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Title: Analysis of loss-of-function mutants in *aspartate kinase* and *homoserine dehydrogenase* genes points to complexity in the regulation of aspartate-derived amino acid contents

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Summary: *Loss-of-function mutations in aspartate kinase and aspartate kinase-homoserine dehydrogenase genes cause changes in downstream amino acids.*
Footnote:

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ABSTRACT

Biosynthesis of aspartate-derived amino acids lysine, methionine, threonine, and isoleucine involves mono-functional aspartate kinases (AKs) and dual-functional aspartate kinase-homoserine dehydrogenases (AK-HSDHs). Four-week-old loss-of-function Arabidopsis thaliana mutants in the AK-HSDH2 gene had increased amounts of aspartate and aspartate-derived amino acids, especially threonine, in leaves. To explore mechanisms behind this phenotype, we obtained single mutants for other AK and AK-HSDH genes, generated double mutants from ak-hsdh2 and ak mutants, and performed free and protein-bound amino acid profiling, transcript abundance, and activity assays. The increases of aspartate, lysine, and methionine in ak-hsdh2 were also observed in ak1-1, ak2-1, ak3-1, and ak-hsdh1-1. However, the threonine increase in ak-hsdh2 was observed in ak-hsdh1-1, but not in ak1-1, ak2-1, or ak3-1. Activity assays demonstrated that AK2 and AK-HSDH1 are the major contributors to overall AK and HSDH activities, respectively. Pairwise correlation analysis revealed positive correlations between the amount of AK transcripts and Lys-sensitive AK activity, and between the amount of AK-HSDH transcripts and both Thr-sensitive AK activity and total HSDH activity. In addition, the ratio of total AK activity to total HSDH activity negatively correlates with the ratio of lysine to the total amount of methionine, threonine, and isoleucine. These data led to the hypothesis that the balance between lysine-sensitive AKs and threonine-sensitive AK-HSDHs is important for maintaining the amounts and ratios of aspartate-derived amino acids.
INTRODUCTION

Humans and other animals rely on plants as a dietary source of essential amino acids (Coruzzi and Last, 2000; Galili et al., 2008). In major field crops, such as corn, rice, soybean, and potato, Asp-derived amino acids Lys, Met, Thr, and Ile are present at levels that limit the nutritional values (Müntz et al., 1998; Debabov, 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). Genetic improvement requires a better understanding of the pathways and regulation of Asp-derived amino acids. The first step of the biosynthetic pathway for Asp-derived amino acids 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 1) (Jander and Joshi, 2009). Asp-4-phosphate is then converted to Asp semialdehyde, the branch-point intermediate for Lys or Met, Thr, and Ile biosynthesis. The committing step leading to Met, Thr, and Ile biosynthesis is the formation of homoserine from Asp semialdehyde, which is catalyzed by dual-functional AK-HSDHs.

Higher plants have multiple genes that encode AKs and AK-HSDHs. For example, the Arabidopsis thaliana nuclear genome contains three AK-encoding genes (AK1, At5g13280; AK2, A5g14060; AK3, At3g02020) and two AK-HSDH-encoding genes (AK-HSDH1, At1g31230; AK-HSDH2, At4g19710) (Jander and Joshi, 2009). Public microarray data indicated that the five enzymes are expressed in all plant tissues (Schmid et al., 2005; Winter et al., 2007). Transcription of these enzymes is repressed by bZIP transcription factors in dark and other low sugar conditions (Ufaz et al., 2011). In addition, both AKs and AK-HSDHs are subject to feedback inhibition by their downstream products. For example, the activity of mono-functional 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). The inhibition is mediated through the two Lys-binding ACT2 domains at the C-terminus of the proteins (Supplemental Figure S1) (Chipman and Shaanan, 2001; Jander and Joshi, 2009). The activity of AK-HSDHs is feedback inhibited by Thr (Muehlbauer et al., 1994; Rognes et al., 2002; Paris et al., 2003; Curien et al., 2005). The inhibition is
mediated through the two Thr-binding ACT2 domains in the middle of the proteins (Supplemental Figure S1) (Chipman and Shaanan, 2001; Jander and Joshi, 2009).

