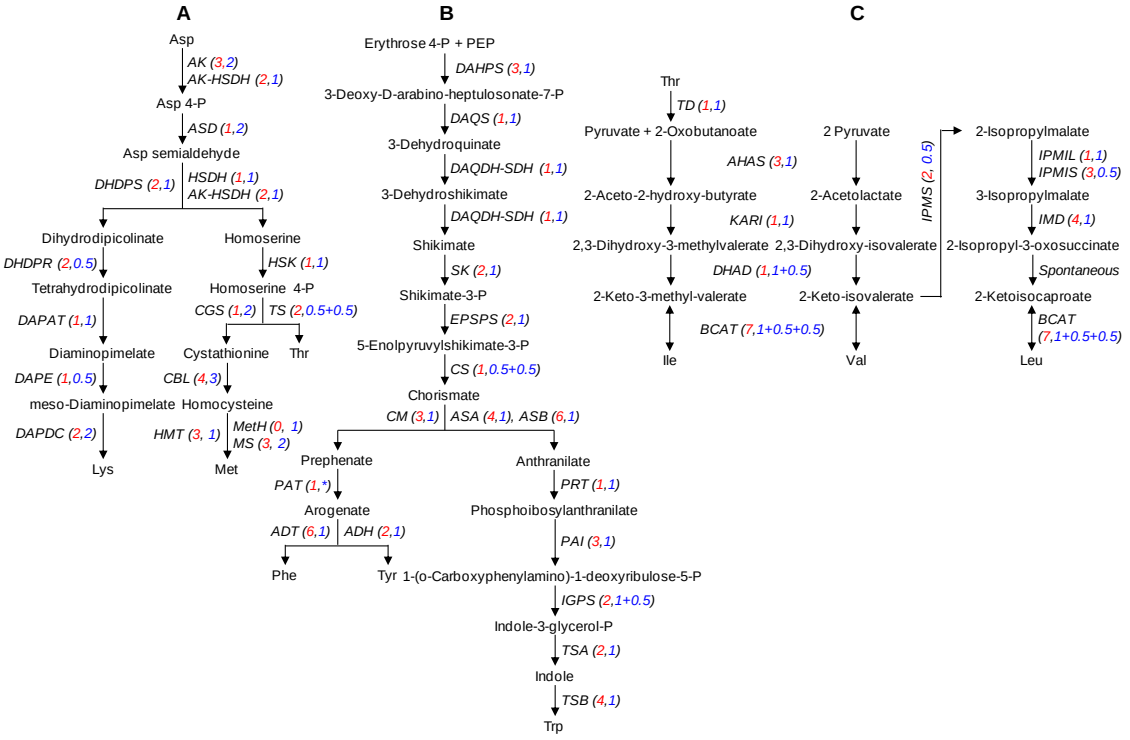


Figure 1.1. Comparison of biosynthetic pathways of Asp-derived, aromatic, and branched-chain amino acids between *A. thaliana* and *N. oceanica*. A, biosynthesis of Asp-derived amino acids Lys, Met, and Thr; B, biosynthesis of aromatic amino acids Phe, Tyr, and Trp; C, biosynthesis of branched-chain amino acids Ile, Leu, and Val. The first numbers (red) in parentheses are numbers of genes per activity in *A. thaliana*; the second numbers (blue) in parentheses are predicted numbers of genes per activity in *N. oceanica*. A value of 0.5 indicates incomplete or partial gene sequence. Because there are a large number of aminotransferases in *N. oceanica* (and *A. thaliana*) and because substrate specificity of these aminotransferases hasn't been experimentally determined, the number of *PAT* genes in *N. oceanica* is not proposed in this figure (indicated by the asterisk). ADH, arogenate dehydrogenase; ADT, arogenate dehydratase; AHAS, acetohydroxyacid synthase; AK, Asp kinase; ASA, anthranilate synthase alpha subunit; ASB, anthranilate synthase beta subunit; ASD, Asp semialdehyde dehydrogenase; BCAT, branched-chain aminotransaminase; CBL, cystathionine beta lyase; CGS, cystathionine gamma synthase; CM, chorismate mutase; CS, chorismate synthase; DAHPS, 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase; DAPAT, diaminopimelate aminotransferase; DAPDC, diaminopimelate decarboxylase; DAPE, diaminopimelate epimerase; DAQDH, dehydroquinate dehydratase; DAQS, dehydroquinate synthase; DHDPR, dihydrodipicolinate reductase; DHAD, dihydroxyacid dehydratase; DHDPS, dihydrodipicolinate synthase; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; HMT, homocysteine S-methyltransferase; HSDH, homoserine dehydrogenase; HSK, homoserine kinase; IGPS, indole-3-glycerol phosphate synthase; IMD, isopropylmalate dehydrogenase; IPMS, isopropylmalate synthase; IPMIL, isopropylmalate isomerase large subunit; IPMIS, isopropylmalate isomerase small subunit; KARI, ketolacid reductoisomerase; MetH, cobalamin-dependent Met synthase;

MS, cobalamin-independent Met synthase; PAI, phosphoribosylanthranilate isomerase; PAT, prephenate aminotransferase; PRT, anthranilate phosphoribosyltransferase; SDH, shikimate dehydrogenase; SK, shikimate kinase; TD, Thr deaminase; TS, Thr synthase; TSA, Trp synthase alpha subunit; TSB, Trp synthase beta subunit.

Annotation of aromatic amino acid biosynthesis-related genes

Similar to Asp-derived amino acids, the biosynthesis of aromatic amino acids also has two branch-point metabolites: chorismate and arogenate. Chorismate is the substrate for chorismate mutase (CM), the committing (or branch-point) enzyme for Phe and Tyr biosynthesis, and anthranilate synthase (ASA and ASB), the committing enzyme for Trp biosynthesis. Arogenate is the substrate for arogenate dehydratase (ADT), the committing enzyme for Phe biosynthesis, and arogenate dehydrogenase (ADH), the committing enzyme for Tyr biosynthesis. Even though some of the open reading frames are incomplete, *N. oceanica* appears to contain all the necessary genes for aromatic amino acid biosynthesis.

Aromatic amino acid biosynthesis is one of the most interesting pathways in *N. oceanica* because five gene pairs in this pathway are predicted to exist as fused rather than separate genes, including *dehydroquinate dehydratase-shikimate dehydrogenase* (*DHQDH-SDH*), *arogenate dehydratase-arogenate dehydrogenase* (*ADT-ADH*), *anthranilate synthase alpha-anthranilate synthase beta* (*ASA-ASB*), *indole-3-glycerol phosphate synthase- phosphoribosylanthranilate isomerase* (*IGPS-PAI*), and *Trp synthase alpha-Trp synthase beta* (*TSA-TSB*) (Figure 1.2). To investigate whether these fusion genes are also present in other organisms, we searched for homologous genes in bacterium *Escherichia coli*, cyanobacterium *Synechocystis sp.*, green alga *Chlamydomonas reinhardtii*, diatoms *Phaeodactylum tricornutum* and *Thalassiosira*

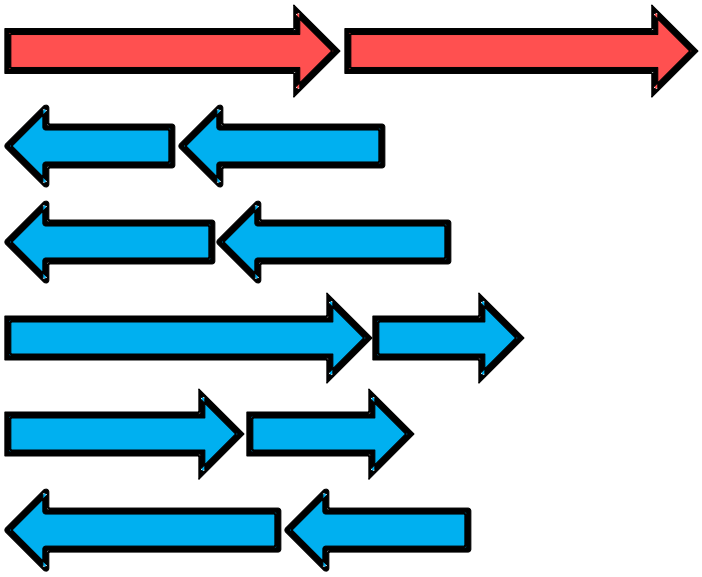


Figure 1.2. Fused genes in essential amino acid biosynthesis in the *N. oceanica* genome. A, Asp kinase and homoserine kinase genes on contig 459; B, dehydroquinate dehydratase and shikimate dehydrogenase genes on contig 166; C, arogenate dehydratase and arogenate dehydrogenase genes on contig 1334; D, anthranilate synthase alpha and beta subunit genes on contig 146; E, indole-3-glycerol phosphate synthase and phosphoribosylanthranilate isomerase genes on contig 454; F, Trp synthase alpha and beta subunit genes on contig 78. Red and blue arrows represent genes in the biosynthetic pathways of Asp-derived and aromatic amino acids, respectively.

pseudonana, and higher plant *A. thaliana* (Table 1.1).

Annotation of branched-chained amino acid biosynthesis-related genes

Like most bacteria, archaea, fungi, higher plants, and other algae, *N. oceanica* synthesizes branched-chain amino acids by a common pathway starting with two molecules of pyruvate to form one molecule of Val or Leu, and one molecule of pyruvate and one molecule of Thr-derived 2-oxobutanotate for Ile (Figure 1.1). An interesting exception is that some prokaryotes use Thr-independent citramalate pathway to form Ile from pyruvate and acetyl-CoA (Akman Gündüz and Douglas, 2009). A unique feature of branched-chain amino acid biosynthesis is that Val and Ile are synthesized in two parallel

Table 1.1. Presence of fused genes in essential amino acid biosynthesis in representative bacteria, cyanobacteria, green algae, diatoms, *Nannochloropsis*, and higher plants

Gene pairs	*E. coli*	***Synechocystis*** *sp.*	*C. reinhardtii*	*P. tricornutum*	*T. pseudonana*	*N. oceanica*	*A. thaliana*
AK-HSDH	+	-	+	+	+	+	+
DHQDH-SDH	-*	-	+	+	+	+	+
ADT-ADH	-	-	-	+	+	+	-
ASA-ASB	-**	-***	+	+	+	+	+
IGPS-PAI	+	-	-	+	+	+	-
TSA-TSB	-	-	-	+	+	+	-

*Although fused *DHQDH-SDH* was not found in *E. coli*, it is present in other bacteria, such as *Acidobacterium sp.* and *Planctomyces maris.*

**Although fused *ASA-ASB* was not found in *E. coli*, it is present in other bacteria, such *Legionella longbeachae* and *Sorangium cellulosum*.

***Although fused *ASA-ASB* was not found in *Synechocystis sp.*, it is present in other cyanobacteria, such as *Anabaena variabilis* and *Nostoc sp*.

The accession numbers for AK-HSDH homologs in *E. coli*, *C. reinhardtii*, *P. tricornutum*, *T. pseudonana*, and *A. thaliana* are ZP_08372323, XP_001695256, XP_002182284, XP_002296299, At1g31230 and At4g19710. The accession numbers for DHQDH-SDH homologs in *Acidobacterium sp.*, *P. maris*, *C. reinhardtii*, *P. tricornutum*, *T. pseudonana*, and *A. thaliana* are ZP_07032873, ZP_01854559, XP_001694346, XP_002179655, XP_002289031, and At3g06350. ADT-ADH homologs in *P. tricornutum* and *T. pseudonana* were obtained using tBLASTn at www.jgi.doe.gov; the corresponding accession numbers are estExt_fgenesh1_pg.C_chr_130260 and estExt_fgenesh1_pg.C_chr_10420. The accession numbers for ASA-ASB homologs in *L. longbeachae*, *S. cellulosum*, *A. variabilis*, *Nostoc sp. C. reinhardtii*, *P. tricornutum*, *T. pseudonana*, and *A. thaliana* are YP_003455732, YP_001615687, YP_325382, NP_484458, XP_001702943, XP_002177062, XP_002287912, and At2g28880. The accession number for IGPS-PAI homolog in *E. coli* is ZP_06938008. IGPS-PAI homologs in *P. tricornutum* and *T. pseudonana* were obtained using tBLASTn at www.jgi.doe.gov; the corresponding accession numbers are estExt_fgenesh1_pg.C_chr_60121 (from DOE-JGI) and fgenesh1_pg.C_chr_10000275. The accession numbers for TSA-TSB homologs in *P. tricornutum* and *T. pseudonana* are XP_002176877 and XP_002294706.

pathways (Figure 1.1). They share a set of four enzymes by using different substrates, and those enzymes include AHAS, ketolacid reductoisomerase (KARI), dihydroxyacid dehydratase (DHAD), and branched-chain aminotransferase (BCAT) (Binder, 2010). The biosynthesis of Leu branches off from the last intermediate, 2-keto-isovalerate, in Val biosynthesis. Leu biosynthesis enzymes include isopropylmalate synthase (IPMS), isopropylmalate isomerase (IPMI), isopropylmalate dehydrogenase (IMDH), and BCAT (Singh and Shaner, 1995; Binder, 2010). Four genes encoding IPMS-like proteins are present in *A. thaliana*; two of them encode true IPMS (Figure 1.1) and the other two encode methylthioalkylmalate synthases (MAM), which control the Met-derived aliphatic

glucosinolate side-chain elongation process (de Kraker *et al.*, 2007; Textor *et al.*, 2007). In prokaryotes, algae, and plants, the IPMI enzyme is a heterodimer composed of a large (IPMIL) and a small subunit (IPMIS) (Binder, 2010). *N. oceanica* appears to have one copy of large subunit and one copy of small subunit (Figure 1.1 and Appendix A). Aside from the fact that some open reading frames are incomplete, *N. oceanica* appears to have all the genes for branched-chain amino acid biosynthesis.

Discussion

N. oceanica *has all the genes for essential amino acid biosynthesis*

Overall, the biosynthesis of Asp-derived, aromatic, and branched-chain amino acids in *N. oceanica* resemble the pathways in *A. thaliana*, but the gene content seems to be simpler. The major variation in branched-chain amino acid biosynthesis is that *A. thaliana* makes glucosinolate via two IPMS-like proteins catalyzing side-chain elongation of Met-derived aliphatic glucosinolate, but *N. oceanica* does not make glucosinolate. Between the three pathways, *A. thaliana* has an average of 2.5 genes per enzyme activity and *N. oceanica* has an average of 1.3 predicted genes per enzyme activity (Figure 1.1). This is consistent with their difference in genome sizes and their places in evolutionary history.