Genetic improvement of Asp-derived amino acid contents in crop plants is complicated and will benefit from the knowledge of the relative importance of different isozymes when overlapping functions exist.
(Galili et al., 2008). However, most of the previous studies on AKs and AK-HSDHs focused on kinetic properties and allosteric regulation of purified enzymes in the absence of other isozymes (Dotson et al., 1989, 1990; Rognes et al., 2002; Curien et al., 2005; Curien et al., 2007). In addition to allosteric regulation, enzyme abundance is key for determining the relative importance of individual enzymes. Therefore, it is difficult to predict the relative significance of individual isozymes solely based on the kinetic properties of the enzymes. On the contrary, characterization of a series of loss-of-function single and double \textit{ak} and \textit{ak-hsdh} mutants will help to elucidate the contributions of individual AK and AK-HSDH enzymes to the biosynthesis of Asp-derived amino acids and how plants respond to the loss of individual enzymes. Understanding how plants adjust resources among competing branches within Asp-derived amino acid biosynthesis in response to the loss of an AK enzyme and/or an AK-HSDH enzyme, will provide additional insights into metabolic regulation of branched pathways in general. Because mono-functional AKs catalyze the committing step leading to Asp-derived amino acid biosynthesis (Figure 1), one would expect that loss-of-function mutations in the \textit{AK} genes result in reduced amounts of Asp-derived amino acids. Similarly, because dual-functional AK-HSDHs catalyze the committing step in Asp-derived amino acid biosynthesis as well as the branching step leading to Met, Thr, and Ile biosynthesis (Figure 1), one would anticipate that loss-of-function mutations in the \textit{AK-HSDH} genes cause reductions in the contents of Asp-derived amino acids, especially Met, Thr, and Ile.

In this work, we described the identification and analysis of two previously uncharacterized Arabidopsis mutants with high levels of Asp and Asp-derived amino acids, especially Thr, in leaves. This observation was further investigated with free and protein-bound amino acid profiling, transcript abundance, and activity assays on a series of single and double loss-of-function \textit{ak} and \textit{ak-hsdh} mutants. Pairwise Pearson’s correlation analysis revealed that the ratio of total AK activity to total HSDH activity negatively correlates with the ratio of Lys to the total amount of Met, Thr and Ile. These results suggested that the balance between Lys-sensitive mono-functional AKs and Thr-sensitive dual-functional AK-HSDHs is important for maintaining the levels and ratios of Asp-derived amino acids.
RESULTS

A T-DNA Mutant Line with Increased Leaf Thr Content

In an attempt to identify functions for chloroplast proteins and mechanisms for processes occurring in the chloroplast, thousands of Arabidopsis T-DNA lines of nuclear-encoded, chloroplast-targeted genes were analyzed for a variety of phenotypes, including leaf and seed free amino acid contents using an HPLC-Tandem Mass Spectrometry method (Lu et al., 2008; Ajjawi et al., 2010; Lu et al., 2011a; Lu et al., 2011b; Lu et al., 2011c; Bell et al., 2012). This screen identified a homozygous mutant (SALK_082155) in the Columbia (Col, CS60000) background with high leaf Thr content (Supplemental Figure S2a). Analysis of the public T-DNA Express database (http://signal.salk.edu/cgi-bin/tdnaexpress; O’Malley and Ecker, 2010) revealed that this mutant contains a T-DNA insertion in the single exon of the At1g55805 gene (Supplemental Figure S2b), which encodes a BolA-like family protein. A second mutant (SALK_059678) in the At1g55805 gene did not have an increased level of Thr in leaves (Supplemental Figure S2a). These results indicated that the phenotype in SALK_082155 is caused by a second-site mutation.
To identify the mutation in SALK_082155 that caused the increases in Asp-derived amino acids, a mapping population was made by crossing a SALK_082155 plant to Landsberg erecta (Ler) wild type. A total of 181 F₂ plants were analyzed for leaf free amino acid contents (Supplemental Figure S2c-d). DNA
from 11 F2 plants with the highest Thr contents and 13 F2 plants with the lowest Thr contents were extracted and used for array mapping. The output from array mapping showed that the mutation is located in a 1.75 mega base pair (Mb) region of chromosome 4 (At4g17440 to At4g21580, Supplemental Figure
Based on gene descriptions of the 492 genes in this region, one candidate gene (AK-HSDH2, At4g19710) was identified. This gene encodes a dual-functional Asp kinase-homoserine dehydrogenase. Targeted genomic DNA sequencing showed that there is a thymine (T) insertion in the fourth exon of the
Mutations in the AK-HSDH2 gene cause the Thr increase.

(a) 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.

(b) Fold change of Thr content in Col wild type and the two mutants. The values (mean ± SE, n = 5) are presented as the ratios to the Thr content in Col wild type. 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).

(c) Relative amounts of the AK-HSDH2 transcript in Col wild type and the two mutants. Total RNA was extracted from mature leaves and analyzed with quantitative RT-PCR. The level of the AK-HSDH2 transcript was normalized to that of the ACTIN2 transcript (At3g18780). The values (mean ± SE, n = 3 - 6) are presented as the ratios to the transcript level in Col wild type. Asterisks indicate significant differences between the mutant and the Col wild type (Student’s t test; *, p < 0.05).

(d) Representative immunoblot of the AK-HSDH2 protein and Coomassie Brilliant Blue G250 stained gel showing the Rubisco large subunit (RbsL, loading control). The lanes were loaded on an equal protein basis.

(e) Images of four-week-old Col wild-type and ak-hsdh2 plants under a 12-h-light/12-h-dark photoperiod.

a predicted truncated protein of 241 amino acids (aa) (Figure 2) instead of the wild-type 961 aa protein.
The truncated protein is predicted to be missing part of the AK domain (aa^{88-371}), the two Thr-binding ACT2 domains (aa^{411-471} and aa^{491-552}), and the HSDH domain (aa^{564-906}).

**The Phenotype Is Confirmed in a Second ak-hsdh2 Mutant**

To confirm that the phenotype in SALK_082155 is caused by the single-base insertion in the AK-HSDH2 gene, we analyzed leaf amino acid contents in a second homozygous mutant (SALK_019023) of the same gene. SALK_019023 has a T-DNA insertion in the last intron of the AK-HSDH2 gene (Figure 2). SALK_019023 was renamed as ak-hsdh2-1. The mutant with a single-base insertion in the AK-HSDH2 gene after genetic background cleanup (SALK_082155) was renamed as ak-hsdh2-2. As shown in Figure 2b, the amount of Thr in four-week-old ak-hsdh2 mutants was four to seven times higher than that in the wild type at the same age. Quantitative reverse transcription (RT)-PCR showed that the AK-HSDH2 transcript was completely abolished in ak-hsdh2-1 and substantially reduced in ak-hsdh2-2 (Figure 2c). The reduction of the AK-HSDH2 transcript in the ak-hsdh2-2 mutant could be due to a nonsense-mediated decay pathway (Kalyna et al., 2011). Although there was still a small amount of the AK-HSDH2 transcript in the ak-hsdh2-2 mutant, the transcript is frame-shifted and the corresponding protein is truncated and probably not functional (Figure 2a). Consistent with the quantitative RT-PCR data, mature AK-HSDH2 protein was detected in wild-type Arabidopsis plants, but was undetectable in either ak-hsdh2 mutant (Figure 2d-e). These results revealed that loss-of-function mutations in the AK-HSDH2 gene cause an increased level of Thr.

**Isolation and Generation of Other ak and ak-hsdh Single and Double Mutants**

To further understand the regulation of mono-functional AKs and dual-functional AK-HSDHs, we obtained ak1-1 (WiscDsLox461-464J6), ak2-1 (SALK_003685), ak3-1 (SALK_043533), and ak-hsdh1-1 (SALK_125957) single mutants (Figure 3), and generated three double homozygous mutants: ak-hsdh2-1 ak1-1, ak-hsdh2-1 ak2-1, and ak-hsdh2-1 ak3-1. Attempts to make the double homozygous ak-hsdh2-1 ak-hsdh1-1 mutant were not successful, thus this combination was not analyzed. About one fifth to one
fourth of seeds from ak-hsdh2-1/ak-hsdh2-1 AK-HSDH1-1/ak-hsdh1-1 plants did not develop into normal seeds, suggesting that the double homozygous ak-hsdh2-1 ak-hsdh1-1 mutant is not viable (Supplemental Figure S3). The six single mutants and three double mutants appeared to be healthy and normal (Figure 2e and Figure 3b).

Free and Protein-Bound Amino Acids in Four-week-old ak and/or ak-hsdh Mutants
We determined free amino acid contents in mature rosette leaves from four-week-old wild type, and single and double mutants. The contents of Asp, Lys, and Met in \(ak1-1\), \(ak2-1\), \(ak3-1\), \(ak\)-hsdh1-1, \(ak\)-hsdh2-1, and \(ak\)-hsdh2-2 were significantly higher than in the wild type (Figure 4). On average, these amino acids increased 93%, 56%, and 101%, respectively, in the six single mutants as compared to the wild type (Figure 4). The Ile level in \(ak1-1\), \(ak3-1\), \(ak\)-hsdh2-1 and \(ak\)-hsdh2-2 was also significantly higher than in the wild type (Figure 4). Similar to the two \(ak\)-hsdh2 mutants, the \(ak\)-hsdh1-1 mutant had an increased amount of Thr (Figure 4). The total amount of Asp-derived amino acids behaved similarly to Thr, which is not surprising since Thr is the most abundant Asp-derived amino acid in Arabidopsis leaves (Figure 4).

The near-unanimous increases of Asp, Lys, and Ile in the single mutants were not observed in the double mutants at the same age; only \(ak\)-hsdh2-1 \(ak2-1\) was found to have increased Asp content (Figure 4). The Thr increase observed in \(ak\)-hsdh1-1, \(ak\)-hsdh2-1, and \(ak\)-hsdh2-2 single mutants was absent in \(ak\)-hsdh2-1 \(ak1-1\) and \(ak\)-hsdh2-1 \(ak2-1\) double mutants (Figure 4). However, the \(ak\)-hsdh2-1 \(ak3-1\) double mutant still had a high amount of Thr (280% higher than the wild type), and all three double mutants had increased Met content (Figure 4).