N. oceanica *is interesting from an evolutionary perspective*

The most important difference in Asp-derived amino acid biosynthesis is that *N. oceanica* has both cobalamin-dependent and cobalamin-independent Met synthases and *A. thaliana* only has cobalamin-independent Met synthase. One hypothesis is different environmental conditions caused algae to retain both forms of Met synthase from their

ancestor, while land plants (and fungi) to lose the cobalamin-dependent Met synthase (Drennan *et al*., 1994; Ravanel *et al*., 2004; Helliwell *et al*., 2011). An important environmental condition is the presence or absence of cobalamin. Transcription of the cobalamin-independent Met synthase is repressed in the presence of cobalamin, which could permit gene loss (Helliwell *et al*., 2011). It is conceivable that a similar mechanism may lead to the loss of the cobalamin-dependent Met synthase.

The key dissimilarity in aromatic amino acid biosynthesis is that *N. oceanica* has fused *DHQDH-SDH*, *ADT-ADH*, *ASA-ASB*, *IGPS-PAI* and *TSA-TSB* genes and *A. thaliana* only has the *DHQDH-SDH* fusion. These five fusion gene pairs are also present in diatoms *P. tricornutum* and *T. pseudonana*, but not in bacterium *E. coli*, cyanobacterium *Synechocystis sp.*, or green alga *C. reinhardtii* (Table 1.1). It was recently proposed that the ancestral state of Trp biosynthesis is composed of separate ASA and ASB, fusion of IGPS and PAI, and fusion of TSA and TSB, and that ASA-ASB fusion in diatoms is the result of an ancient replacement of separate ASA and ASB by fused bacterial ASA-ASB (Jiroutová *et al*., 2007).

Furthermore, both *N. oceanica* and *A. thaliana* have fused *AK-HSDH* genes in their Asp-derived amino acid biosynthesis pathways. AK-HSDH fusion is also present in other organisms, such as bacterium *E. coli*, green alga *C. reinhardtii*, diatoms *P. tricornutum* and *T. pseudonana*, and higher plant *A. thaliana*, but it is not found in cyanobacterium *Synechocystis sp* (Figure 1.2 and Table 1.1). Understanding why *N. oceanica* has a relatively high occurrence of gene fusion events in amino acid biosynthesis is an interesting topic from an evolutionary point of view.

CHAPTER II

TRANSCRIPTIONAL REGULATION OF ASPARTATE-DERIVED AMINO ACID BIOSYNTHESIS

Introduction

Overview of ADAA biosynthesis

The aspartate (Asp)-derived amino acids (ADAAs) include essential amino acids threonine (Thr), methionine (Met), lysine (Lys), and isoleucine (Ile), which are present at growth-limiting levels in major field crops, such as corn, rice, soybean, and potato (Muntz *et al.*, 1998; Debadov, 2003; Pfefferle *et al.*, 2003; Stiller *et al.*, 2007). Increasing the contents of these amino acids in crop plants has long been a major goal of traditional breeding and genetic engineering (Galili *et al.*, 2008; Ufaz and Galili, 2008; Jander and Joshi, 2009). This requires a better understanding of the pathways and regulation of ADAAs.

The majority of the reactions in the biosynthesis of ADAAs occur in the chloroplast (Coruzzi and Last, 2000). The first step of the biosynthetic pathway for ADAAs is the activation of Asp to Asp 4-phosphate by mono-functional Asp kinases (AKs) and dual-functional Asp kinase-homoserine dehydrogenases (AK-HSDHs) (Figure 2.1). Asp 4-phosphate is then converted to Asp semialdehyde, the branching-point intermediate for Lys biosynthesis and Met and Thr biosynthesis. The committing step leading to Met and Thr biosynthesis is the formation of homoserine from Asp semialdehyde by dual-functional AK-HSDHs and the mono-functional HSDH.

Most of the recent studies on the ADAA biosynthesis pathway focused on the

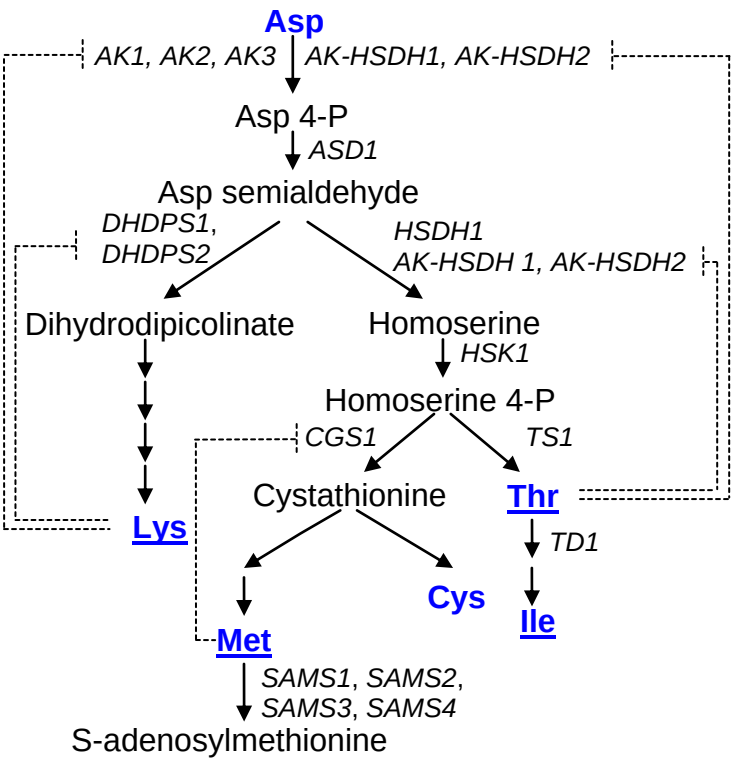


Figure 2.1. Biosynthetic pathway for Asp-derived amino acids (ADAAs) in *A. thaliana*. Key enzymes and metabolites are shown. Essential amino acids Lys, Thr, Met, and Ile are underlined. Amino acids with significant increases in *ak-hsdh2* mutants are shown in blue. Feedback inhibition by Lys, Met, and Thr are shown as dotted lines. AK, Asp kinase; CGS, cystathionine gamma synthase; DHDPS, dihydrodipicolinate synthase; HSDH, homoserine dehydrogenase; SAMS, S-adenosylmethionine synthase; TD, Thr deaminase; TS, Thr synthase.

effect of downstream products on enzyme function, not the effect on gene transcription. For example, Curien *et al.* (2005) studied the effect of seven chloroplast amino acids on *Arabidopsis thaliana* enzymes AK-HSDH1 (At1g31230) and AK-HSDH2 (At4g19710). They investigated whether these amino acids act as activators or inhibitors for both the AK and HSDH activities of the two dual-function enzymes. In addition, Curien *et al.* (2007) tested the effect of amino acids on the activity of mono-functional AKs. There has been at least one study on the transcriptional regulation of ADAA pathway genes. Hacham *et al*. (2007) found that high Lys content reduces the expression level of *S*-

adenosylmethionine synthase (*SAMS*), which leads to an accumulation of Met.

Experimental overview

In this work, we describe two previously uncharacterized *A. thaliana* mutants with high levels of Thr and other ADAAs in mature leaves: SALK_019023 (designated as *ak-hsdh2-1*) and SALK_082155 (designated as *ak-hsdh2-2*) (Figure 2.2). AK-HSDH2 is a protein with separate functional domains for its HSDH and AK activities. The *ak-hsdh2-1* mutant has a T-DNA insertion in the HSDH domain. This should interfere with transcription by activating the nonsense-mediated mRNA decay pathway. The *ak-hsdh2-2* mutant has a single-base insertion in the AK domain, which causes a premature stop codon and a truncated protein (Figure 2.3).

Figure 2.2. Images of wild-type and *ak-hsdh2* *A. thaliana* plants. Col WT, SALK_019023 (*ak-hsdh2-1*), and SALK_082155 (*ak-hsdh2-2*) plants were grown under a 12-h-light/12-h-dark photoperiod for four weeks. Both *ak-hsdh2* mutants are morphologically similar to the wild type.

We confirmed these mutations cause loss of function in the AK-HSDH2 protein using quantitative PCR (QPCR) and immunodetection, as well as measured the leaf free amino acid content in 4-week-old wild type and *ak-hsdh2* mutants. We tested whether over-accumulation of ADAAs will up- or down-regulate ADAA-related genes by supplementing wild-type and *ak-hsdh2-1* mutant plants with exogenous amino acids and measuring the transcript levels of several genes. To identify other genes that may be up-

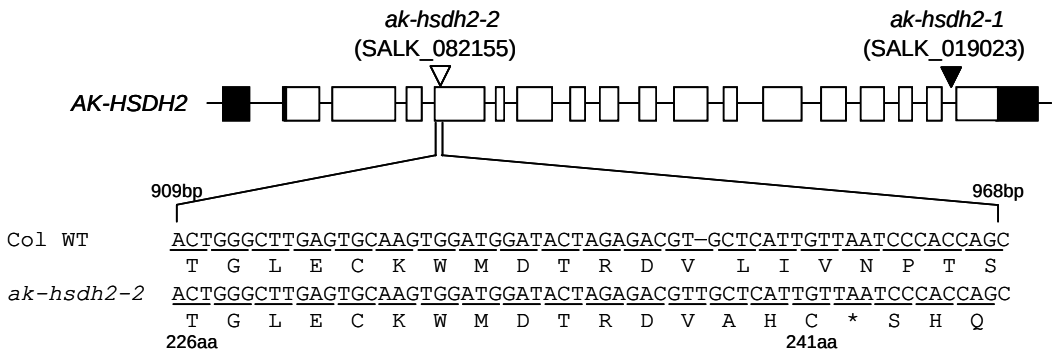


Figure 2.3. Schematic representation of the *AK-HSDH2* gene (At4g19710) and two mutations: SALK_019023 and SALK_082155. Black rectangles represent 5' and 3' untranslated regions; white rectangles represent exons; solid lines represent introns and intergenic regions; the white triangle represents a single-base insertion; the black triangle represents a T-DNA insertion. SALK_019023 (*ak-hsdh2-1*) contains a T-DNA insertion in the last intron of the *AK-HSDH2* gene. SALK_082155 (*ak-hsdh2-2*) contains an extra base of thymine (T) in the fourth exon of the same gene. The location of this extra thymine base was determined by array mapping and targeted genomic DNA sequencing. This single base insertion results in a premature stop codon and a truncated protein of 241 amino acids in size. aa, amino acids, bp; base pairs; WT, wild type.

or down-regulated in the mutants, we used microarray analysis on wild type and *ak-hsdh2-1* mutants. This allowed us to investigate the expression of many genes simultaneously. Genes that were found to be up- or down-regulated by microarray analysis were validated with QPCR.

Methods

Plant materials and growth conditions

Arabidopsis thaliana T-DNA lines (SALK_019023 and SALK_082155) used in this study were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). Both are in the Columbia (Col) ecotype (Alonso *et al.*, 2003). Mutant and wild-type *A. thaliana* plants were grown on soil or on media in a growth chamber on a 12-h-light/12-h-dark photoperiod. The light intensity was 90-110 μmol photons m^{-2} s^{-1}, the

temperature was 20°C, and the relative humidity was 50%.

Media preparation

To test the effect of Thr on transcript levels, wild-type and *ak-hsdh2* mutant plants were grown in Magenta^{TM} boxes (Magenta Corporation, Chicago, IL) on Murashige and Skoog (MS) media supplemented with 0, 10, 100, 1000, or 5000 μM Thr. MS media (pH 5.65-5.80) was prepared with Gamborg's vitamins, 0.025% 4-morpholineethanesulfonic acid (MES) sodium salt, 1% sucrose, and 0.7% agar. Sucrose and Thr were filtered and added post-autoclaving to prevent degradation.

Leaf free amino acid assay by HPLC-MS/MS

Leaf tissues from soil-grown plants for free amino acid measurement were harvested into 2-mL microfuge tubes containing a single 3-mm stainless steel ball. Leaf samples were immediately frozen with dry ice and ground to a fine powder for 2 min on a TissueLyser II bead mill (Qiagen, Valencia, CA). Free amino acids were extracted and analyzed with the HPLC-MS/MS method (Lu *et al.*, 2008). For quantification, mixtures of 20 protein amino acids, gamma-aminobutyric acid, anthranilate, homoserine, hydroxyproline, and S-methyl methionine of varying concentrations plus L-Phe-α,β,β,2,3,4,5,6-d8 (Phe-d8) and L-Val-2,3,4,4,4,5,5,5-d8 (Val-d8) of 0.9 μM were analyzed along with the leaf samples.

RNA extraction

For soil-grown plants, individual leaves were harvested and frozen for RNA extraction. For media-grown plants, the entire aerial portion was harvested. Frozen plants were ground to a fine powder in a mortar with liquid nitrogen. Total RNA was extracted

and purified using the RNeasy plant mini kit (Qiagen), followed by digestion with RNase-free DNase I (Qiagen). RNA concentration was determined using a Thermo Scientific Nanodrop 2000 spectrophotometer (Pittsburgh, PA).

Reverse transcription and QPCR

Reverse transcription (RT) was performed using Promega Moloney murine leukemia virus (M-MLV) reverse transcriptase (Madison, WI) and oligo $dT_{(15)}$ primers. QPCR was performed on a StepOnePlus™ Real-Time PCR system using SYBR Green reagents (Applied Biosystems, Life Technologies, Carlsbad, CA). Transcript quantities were normalized by the transcript level of *ACTIN2*, a constitutively-expressed gene. A list of QPCR primers can be found in Appendix B. The presence of PCR products and the absence of primer dimers were confirmed with agarose gel electrophoresis.

Preparation of DNA standards for QPCR

DNA standards were made by amplifying wild-type cDNA with gene-specific primers. PCR reactions used Sigma RedTaq DNA polymerase (St. Louis, MO). A list of primers can be found in Appendix B. Agarose gel electrophoresis and a Qiagen QIAquick gel extraction kit were used to purify the DNA. DNA concentration was determined using a Thermo Scientific Nanodrop 2000 spectrophotometer, then diluted based on molecular weight.

Microarray analysis

Total RNA from soil-grown wild-type and *ak-hsdh2-1* mutant leaves was extracted and sent to the Research Technology Support Facility (RTSF) at Michigan State University (Lansing, MI) to be used for cDNA production, labeling, hybridization to

Arabidopsis ATH1 gene chips, and scanning.

Immunoblots

Leaf tissues for immunodetection of the AK-HSDH2 protein were harvested, frozen in liquid nitrogen, and ground into a fine powder. Total soluble proteins were extracted in a buffer containing 100 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and 50 mg/mL polyvinylpolypyrrolidone, as described by Lu *et al*. (2006). The protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA), with bovine serum albumin as the standard. A total of 43.8 μg protein per lane was separated on a pre-cast NuPAGE® Novex® 4-12% Bis-Tris Gel (Invitrogen, Life Technologies) according to the manufacturer's instructions. After electrophoresis, the proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane as described by Lu *et al*. (2011). Immunodetection of the AK-HSDH2 protein on the PVDF membrane was performed using the SuperSignal west pico rabbit immunoglobulin G detecting kit (Thermo Scientific) and analyzed by the Gel Logic 1500 imaging system (Kodak, Rochester, NY). The anti-AK-HSDH2 polyclonal antibody was made by Open Biosystems (now part of Thermo Scientific). A 14-amino-acid peptide (corresponding to amino acids 820-833 of the full-length AK-HSDH2 protein) with an additional N-terminal Cys residue, C-DGDLAKERLDAENS, was synthesized, conjugated with keyhole limpet hemocyanin, and used to raise the AK-HSDH2-specific antibody.