In addition to Asp and Asp-derived amino acids, the contents of Asn, Gln, Glu, His, and Leu showed significant increases in the six four-week-old single mutants (Supplemental Figure S4). These amino acids did not show corresponding changes in the three double mutants at the same age (Supplemental Figure S4). Furthermore, the contents of Ala, Gly, Phe, Pro, Trp, and Tyr demonstrated significant increases in four-week-old \(ak1-1\), \(ak2-1\), and \(ak3-1\) mutants (Supplemental Figure S4). These changes were less evident or not observed in \(ak\)-hsdh1-1, \(ak\)-hsdh2 mutants, and the three double mutants (Supplemental Figure S4). Consequently, the total amount of free amino acids was substantially increased in the six single mutants, but it remained unchanged in the three double mutants (Supplemental Figure S4). These observations demonstrated that perturbation in Asp-derived amino acid biosynthesis may cause changes in the contents of other amino acids.
Because free amino acids represent a minor proportion of the total amino acids in plants, we determined the amounts of protein-bound amino acids in the leaves of four-week-old plants after acid hydrolysis. During acid hydrolysis, Asn and Gln are converted to Asp and Glu, respectively, and Met is partially oxidized (Anders, 2002). Therefore, Asn and Asp were reported collectively, Glu and Gln were shown together, and Met was not reported. The only statistically significant differences in these amino acids

**Figure 4.** The contents of free Asp and Asp-derived amino acids in leaves of four-week-old plants. (a) Asp; (b) Lys; (c) Met; (d) Thr; (e) Ile; (f) the total amount of Asp-derived amino acids Lys, Met, Thr and Ile. Values are presented as mean ± SE (n = 5). Values not connected by the same letter are significantly different (Student’s t-test, p < 0.05). Values connected by the same letter are not significantly different.
between the mutants and the wild type were decreases in Asn+Asp and Gln+Glu contents in \(ak3-1\) and \(ak-hsdh1-1\) (Supplemental Table S1).

**Free Amino Acid Profiling in One-, Two-, and Three-Week-Old Plants**

To further investigate the dynamics of Asp-derived amino acid metabolism, we measured leaf free amino acid contents in one-, two-, and three-week-old wild-type and mutant plants. Due to the relative small plant size, the entire above-ground portion of one-week-old plants was used for free amino acid assay and the \(ak1-1\), \(ak3-1\), \(ak-hsdh1-1\), \(ak-hsdh2-1\), and \(ak-hsdh2-2\) mutants showed significant increases in the Lys content (Supplemental Table S2). For two-week-old plants, the first two true leaves were used and the six single mutants showed significant reductions in Lys, Met and Ile contents (Supplemental Table S3). For three-week-old plants, the second, third, and fourth true leaves were used and the six single mutants exhibited significant increases in the contents of Lys, Met, and Ile (Supplemental Table S4), similar to the same mutants at four weeks old (Figure 4). Unlike other Asp-derived amino acids, the Thr increase was only observed in the \(ak-hsdh1-1\), \(ak-hsdh2-1\), and \(ak-hsdh2-2\) mutants among three-week-old plants.

Many of these changes in Asp-derived amino acids were not observed in the three double mutants. For example, the Lys increase in the six one-week-old single mutants was not observed in the three double mutants at the same age (Supplemental Table S2). Also, the unanimous decreases of Lys, Met, and Ile contents in the six two-week-old single mutants were only observed in some double mutants at the same age (Supplemental Table S3). None of the three-week-old mutants showed increases of Lys, Met, or Ile content, unlike the six single mutants at the same age (Supplemental Table S4). Taken together, the amino acid data from plants at
multiple growth stages consistently showed that changes of Asp-derived amino acids in the \textit{ak} and \textit{ak-hsdh} single mutants were often not observed in the double mutants.

**Quantitative RT-PCR Analysis of Select Genes Involved in the Biosynthesis and Catabolism of Asp-derived Amino Acids**

To investigate the transcript levels of the five \textit{AK} or \textit{AK-HSDH} genes in the wild type and nine \textit{ak} and/or \textit{ak-hsdh} mutants, we performed quantitative RT-PCR in mature rosette leaves from four-week-old plants.