Accession numbers

Sequences for genes mentioned in this work can be found in the GenBank database under the following accession numbers: *ACTIN2*, At3g18780; *AK1*, At5g13280; *AK2*, A5g14060; *AK3*, At3g02020; *AK-HSDH1*, At1g31230; *AK-HSDH2*, At4g19710; *HSDH1*, At5g21060; *ASD1*, At1g14810; *DHDPS1*, At3g60880; *DHDPS2*, At2g45440; *HSK1*, At2g17265; *TS1*, At4g29840; *TD1*, At3g10050; *CGS1*, At3g01120; *SAMS1*, At1g02500; *SAMS2*, At4g01850; *SAMS3*, At3g17390; *SAMS4*, At2g36880; *RABD1*, At3g11730; *NADBP*, At5g58750; *PKSP*, At4g00960.

Results

Mature AK-HSDH2 protein is absent in ak-hsdh2-1 *and* ak-hsdh2-2 *plants*

The transcript level for *AK-HSDH2* was measured in *ak-hsdh2* and wild-type plants to confirm that the mutations in *ak-hsdh2-1* and *ak-hsdh2-2* caused loss of functional AK-HSDH2 protein (Figure 2.4). As expected, the level of *AK-HSDH2* transcript is completely abolished in *ak-hsdh2-1* and substantially reduced in *ak-hsdh2-2*. Although there is still a small amount of the *AK-HSDH2* transcript left in the *ak-hsdh2-2* mutant, the transcript is frame-shifted and the corresponding protein is therefore truncated and dysfunctional.

Immunodetection of the AK-HSDH2 protein was performed on wild-type, *ak-hsdh2-1*, and *ak-hsdh2-2* plants to confirm that mature AK-HSDH2 protein (95 kDa) is absent in the mutants (Figure 2.5). As expected, this enzyme was present in the wild type and absent in the two *ak-hsdh2* mutants.

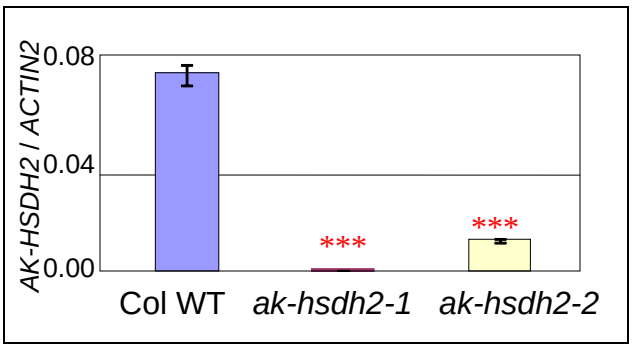


Figure 2.4. Relative amount of the *AK-HSDH2* transcript. Total RNA was extracted from mature leaves and analyzed with quantitative PCR to verify loss of functional AK-HSDH2 transcript in *ak-hsdh2* mutants. The residual transcript in *ak-hsdh2-2* mutants is frame-shifted and not expected to yield a functional protein. The level of *AK-HSDH2* transcript was normalized by the relative abundance of the *ACTIN2* transcript. Values are presented as mean ± SE (n = 6). Asterisks indicate significant differences between the mutant and the Col wild type (Student's *t* test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

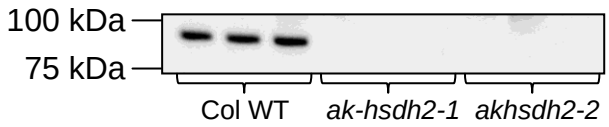


Figure 2.5. Representative immunoblot of the AK-HSDH2 protein. The estimated size of mature AK-HSDH2 protein in *A. thaliana* is 95 kDa. As expected, this protein is present in the wild type and absent in both *ak-hsdh2* mutants. Lanes were loaded on an equal protein basis. The AK-HSDH2-specific antibody was raised from a 14-amino-acid peptide corresponding to amino acids 820-833 of the full-length AK-HSDH2 protein.

The ak-hsdh2 *mutants have increased amino acid levels*

Leaf free amino acid content was measured for 4-week-old wild type and *ak-hsdh2* mutants (Figure 2.6). In congruence with our previous findings, mature *ak-hsdh2* leaves accumulate high levels of Thr. There are also significant increases in Asp, Lys, Met, cysteine (Cys), and Ile levels.

The effects of exogenous Thr on the transcript levels

Transcript levels for three ADAA biosynthesis-related genes (*AK-HSDH2*, *HSK1*,

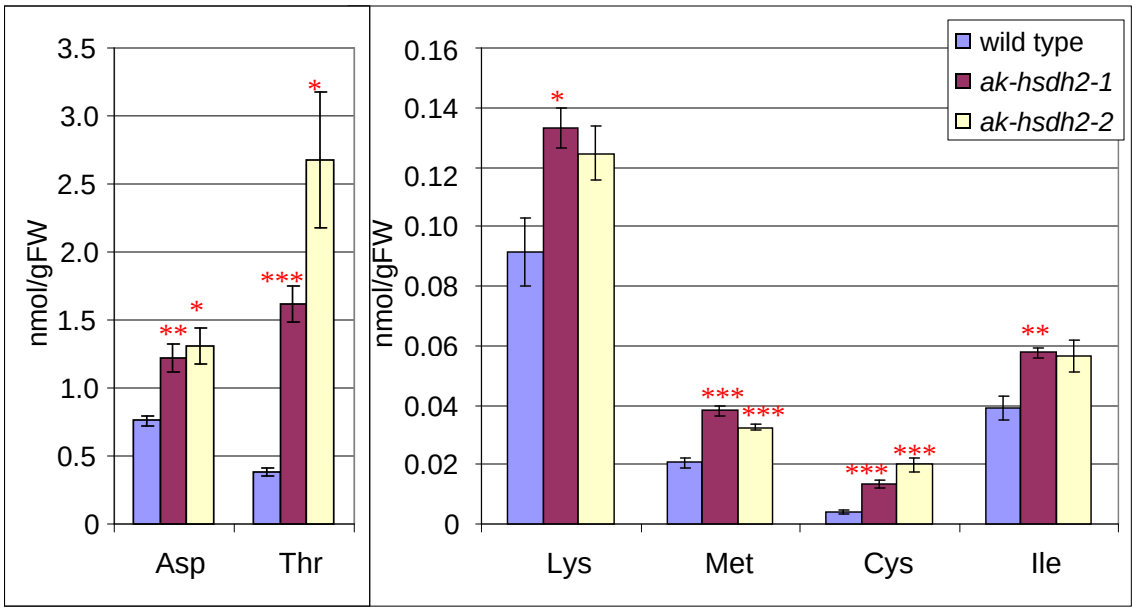


Figure 2.6. Increased levels of amino acids in *ak-hsdh2* mutants. The relative abundance of ADAAs in mature leaves was measured using HPLC-MS/MS and normalized by leaf fresh weight. Values are presented as mean ± SE (n = 5). Asterisks indicate significant differences between *ak-hsdh2* mutants and wild type (Student's *t* test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

and *TS1*) were measured in Thr-supplemented plants (Figure 2.7). As expected, there was no detectable amount of *AK-HSDH2* transcript in the *ak-hsdh2-1* mutant. We found that 10 µM of Thr causes ~3-4 fold increases in transcript levels of *AK-HSDH2*, *HSK1*, and *TS1* in the wild-type plants (Figure 2.8). However, due to the large variation in transcript levels in the wild-type plants treated with 10 µM of Thr, the increases are not statistically significant. Interestingly, the effects of 100, 1000, and 5000 µM of Thr are not as obvious as 10 µM of Thr. The transcript levels for soil-grown plants were also measured as a comparison. It is unknown why the soil-grown plants have fewer transcripts for the three genes as compared to media-grown plants. We also noticed that wild-type and *ak-hsdh2-1* plants growing in 1000 and 5000 µM Thr are visibly smaller than those growing in 0, 10, and 100 µM Thr (Figure 2.7). This observation is consistent with an early report that

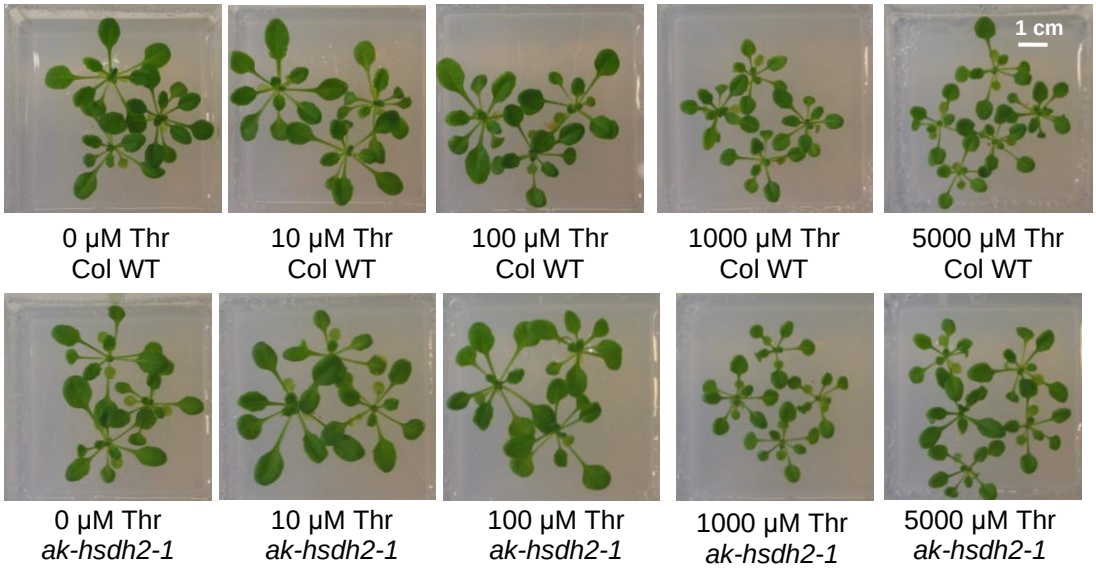


Figure 2.7. Images of wild-type and *ak-hsdh2* *A. thaliana* plants grown with exogenous Thr. Plants were grown on MS media supplemented with 0, 10, 100, 1000, or 5000 µM Thr under a 12-h-light/12-h-dark photoperiod for one month. Plants supplemented with 1000 and 5000 µM Thr are visibly smaller than those supplemented with 0, 10, and 100 µM Thr.

a high amount of Thr in the media has a toxic effect on plant growth (Sarrobert *et al.*, 2000).

Two trials of microarray analysis indicate three seemingly unrelated genes are up- or down-regulated in ak-hsdh2 *mutants*

Microarray analysis was used to determine which genes were up- or down-regulated at least two-fold in the *ak-hsdh2* mutants. To minimize the number of false positives, we performed two microarray analysis trials (Table 2.1). As expected, transcript levels of *AK-HSDH2* were significantly decreased in the *ak-hsdh2-1* mutant. To our surprise, other ADAA biosynthesis-related genes were not significantly altered in mutants, including *AK1*, *AK2*, *AK3*, *AK-HSDH1*, *HSDH1*, *HSK1*, and *TS1* (Table 2.1). However, three non-ADAA biosynthesis-related genes were found to be consistently up- or down-regulated in the mutants. One of them encodes a Rab GTPase (RABD1), one of

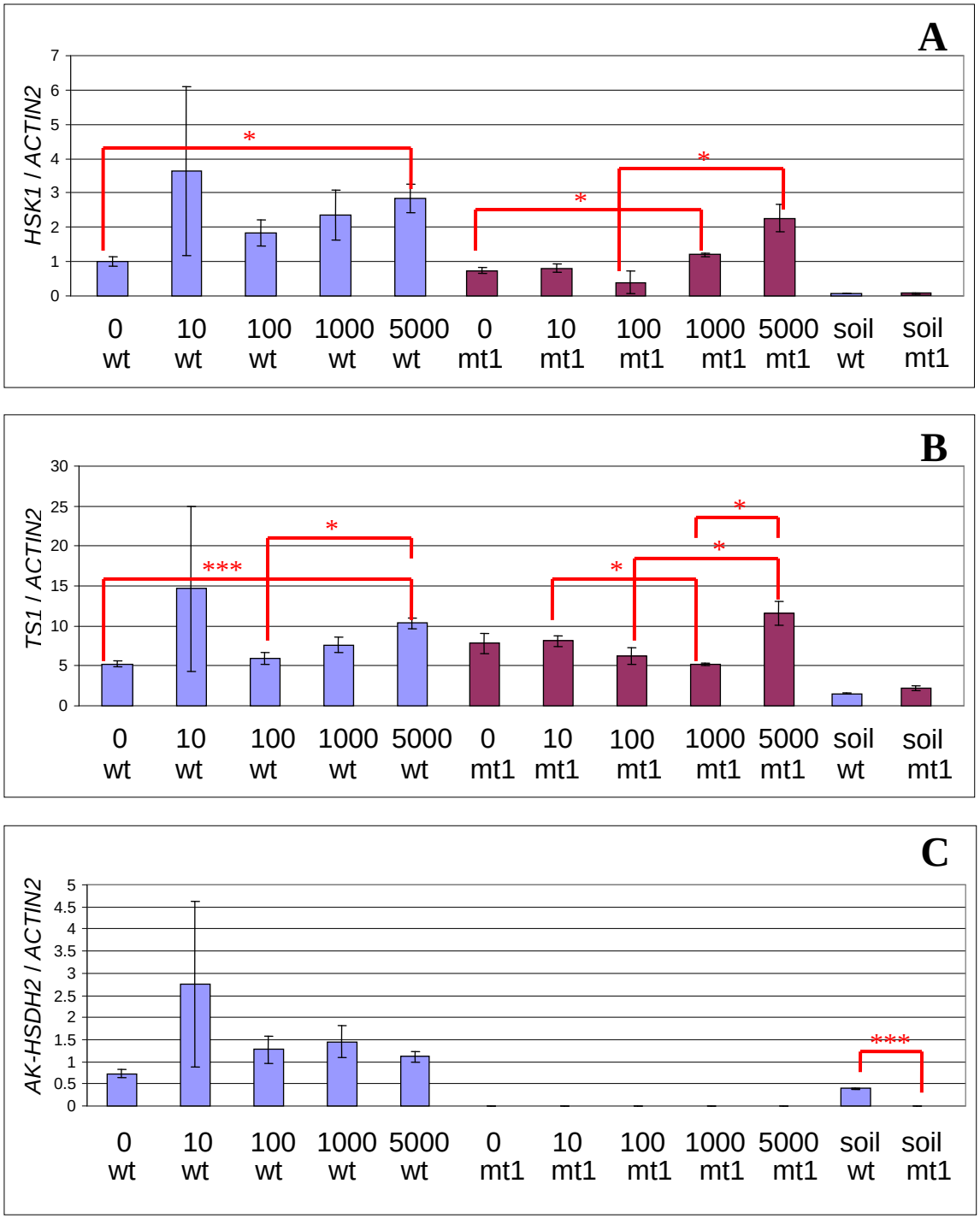


Figure 2.8. Thr does not consistently and significantly affect transcript levels of ADAA biosynthesis-related genes. The relative abundances of *AK-HSDH2* (A), *HSK1* (B), and *TS1* (C) were determined by QPCR, and normalized by the relative abundance of *ACTIN2*. Samples include wild-type *A. thaliana* (wt) and *ak-hsdh2-1* mutants (mt1), grown on media supplemented with 0, 10, 100, 1000, or 5000 µM Thr, or grown on soil. Values are presented as mean ± SE (n = 3). Asterisks indicate significant differences between concentrations of Thr (Student's *t* test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Table 2.1. Microarray analysis results for select ADAA biosynthesis-related genes and three unrelated genes