In the wild type, the \textit{AK2} transcript was more abundant than \textit{AK1} and \textit{AK3}, and the \textit{AK-HSDH1} transcript was more abundant than \textit{AK-HSDH2} (Figure 5). In the \textit{ak1-1} mutant, the \textit{AK1} transcript was completely abolished, which was accompanied by a 46\% increase in \textit{AK3}, and a 22\% increase in \textit{AK-HSDH2} (Figure 5). Overall, there was no significant change in total \textit{AK} or \textit{AK-HSDH} transcripts in \textit{ak1-1}. In the \textit{ak2-1} mutant, there was a 63\% decrease in \textit{AK2}, a 43\% decrease in total \textit{AK} transcripts, and an 11\% decrease in total \textit{AK-HSDH} transcripts (Figure 5). In the \textit{ak3-1} mutant, there was an 87\% reduction in \textit{AK3}, but because \textit{AK3} is the least abundant \textit{AK} transcript, there was no significant change in total \textit{AK} transcripts (Figure 5). In the \textit{ak-hsdh1-1} mutant, there was a 78\% decrease in \textit{AK-HSDH1}, a 47\% increase in total \textit{AK} transcripts, and a 56\% decrease in total \textit{AK-HSDH} transcripts (Figure 5). In the \textit{ak-hsdh2-1} mutant, the \textit{AK-HSDH2} transcript was completely abolished, and there was a 67\% decrease in total \textit{AK-HSDH} transcripts with no net change in total \textit{AK} transcripts (Figure 5). In the \textit{ak-hsdh2-2} mutant, there was a 90\% decrease in \textit{AK-HSDH2}, a 36\% increase in total \textit{AK} transcripts, and a 55\% decrease in total \textit{AK-HSDH} transcripts (Figure 5). Overall, some significant changes in the transcript levels of other \textit{AK} or \textit{AK-HSDH} gene(s) were observed in the six single mutants. However, loss-of-function mutations in one \textit{AK} or \textit{AK-HSDH} gene did not cause systematic up- or down-regulation of other \textit{AK} or \textit{AK-HSDH} gene(s).

In the \textit{ak-hsdh2-1 ak1-1} double mutant, both \textit{AK1} and \textit{AK-HSDH2} transcripts were completely abolished, and there was a 64\% decrease in total \textit{AK-HSDH} transcripts (Figure 5). However, there was no net change in total \textit{AK} transcripts in this double mutant possible because the \textit{AK2} transcript is much more abundant
In the ak-hsdh2-1 ak2-1 double mutant, the AK-HSDH2 transcript was completely abolished, the AK2 transcript was reduced by 62%, total AK transcripts were reduced by 49%, and total AK-HSDH transcripts were reduced by 61% (Figure 5). In the ak-hsdh2-1 ak3-1 double mutant, the AK-
HSDH2 transcript was completely abolished, the AK3 transcript was reduced by 83%, total AK-HSDH transcripts were reduced by 46%, and there was no significant change in total AK transcripts (Figure 5). To summarize, among the three double mutants, ak-hsdh2-1 ak2-1 showed the most pronounced reductions in the amounts of AK and AK-HSDH transcripts.

We investigated the transcript levels of other biosynthetic genes in the nine mutants. The DHDPS1 and DHDPS2 genes encode dihydrodipicolinate synthases, the branch-point enzymes leading to Lys biosynthesis. There were significant increases in total DHDPS transcripts in ak1-1 and ak3-1, but no significant change was observed in other single or double mutants (Supplemental Figure S5). The CGS1 and TS1 transcripts encode cystathionine gamma synthase and Thr synthase, which are the branch-point enzymes leading towards Met and Thr biosynthesis, respectively (Figure 1). The CGS1 and TS1 transcripts did not show significant changes in any of the mutants (Supplemental Figure S5). The TD1 gene encodes Thr deaminase, the branch-point enzyme towards Ile biosynthesis (Figure 1). There were significant increases in the TD1 transcript level in all six single mutants, but there was no significant change in the three double mutants (Supplemental Figure S5).

We also analyzed the transcript levels of select catabolic genes in the nine mutants. The LKR-SDH1 gene encodes a Lys catabolic enzyme: lysine-ketoglutarate reductase-saccharopine dehydrogenase (Figure 1). The LKR-SDH1 transcript level was significantly increased in the ak-hsdh2-1 and ak-hsdh2-2 single mutants and the ak-hsdh2-1 ak1-1 and ak-hsdh2-1 ak2-1 double mutants (Supplemental Figure S6). The SAMS1, SAMS2, SAMS3, and SAMS4 genes encode S-adenosylmethionine (SAM) synthases, which convert Met to SAM (Figure 1). The total amount of SAMS transcripts was increased in ak1-1 and ak2-1, but decreased in ak-hsdh2-1, ak-hsdh2-2, ak-hsdh2-1 ak1-1, and ak-hsdh2-1 ak2-1 (Supplemental Figure S6). The THA1 and THA2 genes encode Thr aldolases, which convert Thr to Gly (Figure 1). The total amount of THA transcripts was only significantly increased in ak-hsdh2-1 (Supplemental Figure S6). Although branched-chain aminotransferases
(BCATs) are involved in both biosynthesis and catabolism of branched-chain amino acids Ile, Leu, and Val, BCAT1 was shown to initiate catabolism (Schuster and Binder, 2005). The BCAT1 transcript level demonstrated universal decreases in the six single mutants and the ak-hsdh2-1 ak1-1 and ak-hsdh2-1 ak2-1 double mutants (Supplemental Figure S6). Taken together, loss-of-function mutations in the AK and/or AK-HSDH gene(s) can result in changes to related anabolic and catabolic genes, such as a near-unanimous decrease of the BCAT1 transcript.