	Trial 1		Trial 2	
Gene	**wild type**	***ak-hsdh2-1***	**wild type**	***ak-hsdh2-1***
AK1	0.82 ± 0.06	0.83 ± 0.10	1.28 ± 0.11	1.10 ± 0.05
AK2 and *AK3*	4.51 ± 0.09	3.98 ± 0.49	3.21 ± 0.01	3.62 ± 0.36
AK-HSDH1	0.88 ± 0.05	0.96 ± 0.14	1.30 ± 0.13	1.35 ± 0.01
AK-HSDH2	1.89 ± 0.15	0.22 ± 0.03***	2.51 ± 0.34	0.23 ± 0.06***
HSDH1	0.90 ± 0.01	1.17 ± 0.02	1.35 ± 0.03	1.38 ± 0.13
ASD1	2.16 ± 0.26	3.20 ± 0.63	5.61 ± 0.29	3.79 ± 0.44
DHDPS1	0.40 ± 0.01	0.39 ± 0.02	0.84 ± 0.24	0.95 ± 0.07
DHDPS2	0.51 ± 0.07	0.65 ± 0.15	0.73 ± 0.04	0.517 ± 0.005
HSK1	0.86 ± 0.08	1.03 ± 0.13	0.95 ± 0.01	0.70 ± 0.11*
TS1	1.08 ± 0.07	1.38 ± 0.31	0.73 ± 0.01	0.65 ± 0.06
TD1	0.54 ± 0.03	0.67 ± 0.01*	0.81 ± 0.07	0.89 ± 0.02
CGS1	10.97 ± 0.64	9.72 ± 0.62	9.89 ± 0.34	9.88 ± 0.23
SAMS1	7.57 ± 0.95	7.48 ± 0.55	8.50 ± 1.43	5.98 ± 0.75
SAMS2	5.84 ± 0.60	6.15 ± 0.34	5.39 ± 0.56	7.06 ± 0.11
SAMS3	11.50 ± 0.48	11.42 ± 1.42	16.51 ± 0.71	20.13 ± 1.33
SAMS4	5.54 ± 0.36	6.31 ± 0.87	8.39 ± 1.47	5.51 ± 0.12
RABD1	0.79 ± 0.04	0.04 ± 0.02***	0.83 ± 0.03	0.015 ± 0.001*
NADBP	0.34 ± 0.08	0.74 ± 0.08*	0.27 ± 0.06	0.82 ± 0.08*
PKSP	0.11 ± 0.01	0.25 ± 0.05	0.12 ± 0.03	0.259 ± 0.003

[a]Values (signals relative to the mean signal) are presented as mean ± SE (n = 3). *Asterisks indicate significant differences between the soil-grown ak-hsdh2-1 mutant and the wild type (Student's *t* test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

them encodes a NAD(P)-binding protein (NADBP), the other encodes a protein kinase superfamily protein (PKSP) (Table 2.1). Compared to those in the wild-type plants, the transcript level of the *RABD1* gene decreased by 20-50 fold in the *ak-hsdh2-1* mutant; the transcript levels of the *NADBP* and *PKSP* genes increased by 2-3 fold in the *ak-hsdh2-1* mutant (Table 2.1). The reduction in the *RABD1* transcript in the *ak-hsdh2-1* mutant was confirmed via QPCR (Figure 2.9a). The transcript level of *RABD1* did not decrease in Thr-supplemented wild-type plants (Figure 2.9b).

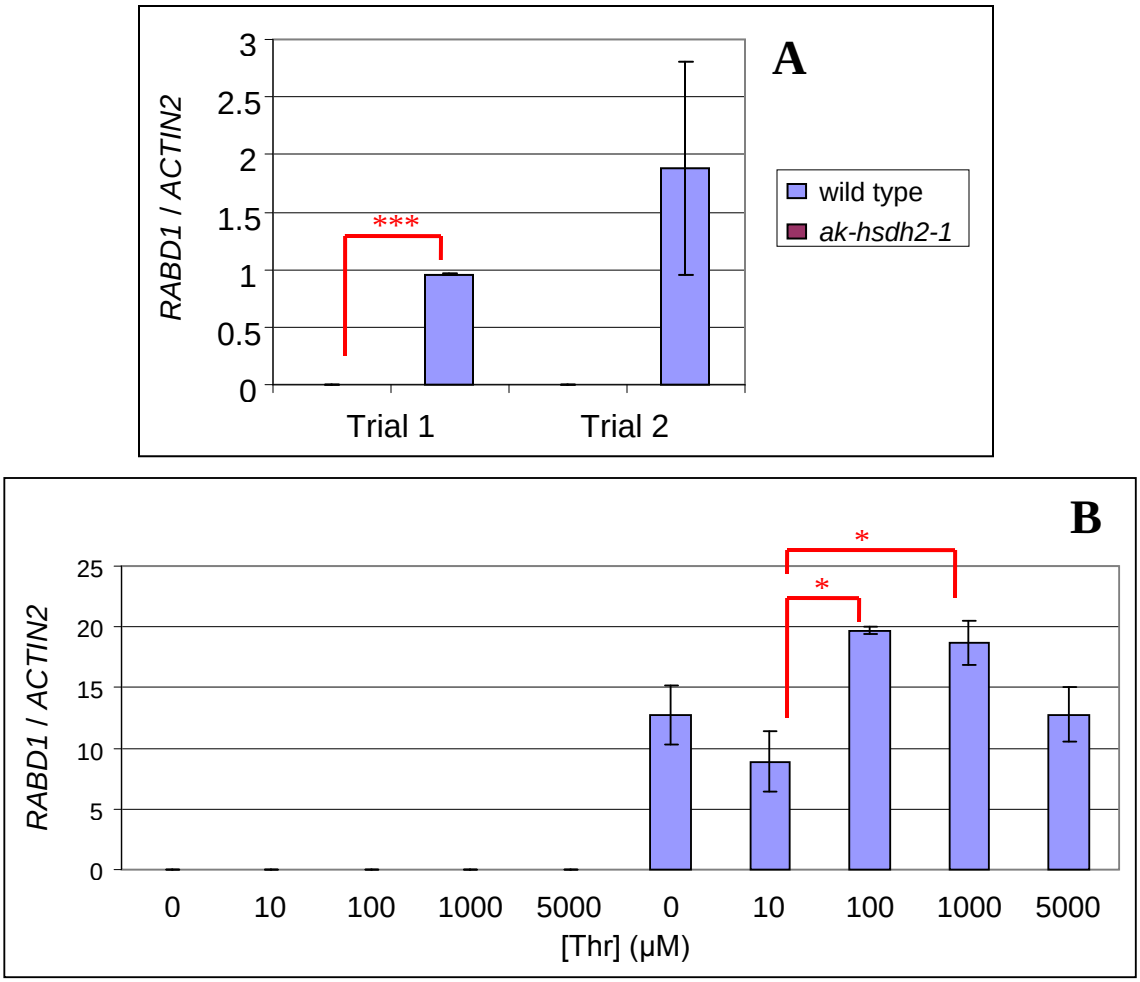


Figure 2.9. Relative amount of the *RABD1* transcript.
(A) The reduction in the *RABD1* transcript in the soil-grown wild type and *ak-hsdh2-1* mutant was confirmed via QPCR. Samples correspond to microarray analysis trials. (B) Plants were grown on media supplemented with 0, 10, 100, 1000, or 5000 µM Thr. The level of *RABD1* transcript is highest at medium levels of Thr. In both (A) and (B), the amount of the *RABD1* transcript in the wild type and *ak-hsdh2-1* mutants was determined by QPCR and normalized by the amount of the *ACTIN2* transcript. Values are presented as mean ± SE (n = 3). Asterisks indicate significant differences between different concentrations of Thr (Student's *t* test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Discussion

Transcriptional characterization of ak-hsdh2 *mutants*

We discovered that loss-of-function mutations in the *AK-HSDH2* gene cause an increase in downstream products Thr, Asp, Lys, Met, Cys, and Ile in *A. thaliana* leaves.

Among these six amino acids, the change in the Thr content is most evident. We first hypothesized that ADAA biosynthesis may be regulated at the transcriptional level in response to the changes in amino acid contents. This hypothesis raised three main questions: (1) which amino acid(s) causes the change in transcript levels; (2) are there transcription factors linking amino acid levels to transcript levels; (3) how do these transcription factors regulate the expression of the affected genes?

Because Thr is substantially increased in *ak-hsdh2* mutants and is important in regulating enzyme function in the ADAA biosynthesis pathway (see Chapter III), we tested the effects of exogenous Thr on the transcript levels of *HSK1* and *TS1*. The HSK1 protein converts homoserine to homoserine 4-phosphate, the precursor of Thr biosynthesis by TS1. The physiological concentration of Thr in wild-type chloroplasts, where Thr is synthesized, is about 300 μM (Curien *et al.*, 2005). In the two *ak-hsdh2* mutants, the estimated concentration of Thr in the leaf is between 1600 and 2700 μM (Figure 2.6). Therefore, we decided to examine the effect of supplementing *A. thaliana* with 0, 10, 100, 1000, or 5000 μM of Thr.

We did not observe consistent and significant changes in transcript levels as the concentration of Thr increased (Figure 2.9). This may have been in part due to the large variation in transcript levels in the wild-type plants treated with 10 μM of Thr. Also, too much Thr can have a toxic effect on plant growth. For example, Sarrobert *et al.* (2000) determined that 25 μM of Thr causes a significant reduction in root growth. This is consistent with our own observations; there is a visible reduction of overall plant growth in the presence of higher Thr concentrations (Figure 2.7).

To identify other genes that are up- or down-regulated in the mutants, we used

microarray analysis on the wild type and *ak-hsdh2-1* mutants. Two trials of microarray analysis indicate the transcript levels of genes associated with ADAA biosynthesis, other than *AK-HSDH2*, were not changed in the *ak-hsdh2-1* mutants. This suggests that ADAA biosynthesis is not regulated at the transcriptional level in response to the changes in amino acid content. However, the transcript level of a seemly unrelated gene, *RABD1*, was substantially reduced in the *ak-hsdh2* mutants. The differences seen in the two trials may be due to slightly different growing conditions or may simply be natural variations in transcript levels. It is unlikely that the decrease in *RABD1* transcript level is due to the T-DNA insertion into *AK-HSDH2* because *AK-HSDH2* and *RABD1* are located on different chromosomes (chromosomes 4 and 3, respectively).

Hypothesis – Transcription of RABD1 *is influenced by amino acid levels*

Although the function of RABD1 is currently unknown, other RAB proteins help regulate the mTORC1 kinase pathway, which leads to cell growth in mammalian cells (Dickinson *et al.*, 2011). High amino acid levels can stimulate this pathway and induce cell growth, but RAB proteins inhibit mTORC1-mediated amino acid signaling (Sancak *et al.*, 2010; Dickinson *et al.*, 2011). The *ak-hsdh2* mutants have increased levels of several amino acids as well as very low *RABD1* transcript levels. We propose that high levels of certain amino acids may inhibit the transcription of RABD1, thereby alleviating the inhibition of the mTORC1 kinase pathway. Thr is not likely to be one of these amino acids: when supplemented by exogenous Thr, wild-type plants showed a tendency to increase *RABD1* transcript levels, rather than decrease them (Figure 2.9b). Leu has been shown to activate the mTORC1 pathway as well as activate ribosomal and putative nutrient sensing proteins (Atherton *et al.*, 2010; Gran and Cameron-Smith, 2011). In

addition, Leu is accumulated in *ak-hsdh2* mutants, particularly in 3-week-old plants (see Chapter III). We hypothesize that Leu may be important in regulating *RABD1* transcription.

Future experimentation

Although *ak-hsdh2* mutants have increased levels of ADAAs, the ADAA biosynthesis-related genes are not consistently up- or down-regulated in the mutants and are thus unlikely to be affected by amino acid levels. Hence, it not necessary to further examine the effect of supplemented amino acids on the transcript levels of ADAA biosynthesis-related genes. Instead, we intend to measure the level of *RABD1* transcripts in wild-type plants supplemented by amino acids such as Leu. Also, we will assay the amino acid content of 1-month-old *rabd1* mutants. These experiments will enable testing of our hypothesis that high amino acid levels suppress *RABD1* transcription.

CHAPTER III

AMINO ACID ACCUMULATION DUE TO LOSS OF AK AND HSDH ISOZYMES

Introduction

Lys, Thr, and Met biosynthesis and regulation in Arabidopsis thaliana

The aspartate (Asp)-derived amino acids (ADAAs) include essential amino acids threonine (Thr), methionine (Met), lysine (Lys), and isoleucine (Ile) (see Chapter II). The first step in the ADAA biosynthesis pathway is a kinase reaction from Asp to Asp 4-phosphate. In the model plant *Arabidopsis thaliana*, this reaction is catalyzed by dual-functional enzymes Asp kinase-homoserine dehydrogenase 1 (AK-HSDH1) and AK-HSDH2 (Vieler *et al*., 2012) as well as mono-functional enzymes AK1, AK2, and AK3 (Jander and Joshi, 2009; Vieler *et al*., 2012). Both AKs and AK-HSDHs are subject to feedback inhibition by their downstream products. The activity of AKs is feedback-inhibited by Lys (Relton *et al*., 1988; Dotson *et al*., 1989; Frankard *et al*., 1997; Tang *et al*., 1997; Curien *et al*., 2007; Wang *et al*., 2007). This inhibition is mediated through the two Lys-binding ACT2 domains at the C-terminus of the proteins (Chipman and Shaanan, 2001; Jander and Joshi, 2009). Additionally, AK1 is synergistically inhibited by Lys and S-adenosyl-l-methionine (SAM). SAM appears to increase the apparent affinity of AK1 for Lys (Curien *et al*., 2007).