**AK2 Is the Major Contributor to the Overall AK Activity**

To dissect the relative importance of different AK and AK-HSDH isozymes, we measured the overall AK activity in leaf protein extracts from the wild type and six single mutants. In the absence of allosteric effectors, the ak1-1, ak2-1, ak3-1, ak-hsdh1-1, and ak-hsdh2 mutants demonstrate 17%, 56%, 22%, 34%, and 15 to 22% reductions in the overall AK activity, respectively (Figure 6). If we use the average reduction of the two ak-hsdh2 mutants and scale the total amount of reductions caused by different mutations in the five genes to 100%, the individual contributions from AK1, AK2, AK3, AK-HSDH1, and AK-HSDH2 can be calculated as 12%, 38%, 15%, 23%, and 12%, respectively. Thus, the group contributions of AKs and AK-HSDHs were 65% and 35%, respectively. Taken together, AK2 is the major contributor to the overall AK activity, and mono-functional AKs collectively contribute more to the overall AK activity than dual-functional AK-HSDHs, which is consistent with their relative transcript abundance in leaves (Figure 5).
AKs are feedback-inhibited by Lys, and AK-HSDHs are feedback-inhibited by Thr. In addition, Curien et al. (2005) reported that the AK activity of AK-HSDH1 and AK-HSDH2 is activated by Ala, Cys, Ile, Ser, and Val, and inhibited by Leu (Curien et al., 2005). Among the six additional effectors, Ala is the most...
abundant in chloroplast stroma (Curien et al., 2005). Moreover, Curien et al. (2007) reported that AK1 is synergistically inhibited by Lys and SAM (Curien et al., 2007). Therefore, we measured the AK activity in near-physiological concentrations of Lys, Thr, Ala, and SAM, which are 70 μM, 300 μM, and 400 μM, respectively.

**Figure 6.** AK and HSDH activities in leaf protein extracts.
(a) The AK activity measured with the hydroxamate-ferric chloride method.
(b) The HSDH activity measured with the NADP+ reduction method. Values are represented as mean ± SE (n = 3). The AK and HSDH activities in physiological concentrations of effectors were measured in the presence of 70 μM Lys, 300 μM Thr, 400 μM Ala and 20 μM SAM. Values not connected by the same letter are significantly different (Student’s t-test, p < 0.05). Values connected by the same letter are not significantly different.

Moreover, Curien et al. (2007) reported that AK1 is synergistically inhibited by Lys and SAM (Curien et al., 2007). Therefore, we measured the AK activity in near-physiological concentrations of Lys, Thr, Ala, and SAM, which are 70 μM, 300 μM, and 400 μM, respectively.
and 20 μM, respectively (Curien et al., 2009). Under this condition, the mutation in AK1, AK2, AK3, AK-HSDH1, or AK-HSDH2 gene reduced the overall AK activity by 28%, 55%, 17%, 21%, and 27 to 35%, respectively (Figure 6a). In the same way as we calculated the AK activity measured in the absence of allosteric effectors, we can calculate the individual contributions from AK1, AK2, AK3, AK-HSDH1, and AK-HSDH2 to be 18%, 36%, 11%, 14%, and 20%, respectively, and the group contributions of AKs and AK-HSDHs to be 66% and 34%. This is in close agreement with the AK activity data measured in the absence of allosteric effectors.

Simultaneous Loss of AK-HSDH2 and Mono-functional AK1 or AK2 Has an Additive Effect on the Reduction of the Overall AK Activity

To test the impact of simultaneous loss of AK-HSDH2 and a mono-functional AK on the overall AK activity, we measured the AK activity in ak-hsdh2-1 ak1-1, ak-hsdh2-1 ak2-1, and ak-hsdh2-1 ak3-1 double mutants. Because the overall AK activity is contributed by both mono-functional AKs and dual-functional AK-HSDHs, one would expect that simultaneous loss of the AK-HSDH2 activity and a mono-functional AK activity would have an additive effect on the reduction of the overall AK activity. Indeed, in the absence of allosteric effectors, the overall AK activity in the ak-hsdh2-1 ak1-1 double mutant was 18% and 20% lower than that in the ak1-1 and ak-hsdh2-1 single mutants, respectively (Figure 6a). Under the same condition, the overall AK activity in the ak-hsdh2-1 ak2-1 double mutant was 30% and 64% lower than that in the ak2-1 and ak-hsdh2-1 single mutants, respectively (Figure 6a). In near-physiological concentrations of Lys, Thr, Ala, and SAM, the overall AK activity in the ak-hsdh2-1 ak1-1 double mutant was ~30% lower than that in the ak1-1 and ak-hsdh2-1 single mutants, and the overall AK activity in the ak-hsdh2-1 ak2-1 double mutant is 49% and 69% lower than that in the ak2-1 and ak-hsdh2-1 single mutants (Figure 6a). To summarize, these data showed that simultaneous loss of AK-HSDH1 and mono-functional AK1 or AK2 has an additive effect on the reduction of the overall AK activity.
The AK activity in the *ak-hsdh2-1 ak3-1* double mutant was quite different from that in the other two double mutants. In the absence of allosteric effectors, the overall AK activity in the *ak-hsdh2-1 ak3-1* double mutant was 28% and 25% higher than that in the *ak3-1* and *ak-hsdh2-1* single mutants, respectively (Figure 6a). In near-physiological concentrations of Lys, Thr, Ala, and SAM, the overall AK activity in the *ak-hsdh2-1 ak3-1* double mutant was ~30% higher than that in the *ak3-1* and *ak-hsdh2-1* single mutants (Figure 6a). It is intriguing that the *ak-hsdh2-1 ak3-1* double mutant demonstrated an unexpectedly higher AK activity than the corresponding single mutants.

**AK-HSDH1 Is the Major Contributor to the Overall HSDH Activity**

The relative contributions of AK-HSDH1 and AK-HSDH2 to the overall HSDH reaction were estimated by comparing the HSDH activity in protein extracts from wild-type, *ak-hsdh1-1*, *ak-hsdh2-1*, and *ak-hsdh2-2* leaves. In the absence of Lys, Thr, Ala, or SAM, the mutation in the AK-HSDH1 gene decreased the activity by 64%, while the mutation in the AK-HSDH2 gene decreased the activity by 18% in the *ak-hsdh2-1* mutant and 24% in the *ak-hsdh2-2* mutant (Figure 6b). In near-physiological concentrations of Lys, Thr, Ala, and SAM, the *ak-hsdh1-1*, *ak-hsdh2-1*, and *ak-hsdh2-2* mutants demonstrated 50%, 24%, and 32% reductions in the overall HSDH activity, respectively. These results suggested that AK-HSDH1 is the primary contributor to the overall HSDH activity. We also measured the overall HSDH activity in the three *ak* single mutants and three double mutants. In the absence or presence of Lys, Thr, Ala, and SAM, the overall HSDH activity in the *ak1*, *ak2*, and *ak3* mutants was not statistically different from that in the corresponding wild type (Figure 6b). The overall HSDH activity in the *ak-hsdh2-1 ak1-1*, *ak-hsdh2-1 ak2-1*, and *ak-hsdh2-1 ak3-1* double mutants was not significantly different from that in the single *ak-hsdh2-1* mutant either (Figure 6b). These observations are consistent with the fact that mono-functional AKs do not have an HSDH domain (Supplemental Figure S1).

**Correlations Exist Among Transcript Levels, Enzyme Activities, Amino Acid Contents, and Their Ratios**
To look for systematic patterns among transcript levels, enzyme activities, and amino acid contents, we performed pairwise Pearson’s correlation analysis among these parameters and their ratios. A total of 7381 correlations were analyzed (Supplemental Table S5). For simplicity reasons, only select correlations are described below.

First, we focused on the relationships between transcript levels and enzyme activities (Supplemental Table S5). The total amount of AK transcripts had a moderate positive correlation with the Lys-sensitive AK activity ($r = 0.6449$, $p = 0.0441$). The total amount of AK-HSDH transcripts had a moderate positive correlation with the Thr-sensitive AK activity ($r = 0.537$, $p = 0.1095$); it also had a moderate positive correlation with the HSDH activity measured in the absence of allosteric effectors ($r = 0.5897$, $p = 0.0728$).