The activity of AK-HSDHs is feedback-inhibited by Thr (Muehlbauer *et al*., 1994; Paris *et al*., 2003; Rognes *et al*., 2003; Curien *et al*., 2005). This inhibition is mediated through the two Thr-binding ACT domains between the AK and HSDH functional domains (Chipman and Shaanan, 2001; Jander and Joshi, 2009). Other amino

acids have been found to either inhibit or activate AK-HSDHs, but most are not normally found at sufficient concentrations in the chloroplast (Paris *et al*., 2002b; Rognes *et al*., 2002; Paris *et al*., 2003; Curien *et al*., 2005).

In the second step in the ADAA biosynthesis pathway, Asp 4-phosphate is converted to Asp semialdehyde, the branching-point intermediate for Lys biosynthesis and Met and Thr biosynthesis (Paris *et al.*, 2002a). The committing step leading to Lys biosynthesis is performed by dihydrodipicolinate synthase (DHDPS). In *A. thaliana*, there are two DHDPSs, both of which are feedback-inhibited by Lys (Vauterin and Jacobs, 1994; Vauterin *et al*., 1999; Craciun *et al*., 2000; Sarrobert *et al*., 2000). This reaction is considered to be the primary point of regulation for Lys biosynthesis because DHDPSs are more sensitive to Lys than AKs (Vauterin *et al*., 2000; Galili, 2002).

The committing step leading to Met and Thr biosynthesis is the formation of homoserine from Asp semialdehyde. In addition to AK-HSDHs, this step may be performed in *A. thaliana* by a putative mono-functional HSDH — HSDH1 (Vieler *et al*., 2012). Unlike AKs or AK-HSDHs, mono-functional HSDHs do not contain any ACT domains and are not subject to feedback inhibition by Lys or Thr (Walter *et al*., 1979; Schroeder *et al*., 2010). However, it has not yet been demonstrated that this enzyme is functional or that it is localized to the chloroplast, the location of ADAA biosynthesis (Coruzzi and Last, 2000).

Homoserine kinase (HSK) converts homoserine to homoserine 4-phosphate, the final branching-point intermediate in the ADAA biosynthesis pathway (Lee and Leustek, 1999). Cystathionine γ-synthase (CGS) and Thr synthase (TS) compete for this substrate for Met and Thr biosynthesis, respectively (Amir *et al*., 2002). SAM, a catabolite of Met,

partly controls the flux of this branching-point by inhibiting the translation of CGS and allosterically activating TS (Curien *et al*., 1998; Laber *et al*., 1999; Amir *et al*., 2002; Chiba *et al*., 2003; Onouchi *et al*., 2004).

Some enzymes have other forms of regulation as well. Matthews *et al.* (1989) found that the carrot HSDH interconverts between homodimer and homotrimer forms. This appears to be a form of regulation because only the homotrimer is sensitive to Thr. This HSDH enzyme was later shown to be a dual-functional AK-HSDH enzyme (Wilson *et al.*, 1991).

Certain mutations are often associated with amino acid accumulation

Increasing the amino acid content of important crop plants has long been a major goal of traditional breeding and genetic engineering (Galili *et al*., 2008; Ufaz and Galili, 2008; Jander and Joshi, 2009). In recent years, there have been many studies on the effects of reducing feedback inhibition, introducing feedback-resistant enzymes, overexpressing particular enzymes, and some loss-of-function mutations (Azevedo *et al*., 1997 and references therein; Azevedo and Arruda, 2010). A number of these studies have discovered or created plants with accumulated amino acids.

Mutants containing an AK with decreased Lys-sensitivity often accumulate high levels of Thr (Dotson *et al*., 1990; Rognes *et al*., 1983; Frankard *et al*., 1991). This is thought to occur due to increased flux into the ADAA biosynthesis pathway, generating more Asp semialdehyde to be used in the Lys and/or Thr branches. Because DHDPS is very sensitive to Lys, Lys does not accumulate. Similarly, Met biosynthesis is feedback-inhibited by SAM and Met; Met either does not accumulate or accumulates to a lesser degree than Thr. If a Lys-insensitive DHDPS is introduced into a plant, Lys accumulates

while the levels of Thr and other ADAAs usually decrease, likely due to the increased flux into the Lys branch (Frankard *et al*., 1992; Kwon *et al.*, 1995; Bittel *et al.*, 1996). When both AK and DHDPS are less sensitive to Lys, there is an even greater accumulation of Lys (Frankard *et al*., 1992; Shaul and Galili, 1993; Falco *et al.* 1995).

Previously, our lab discovered that *A. thaliana* plants with loss-of-function mutations in the *AK-HSDH2* gene cause an increase in downstream products Asp, Thr, Met, cysteine (Cys), and Ile (see Chapter II). This is of great interest because most Thr-accumulation findings have been due to a decrease in the sensitivity of AK to Lys. There have been very few accumulation studies on Thr-sensitive AK or HSDH isozymes such as AK-HSDH2. It is also possible that the loss of an AK or HSDH isozyme causes increases in downstream products. The purpose of our research is to understand why *ak-hsdh2* mutants accumulate downstream amino acids.

Experimental overview

To better understand how *ak-hsdh2* mutants accumulate downstream amino acids, we measured and compared the overall AK and HSDH activities in mutant and wild-type *A. thaliana*. We also attempted to determine the contribution of each *A. thaliana* AK and HSDH isozyme to their respective reactions. Furthermore, we measured the leaf free amino acid content for 1, 2, 3, and 4-week-old wild type and mutants with loss-of-function mutations in *AK-HSDH2* and/or another *AK* or *HSDH* gene. This will enable us to test if the accumulation occurs gradually over time.

Methods

Plant materials, genotyping, and growth conditions

A. thaliana T-DNA lines (SALK_003685, SALK_019023, SALK_043533, SALK_059678, SALK_072653, SALK_073176, SALK_082155, SALK_125957, and WiscDsLox461-464J6) used in this study were obtained from the Arabidopsis Biological Resource Center (Columbus, OH). All of them are in the Columbia (Col) ecotype (Alonso *et al.*, 2003; Woody *et al.*, 2007). Homozygosity was confirmed by PCR, as described in Ajjawi *et al*. (2011). Briefly, leaf DNA was archived on Whatman FTA™ cards (GE Healthcare Life Sciences, Little Chalfont, United Kingdom), then punched and washed as directed by the manufacturer. For each gene to be genotyped, two PCR reactions were performed. The first reaction had primers designed to detect wild-type DNA sequences. The second reaction used primers designed to detect T-DNA insertions. Homozygous mutants would only amplify the second reaction, as visualized on an agarose gel. A list of genotyping primers can be found in Appendix B.

Mutant and wild-type *A. thaliana* plants were grown in a growth chamber on a 12-h-light/12-h-dark photoperiod for tissue harvesting or 16-h-light/8-h-dark photoperiod for crossing and seed harvesting. The light intensity was 90-110 μmol photons m^{-2} s^{-1}, the temperature was 20°C, and the relative humidity was 50%. Unless otherwise stated, plants used for activity assays and free amino acid measurements were four weeks old.

Crossing

Single *ak* and *hsdh* mutants were crossed with *ak-hsdh2-1* mutants to generate double mutants. Prior to crossing, both mother and father plants were genotyped to

ensure homozygosity. Flowers from mother plants were masked by removing all sepals, petals, and stamens with fine-point forceps when the petals had just started to emerge from their bud. Once the stigma became sticky, masked flowers were pollinated by rubbing detached flowers from father plants against the stigma. Mature seeds were collected, grown, and selected for double-heterozygosity via genotyping. Seeds from double-heterozygotes were collected, grown, and selected for double-homozygosity.

Enzyme extraction for AK and HSDH activity assays

The aerial parts of plants were harvested and stored at -80°C. Protein samples for AK and HSDH activity assays were prepared as described by Di Marco and Grego (1975) and Matthews *et al.* (1975). Leaf tissues were frozen in liquid nitrogen and ground into a fine powder. Soluble proteins were homogenized in the extraction buffer (0.2 M Tris-HCl (pH 8.5), 0.1 M KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1.4 mM 2-mercaptoethanol, 5 mM Thr, and 30% (v/v) glycerol) in a ratio of 2.5 mL/g fresh weight. Residual ungrounded material was removed by filtering through two layers of cheesecloth and centrifuging at 6°C for 30 min at 20,000 g. Protein extracts were partially purified by adding ammonium sulfate to 50% saturation (0.27 g/mL) and shaking on ice for 30 - 60 min. Proteins were collected by centrifuging at 6°C for 10 min at 3,000 rpm and re-dissolved in the resuspension buffer (50 mM potassium phosphate (pH 7.5), 1.4 mM 2-mercaptoethanol, 1 mM EDTA, and 20% (v/v) glycerol). To prevent the loss of HSDH activity, the resuspension buffer for protein samples to be used in HSDH activity assays contained 0.5 mM Thr (Bryan, 1969). The protein concentration was determined using the Bradford method (Bio-Rad, Hercules, CA), with bovine serum albumin as the standard.

AK in-solution activity assay

The AK activity was quantified using the hydroxyamate-ferric chloride method, as described by Dotson *et al.* (1989) and Paris *et al.* (2002b). The product of the AK reaction, Asp 4-phosphate, has an ester group that undergoes a substitution reaction with hydroxylamine (Pechere and Capony, 1968). This results in the formation of a hydroxamate acid. When ferric chloride is added, the iron binds to the hydroxamate acid and induces a color change (Pechere and Capony, 1968). The reaction mixtures contained 100 mM Tris-HCl (pH 8.0), 400 mM NH_2OH-HCl, 515 mM KOH, 20 mM $MgCl_2$, 150 mM KCl, 40 mM adenosine-5'-triphosphate (ATP), and 100 μL of enzyme extracts. To determine the activities contributed by mono-functional AKs, which are sensitive to Lys inhibition, and the activities contributed by dual-functional AK-HSDHs, which are sensitive to Thr inhibition, a set of reactions were run in the presence of 20 mM Thr and/or 20 mM Lys. The reactions were initiated by the addition of 50 mM Asp in a final reaction volume of 0.5 mL. After a 1-h incubation at 37°C, the assays were stopped by the addition of 0.25 mL of 20% TCA, 370 mM $FeCl_3$, and 360 mM HCl. To prepare the blank reactions, Asp was omitted during the enzyme incubation and added to the reaction mixture immediately before the reaction was stopped. Stopped reactions were vortexed briefly and centrifuged for 15 min at 10,000 g. The absorbance of the supernatant at 505 nm was measured immediately after centrifuging. An extinction coefficient of 750 $M^{-1}cm^{-1}$ was used to convert absorbance to enzyme activity (1 unit = 1 μmol / min) (Paris *et al.*, 2002b).

HSDH in-solution activity assay

The HSDH activity was quantified in the reverse direction by measuring the rate

of nicotinamide adenine dinucleotide phosphate ($NADP^+$) reduction (Matthews *et al.*, 1975; Paris *et al.*, 2002b). The reaction mixtures contained 100 mM Tris-HCl (pH 8.0), 150 mM KCl, 50 mM homoserine, 1 mM $NADP^+$, and 112.5 μL of enzyme extracts in a final volume of 0.75 mL. To determine the effects of Thr on the HSDH activity, the assays were performed at three Thr concentrations: 0, 1000, and 5000 μM. Reactions were initiated by the addition of enzyme extracts, which had been warmed to room temperature and centrifuged briefly to remove suspended particles. Reactions were allowed to proceed for 2 min before the absorbance at 340 nm was measured every 15 sec for 3 min. An extinction coefficient of 6250 $M^{-1}cm^{-1}$ was used to convert absorbance to enzyme activity (1 unit = 1 μmol / min) (Paris *et al.*, 2002b).

AK in-gel activity assay

The AK activity was also semi-quantitatively measured with the calcium phosphate precipitation method (Walter *et al.*, 1979; Dotson *et al.*, 1989). Proteins were separated using native PAGE, where the separating gel contained 375 mM Tris-HCl (pH 8.5), 8% (w/v) acrylamide, 0.1% (v/v) Triton X-100, 15% (v/v) glycerol, 0.1% ammonium persulfate, 10 μM Thr, and 0.035% tetramethylethylenediamine (TEMED). The gel was equilibrated overnight at 4°C in 0.112 M Tris (pH 6.4), 0.112 M acetate, 0.1% (v/v) Triton X-100, and 15% (v/v) glycerol. The electrophoresis buffer included 2 mM Thr and 1 mM 2-mercaptoethanol. After electrophoresis, the gel was incubated in calcium phosphate buffer (25 mM bis-tris propane, 20% (v/v) glycerol, 75 mM KCl, 50 mM $MgCl_2$, 100 mM $CaC1_2$, 10 mM ATP, and 70 mM Asp) for up to 3 hours at room temperature. The product of the AK reaction, Asp 4-phosphate, has a half-life of 30 min at 30°C and breaks down into aspartic acid and inorganic phosphate (Relton *et al.*, 1988).

The latter compound is then trapped in the gel as insoluble calcium phosphate (Nimmo and Nimmo, 1982). White precipitates indicate enzyme activity, excluding the leading edge that contains inorganic phosphate from the enzyme extract (Relton *et al.*, 1988; Nimmo and Nimmo, 1982).

HSDH in-gel activity assay

The HSDH activity was also semi-quantitatively measured with the nitroblue tetrazolium (NBT) precipitation method (Matthews *et al.*, 1975; Walter *et al.*, 1979). Proteins were separated using native PAGE, where the separating gel contained 375 mM Tris-HCl (pH 8.5), 8% (w/v) acrylamide, 0.1% (v/v) Triton X-100, 15% (v/v) glycerol, 0.1% ammonium persulfate, 100 μM Thr, and 0.035% TEMED. The gel was equilibrated overnight at 4°C in 125 mM Tris and 1.25 M glycine. The electrophoresis buffer included 2 mM Thr and 1 mM 2-mercaptoethanol. After electrophoresis, the gel was incubated in the dark at 30°C for 90 min in 60 mM Tris-HCl (pH 9), 0.15 mM EDTA, 0.21 mM 2-mercaptoethanol, 150 mM KCl, 32 mM L-homoserine, 0.266 mg/mL NBT, 0.025 mg/mL phenazine methosulfate, and 0.96 mM $NADP^+$. During the oxidation of NADPH, electrons are transferred to NBT (Esfandiari *et al.*, 2003). This reduces NBT to formazan, which is insoluble (Gordon *et al.*, 1973; Esfandiari *et al.*, 2003). Blue precipitates indicate enzyme activity.

Leaf free amino acid assay by HPLC-MS/MS

Leaf tissues for free amino acid measurement were harvested into 2-mL microfuge tubes containing a single 3-mm stainless steel ball. Leaf samples were immediately frozen with dry ice and ground to a fine powder for 2 min on a TissueLyser

II bead mill (Qiagen, Valencia, CA). Free amino acids were extracted and analyzed with the HPLC-MS/MS method (Lu *et al.*, 2008). For quantification, mixtures of 20 protein amino acids, gamma-aminobutyric acid, anthranilate, homoserine, hydroxyproline, and S-methyl methionine of varying concentrations plus L-phenylalanine-α,β,β,2,3,4,5,6-d8 (Phe-d8) and L-valine-2,3,4,4,4,5,5,5-d8 (Val-d8) of 0.9 μM were analyzed along with the leaf samples.

Accession numbers

Sequences for genes mentioned in this work can be found in the GenBank database under the following accession numbers: *AK1*, At5g13280; *AK2*, A5g14060; *AK3*, At3g02020; *AK-HSDH1*, At1g31230; *AK-HSDH2*, At4g19710; *HSDH1*, At5g21060.

Results

Overall AK activity is not significantly reduced in ak-hsdh2 *mutants*

The relative contributions of AK-HSDH1, AK-HSDH2, and the three mono-functional AKs to the overall AK reaction were determined by comparing the in-solution AK activity of wild-type and *ak-hsdh2* partially-purified protein extracts (Figure 3.1). In an uninhibited environment, wild-type extracts have an average activity of 3.11 units/g total protein. When AK-HSDH1 and AK-HSDH2 are abolished by 20 mM Thr, the overall AK activity in wild-type samples decreases by 0.83 units/g (27%). Abolishing the mono-functional AKs with 20 mM Lys decreases wild-type activity by 1.85 units/g (59%). In the presence of both 20 mM Lys and 20 mM Thr, wild-type activity decreases by 3.02 units/g (97%). The contribution of AK-HSDH2 can be estimated as 0.22 units/g

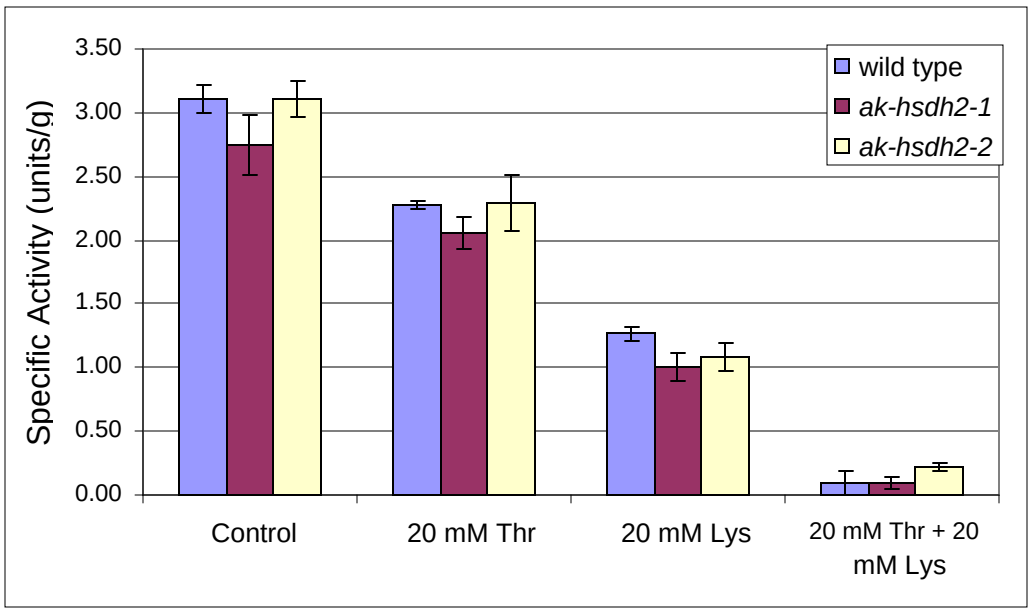


Figure 3.1. In-solution AK activity under non-inhibiting and inhibiting conditions. AK activity was measured with the hydroxyamate-ferric chloride method in wild-type, *ak-hsdh2-1*, and *ak-hsdh2-2* plants. Mono-functional AK and dual-functional AK-HSDH activities can be abolished by addition of 20 mM Lys and 20 mM Thr, respectively. Values are presented as mean ± SE (n = 3). The change in activity was not significant between the mutant and the wild type.

(7%) by comparing Lys-inhibited wild-type and average *ak-hsdh2* activities. In all four environments, there is no significant difference in the overall AK activity between the wild type and *ak-hsdh2*.

The AK in-gel assay also shows that a large amount of activity is performed by the AKs and very little by the AK-HSDHs (Figure 3.2). However, it appears that wild-type activity is greater than *ak-hsdh2*, but the lanes are overloaded with protein and this result has not been replicated. In contrast, the in-solution assay results have been replicated and are considered more reliable than the in-gel assay results.

Overall HSDH activity is significantly reduced in ak-hsdh2 *mutants*

The relative contribution of AK-HSDH2 to the overall HSDH reaction was

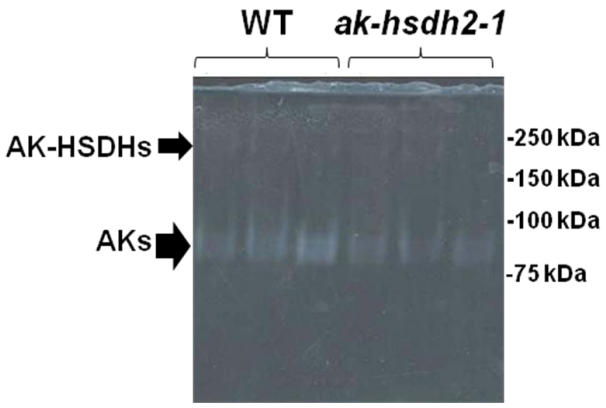


Figure 3.2. In-gel AK activity in wild-type and *ak-hsdh2-1* plants. The AK activity was assayed with the calcium phosphate precipitation method. The lanes were loaded on an equal protein basis. White precipitates indicate AK activity. Most of the AK activity is associated with the mono-functional AK isozymes.

estimated by comparing the uninhibited in-solution HSDH activities of wild-type and *ak-hsdh2* partially-purified protein extracts (Figure 3.3). It appears that AK-HSDH2 contributes between 0.82 (in *ak-hsdh2-2*) and 1.4 units/g (in *ak-hsdh2-1*), which accounts for 15-25% of the total wild-type activity (5.52 units/g). The decrease in overall uninhibited activity is statistically significant in *ak-hsdh2-2* mutants. Overall HSDH activity was also measured in the presence of 1 or 5 mM Thr; the activity in both mutants was significantly less than in wild type. Our in-gel assay also supports that overall HSDH activity is significantly reduced in *ak-hsdh2-1* mutants (Figure 3.4).

Calculation of relative contributions by AK-HSDH1 and HSDH1

The HSDH activities of AK-HSDH1 and AK-HSDH2 are differentially inhibited by Thr (Curien *et al.*, 2005), while monofunctional HSDH1 is not. According to Curien *et al.* (2005), AK-HSDH1 retains ~40% of its activity in 1 mM Thr, while AK-HSDH2 retains ~95%. In 5 mM Thr, AK-HSDH1 and AK-HSDH2 retain ~20% and 75%, respectively. These retention values for AK-HSDH1 can be used to estimate the relative

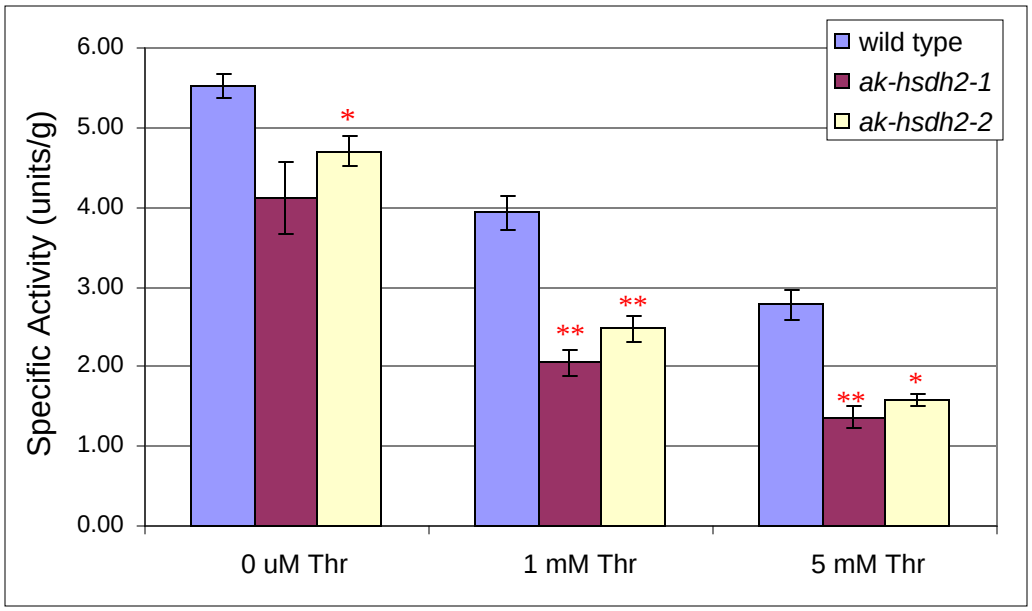


Figure 3.3. In-solution HSDH activity under non- and partially-inhibiting conditions. HSDH activity was measured with the $NADP^+$ reduction method in wild-type, *ak-hsdh2-1*, and *ak-hsdh2-2* plants. Dual-functional AK-HSDH activity was partially-inhibited by addition of 1 or 5 mM Thr. The HSDH activity is decreased in *ak-hsdh2* mutants at all Thr concentrations. Values are presented as mean ± SE (n = 3). Asterisks indicate significant differences between the mutant and the wild type (Student's *t* test; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

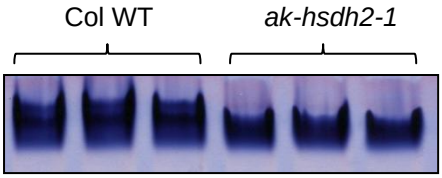


Figure 3.4. In-gel HSDH activity in wild-type and *ak-hsdh2-1* plants. The HSDH activity was assayed with the nitroblue tetrazolium precipitation method. The lanes were loaded on an equal protein basis. Blue precipitates indicate HSDH activity. The HSDH activity is decreased in *ak-hsdh2* mutants.

contributions of AK-HSDH1 and HSDH1. We can construct equations where the left side of each equation is the total HSDH activity at a given concentration of Thr, and the right side is the sum of the enzyme activities (A) retained at that concentration. The equations for *ak-hsdh2-1* are

$$\begin{cases} 2.05 = A_{HSDH1} + 0.4\ A_{AK\text{-}HSDH1} & (1) \\ 1.37 = A_{HSDH1} + 0.2\ A_{AK\text{-}HSDH1}\,. & (2) \end{cases}$$

Solving these two equations yield activity contribution estimates of 3.40 and 0.69 units/g for AK-HSDH1 and HSDH1, respectively.

These estimates can be validated by comparing their sum (4.09 units/g) with the total uninhibited *ak-hsdh2-1* activity (4.12 ± 0.45 units/g). Percent contributions can be obtained by dividing these estimates by the total uninhibited activity. In *ak-hsdh2-1* mutants, we find that AK-HSDH1 contributes ~83% of the overall HSDH activity, while HSDH1 contributes ~17%. In the wild type, we find that AK-HSDH1 contributes ~62% and HSDH1 contributes ~13%. Similar results can be derived using *ak-hsdh2-2*, but at the validation step, they are found to be within 1-2 standard deviations of the mean.

An alternative method of calculating the wild-type percent contributions of the individual isozymes is by constructing of system of equations for wild-type activity. In the wild type, there are three enzymes that contribute to the overall HSDH activity: HSDH1, AK-HSDH1, and AK-HSDH2. Therefore, we constructed three equations for wild type, utilizing all three tested environments:

$$\begin{cases} 5.52 = A_{HSDH1} + A_{AK\text{-}HSDH1} + A_{AK\text{-}HSDH2} & (3) \\ 3.94 = A_{HSDH1} + 0.4\ A_{AK\text{-}HSDH1} + 0.95\ A_{AK\text{-}HSDH2} & (4) \\ 2.78 = A_{HSDH1} + 0.2\ A_{AK\text{-}HSDH1} + 0.75\ A_{AK\text{-}HSDH2} & (5) \end{cases}$$

When these equations are solved, we find that HSDH1, AK-HSDH1, and AK-HSDH2 contribute -0.28, 2.35, and 3.45 units/g, respectively. Repetitions of this experiment have consistently generated a negative contribution by HSDH1 from the wild-type system of equations.

The ak-hsdh2 *mutants accumulate Lys and Met at younger stages*

Leaf free amino acid content was measured for 1, 2, 3, and 4-week-old wild-type *A. thaliana* and mutants with loss-of-function mutations in *AK-HSDH2* and/or another ADAA biosynthesis-related gene (Figures 3.5–3.7). In congruence with our previous findings, mature *ak-hsdh2* leaves accumulate high levels of Thr. However, when this mutation is accompanied by a loss-of-function mutation in *AK1* or *AK2*, the amount of Thr accumulated is significantly reduced. When accompanied by a loss-of-function mutation in *AK3* or *HSDH1*, Thr accumulation still occurs.

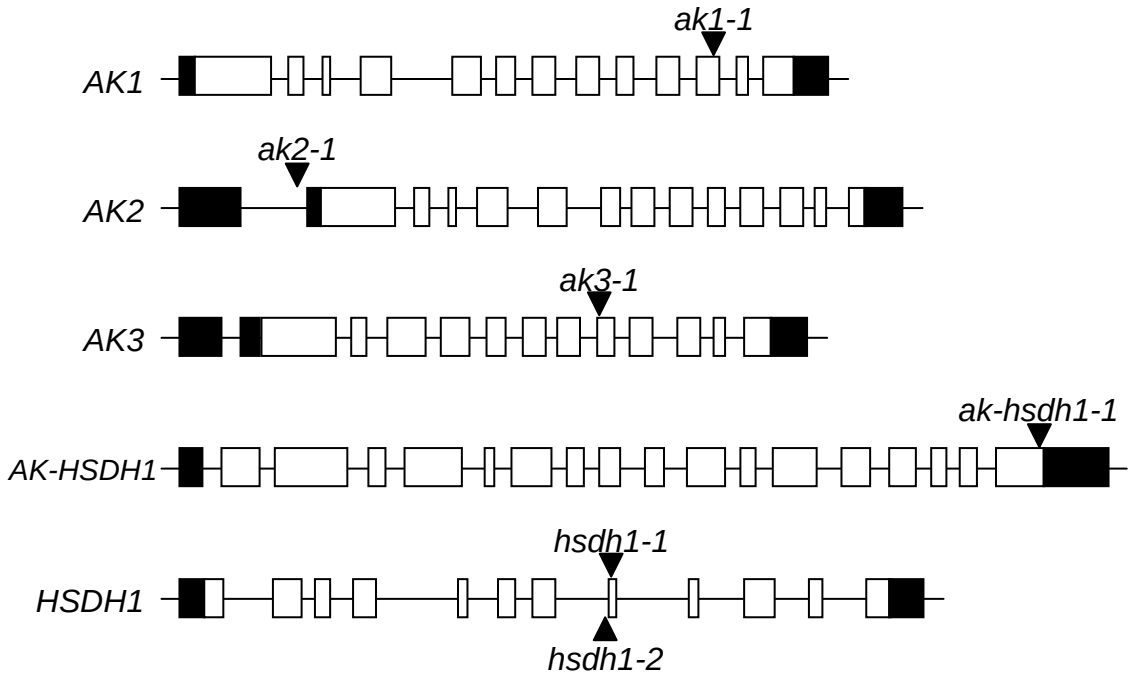


Figure 3.5. Schematic representation of the *AK1, AK2, AK3, AK-HSDH1,* and *HSDH1* genes and six T-DNA insertion alleles. Black rectangles represent 5' and 3' untranslated regions; white rectangles represent exons; solid lines represent introns and intergenic regions; black triangles represent T-DNA insertions. Plant lines: *ak1-1*, WiscDsLox461-464J6; *ak2-1*, SALK_003685; *ak3-1*, SALK_043533; *ak-hsdh1-1*, SALK_125957; *hsdh1-1*, SALK_072653; *hsdh1-2*, SALK_073176.

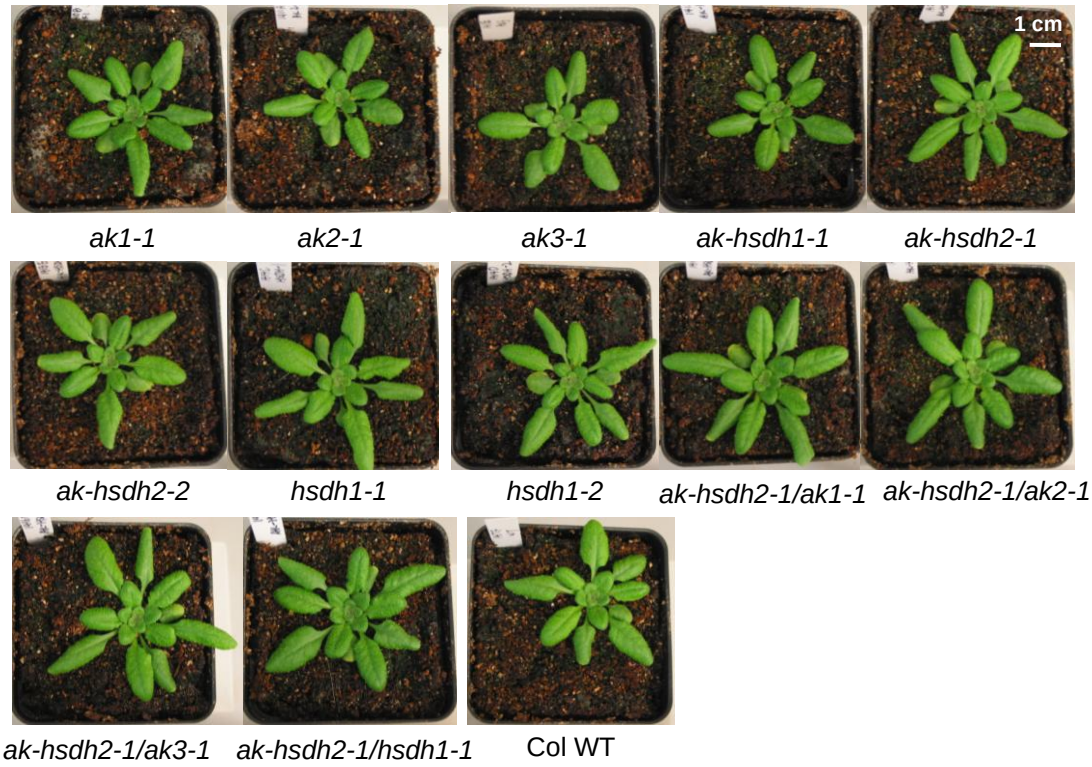
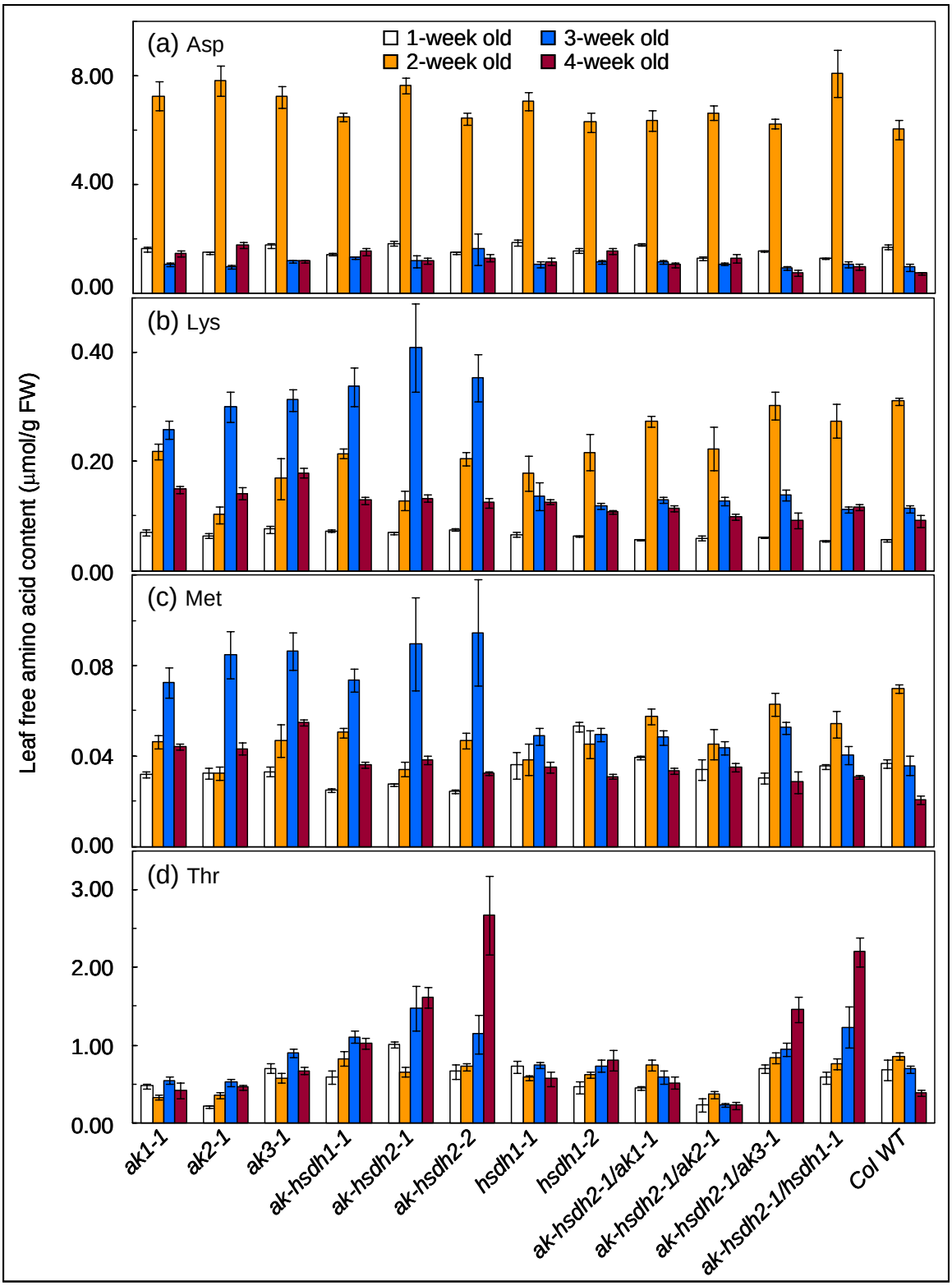


Figure 3.6. Images of 4-week-old wild-type and single and double mutant *A. thaliana* plants. Plants were grown under a 12-h-light/12-h-dark photoperiod. All 13 lines are morphologically similar.

Asp levels were very high at week one, somewhat lower at week two, and very low at three and four weeks. The difference in Asp level between the wild type and most mutants was only statistically significant at one or four weeks. At four weeks, there was an average of 60-70% more Asp in *ak-hsdh2* leaves than in wild-type leaves.

In addition, we found that in wild-type leaves, there is a significant peak in Lys concentration at week two, after which the concentration remains relatively constant. Similarly, the concentration of Met in wild-type leaves peaks at week two, but there is a significant decrease in Met from week three to week four. In *ak-hsdh2* mutants, the Lys and Met peaks are shifted from week two to week three. At week four, the concentration of Met is greater in both *ak-hsdh2* mutants than in the wild type. Single mutants of the


(a) Asp
1-week old
2-week old
3-week old
4-week old
8.00
4.00
0.00
(b) Lys
0.40
0.20
0.00
(c) Met
0.08
0.04
0.00
(d) Thr
3.00
2.00
1.00
0.00
Leaf free amino acid content (µmol/g FW)
ak1-1
ak2-1
ak3-1
ak-hsdh1-1
ak-hsdh2-1
ak-hsdh2-2
hsdh1-1
hsdh1-2
ak-hsdh2-1/ak1-1
ak-hsdh2-1/ak2-1
ak-hsdh2-1/ak3-1
ak-hsdh2-1/hsdh1-1
Col WT

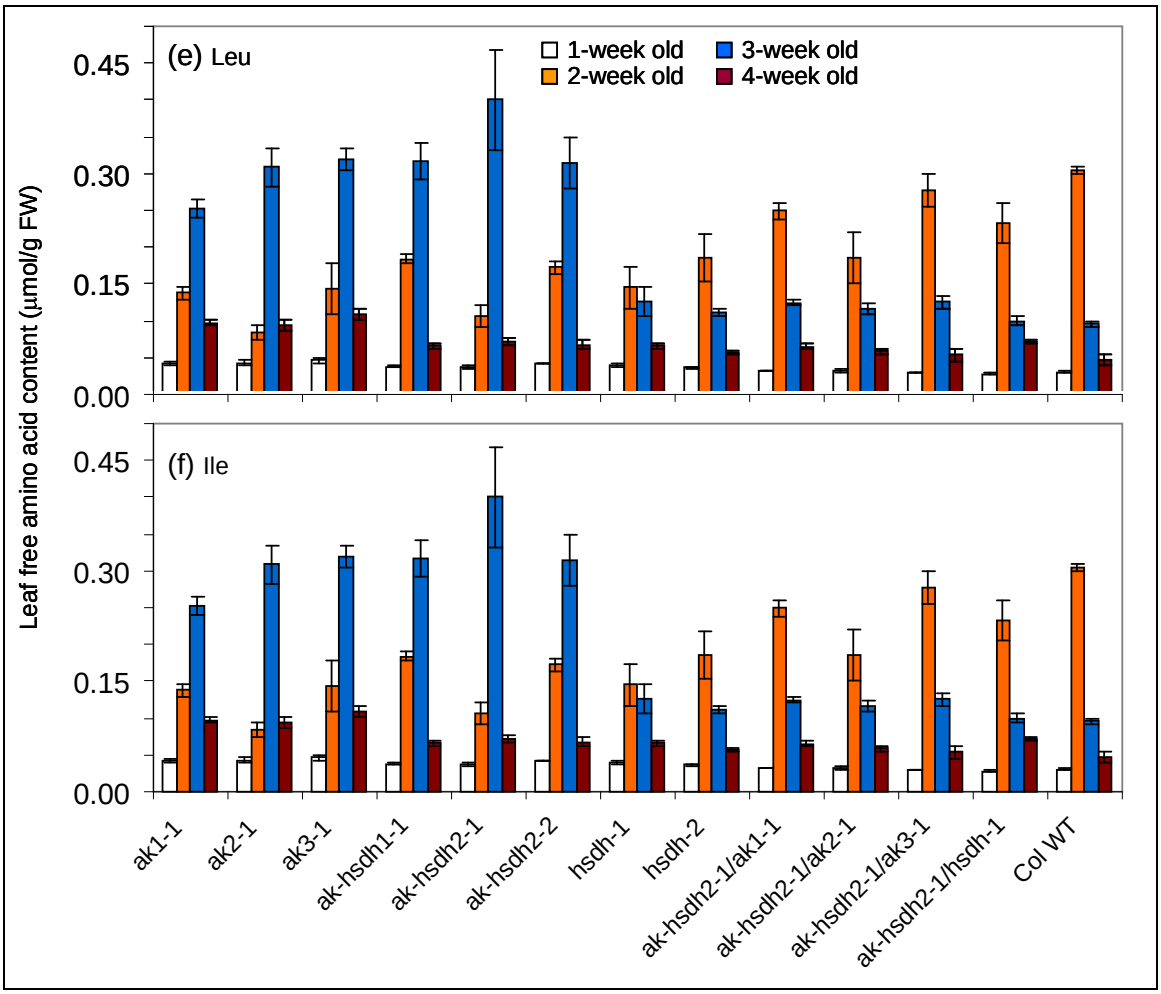


Figure 3.7. Leaf Asp, Lys, Met, Thr, Leu, and Ile contents in 1-, 2-, 3-, and 4-week-old plants. Values are presented as mean ± SE (n = 5). White bars represent 1-week-old plants; yellow bars represent 2-week-old plants; blue bars represent 3-week-old plants; dark red bars represent 4-week-old plants.

other AKs have Met and Lys concentration trends similar to *ak-hsdh2* mutants. However, when the deficiency in AK is accompanied with a loss-of-function mutation in the *AK-HSDH2* gene, the Met and Lys concentration trends resemble wild-type trends. Profiling results for Leu and Ile follow the same pattern as Lys, for all lines tested.

Discussion

Either AK-HSDH2 does not contribute much to the overall AK activity, or the activity of other AKs is up-regulated to compensate for the loss of AK-HSDH2

There is no significant difference between wild-type and *ak-hsdh2* AK activity from 4-week-old plants in any of our assays. In wild-type *A. thaliana* leaves, most of the activity (~59%) appears to be performed collectively by the mono-functional AKs, while AK-HSDH1 contributes ~20% and AK-HSDH2 only contributes ~7%. Furthermore, the AK activity of AK-HSDH2 is very sensitive to Thr inhibition (Curien *et al.*, 2005). Mature wild-type leaves have an average of 0.38 mM Thr (Figure 3.7). This concentration was found to inhibit the AK activity of AK-HSDH2 by ~95% (Curien *et al.*, 2005). Thus, *ak-hsdh2* mutants would not have impaired AK activity because AK-HSDH2 was never a significant contributor. Alternatively, the plant may be capable of up-regulating the activity of other AKs to compensate for the loss of AK-HSDH2.

AK-HSDH2 is an important contributor of the overall HSDH activity

Our HSDH activity assays show there is a significant decrease in overall HSDH activity in 4-week-old *ak-hsdh2* mutants over a range of Thr concentrations (0 to 5 mM Thr). This range encompasses wild-type and *ak-hsdh2* mutant levels of Thr (0.38 and 1.6-2.7 mM, respectively). The loss of AK-HSDH2 activity in the *ak-hsdh2-1* mutants resulted in a 50% reduction of overall activity in the presence of 1 or 5 mM Thr (Figure 3.3).

We estimate that AK-HSDH2 performs up to 25% of the overall HSDH activity in an uninhibited environment. This percentage may be greater *in vivo*; it has not yet been demonstrated whether mono-functional HSDH1 is located in the chloroplast or otherwise

involved in ADAA biosynthesis (Sainis *et al*., 1981; Coruzzi and Last, 2000). Additionally, the HSDH activity of AK-HSDH1 is more sensitive to Thr than AK-HSDH2. At wild-type levels of Thr, AK-HSDH1 only retains ~60% of its potential activity, while AK-HSDH2 retains ~95% (Curien *et al.*, 2005). Consequently, we propose that AK-HSDH2 is a significant contributor of the overall HSDH activity *in vivo*.

Possible explanations for a negative contribution of HSDH activity by HSDH1

When equations are constructed using our wild-type HSDH activity assay data and estimations of percent activity retained under Thr inhibition (Curien *et al.*, 2005), the solution consistently assigns a negative activity contribution to mono-functional HSDH1. We propose two possible explanations of this phenomenon.

First, the three equations for wild-type HSDH activity (Equations 3-5) were constructed under the assumption that the individual enzymes do not affect each other. However, it is possible that AK-HSDH1 and AK-HSDH2 are able to form heterooligomers with different regulatory properties than homooligomers. Several dual-functional plant AK-HSDH enzymes, including *A. thaliana* AK-HSDH2, have been observed to form homooligomers (Wilson *et al*., 1991; Azevedo *et al*., 1992; Paris *et al*., 2002b). In carrots, homodimers and homotrimers have different sensitivities to Thr (Matthews *et al*., 1989; Wilson *et al*., 1991). It is possible that heterooligomers may be another form of regulation. Further studies need to be done to investigate whether AK-HSDH1 and AK-HSDH2 have the ability to form heterooligomers and how the AK and HSDH activities of the heterogoligomers are regulated by downstream amino acids, such as Lys and Thr.

A second explanation is that A_{HSDH1} is negative because our HSDH activity assay

measures activity in the reverse direction. It is possible that the mono-functional HSDH1 is more efficient in the forward direction and more sensitive to the presence of Asp semialdehyde. Hence, if HSDH activity was measured in the forward direction, the system of equations would generate positive activity contributions for all three enzymes. In this case, our current estimations of activity contribution may be inaccurate. Preliminary in-solution activity assays of *hsdh1-1* mutants support this hypothesis; their activity is greater than wild-type.

The amounts of AK and HSDH isozymes might be in excess in the wild type

Loss-of-function mutations in the *AK-HSDH2* gene do not cause any reduction in ADAAs. This indicates that the amounts of AK and HSDH isozymes might be in excess in wild-type *A. thaliana* leaves and the reactions are limited by the substrates. Consistent with this hypothesis, in the presence of 100 µM Lys and 380 µM Thr (*i.e.*, at physiological concentrations of Lys and Thr in wild-type *A. thaliana* leaves), ~5% of the AK activity from AK1 is inhibited, >95% of the AK activities from dual-functional AK-HSDHs, AK2, and AK3 are inhibited, ~50% of the HSDH activity from AK-HSDH1 is inhibited, and ~5% of the HSDH activity from AK-HSDH2 is inhibited (Curien *et al*., 2005; Curien *et al*., 2006).

Because DHDPS, the enzyme for the committing step leading to Lys biosynthesis, is more sensitive to Lys than the mono-functional AKs, Lys-sensitive AK activity is never completely diminished under wild-type levels of Lys (Vauterin *et al*., 2000; Galili, 2002). We found that *ak-hsdh2* mutants produce only slightly more Lys than the wild type (Figure 3.7). Similarly, HSDH1 is insensitive to Thr. Consequently, compensation of the AK and HSDH activities in the *ak-hsdh2* mutants may change how the pathway is

regulated by downstream products, leading to the accumulation of Lys, Met, Thr, and Ile.

The accumulation of ADAAs in the ak-hsdh2 *mutants is a dynamic process*

Peak levels of Lys and Met in *ak* mutants are shifted to week three from week two in the wild type (Figure 3.7). This indicates that compensation for a missing AK isozyme takes time and explains why amino acid accumulation occurs in 3- and 4-week-old plants. In barley and maize, the HSDH activity becomes less sensitive to Thr as the plant ages (Matthews *et al*., 1975; Sainis *et al.*, 1981). A similar phenomenon may occur in *A. thaliana* and contribute to the accumulation.

Although we proposed a mechanism for this accumulation, there are still several unanswered questions. For instance, if the plant is able to compensate for a missing AK isozyme, why do *ak* and *ak-hsdh1* mutants not accumulate Thr to the same degree as *ak-hsdh2*? Among ADAAs, why does Thr have the most evident increase in the *ak-hsdh2* mutants? Also, why do *ak* and *ak-hsdh2* single mutants have delayed peak levels of Lys and Met, but *ak-hsdh2-1/ak1-1* and *ak-hsdh2-1/ak2-1* double mutants revert to the wild-type levels of Lys and Met?

In conclusion, *ak-hsdh2* mutants have increased levels of Asp, Thr, Met, Cys, and Ile in 4-week-old leaves. Met and Lys accumulation peaks in 3-week-old mutants while Thr accumulation peaks in 4-week-old mutants. The over-accumulation of ADAAs in the mutants could be the result of the combination of different biological events. First, AK-HSDH2 is not a major contributor of the overall AK activity. Therefore, the loss of AK-HSDH2 activity did not lead to a reduction of overall AK activity and the flux into the biosynthesis of Lys, Met, and Thr. Second, the amounts of AK and HSDH isozymes might be in excess in the wild type. Therefore, a reduction in the overall HSDH activity

did not result in a reduction in the flux into the biosynthesis of ADAAs. Third, the accumulation of ADAAs in the *ak-hsdh2* mutants is a dynamic process. Furthermore, the overall flux into this pathway might be elevated, which is consistent with the increase in Asp, Lys, Met, and Thr contents in 3- and 4-week-old mutants. Understanding how loss-of-function mutations affect the ADAA biosynthesis pathway may provide insights into the development of novel approaches to increase Lys, Met, and Thr content in plant-based food.

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Appendix A

Predicted genes in the biosynthetic pathways of Asp-derived, aromatic and branched-chain amino acids in CCMP1779

Appendix A. Predicted genes in the biosynthetic pathways of Asp-derived, aromatic, and branched-chain amino acids in CCMP1779

Description	Name	ID
Biosynthesis of Asp-derived amino acids		
Asp kinase	AK	CCMP1779|3150
Asp kinase - homoserine dehydrogenase	AK-HSDH*	CCMP1779|10446
Homoserine dehydrogenase	HSDH	augustus_masked-nanno_5085-abinit-gene-0.2-*****
Asp semialdehyde dehydrogenase	ASD	CCMP1779|11914
Asp semialdehyde dehydrogenase	ASD	CCMP1779|6330
Dihydrodipicolinate synthase	DHDPS	CCMP1779|11357
Dihydrodipicolinate reductase	DHDPR**	CCMP1779|4610
Diaminopimelate aminotransferase	DAPAT	augustus_masked-nanno_2338-abinit-gene-0.3-*****
Diaminopimelate epimerase	DAPE**	CCMP1779|10579
Diaminopimelate decarboxylase	DAPDC	CCMP1779|2505
Diaminopimelate decarboxylase	DAPDC	CCMP1779|8817
Homoserine kinase	HSK	CCMP1779|8751
Cystathionine gamma synthase	CGS	CCMP1779|10900
Cystathionine gamma synthase	CGS	CCMP1779|4094
Cystathionine beta lyase	CBL	CCMP1779|9018
Cystathionine beta lyase	CBL	CCMP1779|9158
Cystathionine beta lyase	CBL	CCMP1779|3085
Homocysteine S-methyltransferase	HMT	CCMP1779|1690
Cobalamin-dependent Met synthase	MetH	CCMP1779|2985
Cobalamin-independent Met synthase	MS	CCMP1779|5185
Cobalamin-independent Met synthase	MS	CCMP1779|8229
Thr synthase	TS***	CCMP1779|10068
Thr synthase	TS**	CCMP1779|4990
Biosynthesis of aromatic amino acids		
3-Deoxy-D-arabino-heptulosonate-7-phosphate synthase	DAHPS	CCMP1779|7898
Dehydroquinate synthase	DHQS	augustus_masked-nanno_505-abinit-gene-0.2-mRNA-1*****
Dehydroquinate dehydratase - shikimate dehydrogenase	DHQDH-SDH*	CCMP1779|5703
Shikimate kinase	SK	CCMP1779|10591
5-Enolpyruvylshikimate-3-phosphate synthase	EPSPS	CCMP1779|6038
Chorismate synthase	CS***	CCMP1779|8941
Chorismate synthase	CS***	CCMP1779|2899
Chorismate mutase	CM	CCMP1779|234
Arogenate dehydratase - arogenate dehydrogenase	ADT-ADH*	CCMP1779|11837

Appendix A—Continued

Description	Name	ID
Biosynthesis of aromatic amino acids		
Anthranilate synthase alpha and beta subunits	ASA-ASB*	CCMP1779|4984
Anthranilate phosphoribosyltransferase	PRT	CCMP1779|4197
Indole-3-glycerol phosphate synthase - phosphoribosylanthranilate isomerase	IGPS-PAI*	CCMP1779|5546
Indole-3-glycerol phosphate synthase	IGPS****	CCMP1779|398
Trp synthase alpha and beta subunits	TSA-TSB*	CCMP1779|102
Biosynthesis of branched-chain amino acids		
Thr deaminase	TD	CCMP1779|7883
Acetohydroxyacid synthase	AHAS	CCMP1779|3427
Ketolacid reductoisomerase	KARI	CCMP1779|6007
Dihydroxyacid dehydratase	DHAD	CCMP1779|4633
Dihydroxyacid dehydratase	DHAD***	CCMP1779|7539
Isopropylmalate synthase	IPMS***	CCMP1779|4567
Isopropylmalate isomerase large subunit	IPMIL	CCMP1779|2773
Isopropylmalate isomerase small subunit	IPMIS***	CCMP1779|5262
Isopropylmalate dehydrogenase	IMD	CCMP1779|7869
Branched-chain aminotransferase	BCAT	CCMP1779|8799
Branched-chain aminotransferase	BCAT***	CCMP1779|5665
Branched-chain aminotransferase	BCAT**	CCMP1779|10881

Genes in the biosynthetic pathways of Asp-derived, aromatic and branched-chain amino acids were manually annotated. For each *N. oceanica* locus, the gene function was predicted based on BLAST similarity to homologous genes in *A. thaliana*. The accuracy of gene function prediction was evaluated by PFAM domain support and reverse blast searches of *A. thaliana* homologs with *N. oceanica* genes.
* These genes are predicted to be fused with other genes in the same pathway in *N. oceanica*.
**These predicted genes are missing the start codon.
***These predicted genes are missing the stop codon.
****This IPGS gene appears to be fused with some unrelated gene(s).
*****This gene model is from augustus or snap gene annotation and was found superior to the final maker annotation after manual examination.

Appendix B

Primers used in this study

Appendix B. Primers used in this study

Sequence	Purpose
5'-AGCCGAGCTAGCTTATTTTGG-3'	genotyping primer for AK1_At5g13280
5'-TTGCCAAGTCTGGGTTGTATC-3'	genotyping primer for AK1_At5g13280
5'-CTCCAAGCTCATGAGCAGTTC-3'	genotyping primer for AK2_A5g14060
5'-TTGAATCTGAACCGTCCATTC-3'	genotyping primer for AK2_A5g14060
5'-TGAGATCGGGATTATCACCAC-3'	genotyping primer for AK3_At3g02020
5'-TGATCAAGCTCCTGTCACATG-3'	genotyping primer for AK3_At3g02020
5'-AGCTGTTCTAGAATCGGGAGC-3'	genotyping primer for AK-HSDH1_At1g31230
5'-TTCCAGTCCAGAATCTTGTGC-3'	genotyping primer for AK-HSDH1_At1g31230
5'-TGCTCCTTTTTATTAAATATCAAATCG-3'	genotyping primer for AK-HSDH2_At4g19710
5'-AAAACCCTTCTGTTTGTGCAG-3'	genotyping primer for AK-HSDH2_At4g19710
5'-TTGGATGAGCAGCTTAGAACC-3'	genotyping primer for AK-HSDH2_At4g19710
5'-TGTTCCAACACACAATTCCAG-3'	genotyping primer for AK-HSDH2_At4g19710
5'-CTCTCATCTTGTCTTCTGCGC-3'	genotyping primer for HSDH1_At5g21060
5'-AGTCGAGCAAGAATCAAACCC-3'	genotyping primer for HSDH1_At5g21060
5'-CTCTCATCTTGTCTTCTGCGC-3'	genotyping primer for HSDH1_At5g21060
5'-AGTCGAGCAAGAATCAAACCC-3'	genotyping primer for HSDH1_At5g21060
5'-TGGTTCACGTAGTGGGCCATCG-3'	genotyping primer for SALK lines
5'-AACGTCCGCAATGTGTTATTAAGTTGTC-3'	genotyping primer for WiscDsLox lines
5'-CTCGTACCAGAACCTCTAAA-3'	QPCR primer for AK-HSDH2_At4g19710
5'-CACCACTCCAACATATCTCA-3'	QPCR primer for AK-HSDH2_At4g19710
5'-GTATTGTAGAAGAAGCCACAG-3'	QPCR primer for TS1_At4g29840
5'-TTGATTCCTCAGCTTGAACA-3'	QPCR primer for TS1_At4g29840
5'-TGGATGTACTATTAGCGGAG-3'	QPCR primer for HSK1_At2g17265
5'-CACAGAAGCAACAGATTTCA-3'	QPCR primer for HSK1_At2g17265
5'-AATTAGGACGATTGAGCAGG-3'	QPCR primer for RABD1_At3g11730
5'-CTTTCCATCTCAGTACAGTCA-3'	QPCR primer for RABD1_At3g11730
5'-GAGATTGTTGTGAAGGAGAAG-3'	QPCR primer for NADBP_At5g58750
5'-CCTCTTAAACCCAAACCTATC-3'	QPCR primer for NADBP_At5g58750
5'-TTTAGTTTCGGTGTACTAGTCC-3'	QPCR primer for PKSP_At5g58750
5'-TTCAACACTTCTCCTTCTCT-3'	QPCR primer for PKSP_At5g58750
5'-CAAAGGCCAACAGAGAGAAGA-3'	QPCR primer for ACTIN2_At3g18780
5'-ATCACCAGAATCCAGCACAA-3'	QPCR primer for ACTIN2_At3g18780