Second, we studied the relationships between enzyme activities and free amino acid contents (Supplemental Table S5). The AK activity measured in the absence of allosteric effectors had a moderate positive correlation ($r = 0.5918$, $p = 0.0715$) with the ratio of free Asp-derived amino acids to free Asp, i.e., free (Lys+Met+Thr+Ile)/Asp. This is in agreement with the AK activity being responsible for the committing enzymatic step leading to the biosynthesis of Asp-derived amino acids (Figure 1) (Jander and Joshi, 2009). Free Lys had a weak negative correlation ($r = -0.3984$, $p = 0.2541$) with Lys-sensitive AK activity, consistent with Lys being a major inhibitor of mono-functional AKs (Jander and Joshi, 2009). Free Thr had a moderate negative correlation ($r = -0.5430$, $p = 0.1048$) with the Thr-sensitive AK activity. These correlations are in agreement with Thr being a major inhibitor of dual-functional AK-HSDHs. The ratio of the AK activity to the HSDH activity measured in the absence of allosteric effectors had a moderate negative correlation with the free Lys/(Met+Thr+Ile) ratio ($r = -0.6433$, $p = 0.0448$). This negative correlation remained when the AK and HSDH activities were measured in the presence of allosteric effectors ($r = -0.7320$, $p = 0.0161$). These data suggested that the balance between Lys-sensitive AKs and Thr-sensitive AK-HSDHs is important to maintain the ratio between Lys and the total amount of Met, Thr, and Ile.
Next, we analyzed whether the transcript levels of genes encoding the committing/branch-point enzymes have any relationships with the amino acid products (Supplemental Table S5). We found that the total amount of AK transcripts had a moderate positive correlation ($r = 0.6144$, $p = 0.0588$) with the total amount of free Asp-derived amino acids. This is consistent with the AK gene products being the committing enzymes in the biosynthetic pathway of Asp-derived amino acids. The total amount of DHDPs transcripts had a strong positive correlation ($r = 0.7704$, $p = 0.0091$) with free Lys, which is in agreement with the DHDPs gene products being the branch-point enzymes leading to Lys biosynthesis.

The total amount of AK-HSDH transcripts had a weak negative correlation ($r = -0.4547$, $p = 0.1868$) with the total amount of free Met, Thr, and Ile. This could be the result of two antagonistic factors: the HSDH activity of AK-HSDHs catalyzes the branch-point step leading to the biosynthesis of Met, Thr, and Ile, but it is feedback-inhibited by Thr. The CGS1 transcript had a weak positive correlation ($r = 0.3576$, $p = 0.3103$) with free Met, which is consistent with the CGS1 gene product being the committing enzyme of Met biosynthesis. The TD1 transcript had a moderate positive correlation ($r = 0.5921$, $p = 0.0713$) with free Ile, which is in agreement with the TD1 gene product being the branch-point enzyme leading to Ile biosynthesis.

Finally, we investigated whether the transcript levels of genes encoding the catabolic enzymes have any relationships with the amino acid substrates. We found that the SAMS1 and SAMS2 transcripts had moderate positive correlations with free Met ($r = 0.6725$, $p = 0.0331$ and $r = 0.4836$, $p = 0.1568$, respectively). The THA2 transcript had a moderate positive correlation with free Thr ($r = 0.5148$, $p = 0.1279$). The BCAT1 transcript had a moderate negative correlation with free Ile ($r = -0.4659$, $p = 0.1748$). This is surprising because BCAT1 has been shown to initiate catabolism of branched-chain amino acids, including Ile (Schuster and Binder, 2005).
DISCUSSION

Asp-derived amino acids Lys, Met, Thr, and Ile are essential to humans and other animals, but they are present in major food crops at suboptimal levels (Müntz et al., 1998; Debabov, 2003; Pfefferle et al., 2003; Stiller et al., 2007). Genetic engineering of crop species with improved contents of Asp-derived amino acids requires a deep and comprehensive understanding of the metabolic pathways of these essential amino acids (Galili et al., 2008). Although the biosynthesis of Asp-derived amino acids has been previously studied, there is still much to learn about the regulation and components of this pathway (Jander and Joshi, 2009). In this work, we reported that loss-of-function mutations in the \textit{AK-HSDH2} gene cause increased contents of Asp and Asp-derived amino acids, especially Thr, in leaves. Further analyses revealed that increases in Lys and Met are also observed in other \textit{ak} and \textit{ak-hsdh} single mutants. These findings underscore the complexity and previously unknown features of this pathway.

\textbf{Accumulation of Downstream Amino Acid Products}

It is common for loss-of-function mutants of a downstream enzyme to accumulate upstream products. For example, Zhu et al. (2001) reported that deficiency in a Lys catabolic enzyme (Lys ketoglutarate reductase-saccharopine dehydrogenase) results in Lys over-accumulation in seeds. Recently, it was reported that loss-of-function mutations in genes encoding branched-chain amino acid catabolic enzymes (isovaleryl-CoA dehydrogenase, methylcrotonyl-CoA carboxylase $\alpha$ and $\beta$ subunits, and hydroxymethylglutaryl-CoA lyase) cause over-accumulation of branched-chain amino acids in seeds (Gu et al., 2010; Lu et al., 2011a). Moreover, it is not uncommon for feedback-insensitive mutants of an upstream enzyme to accumulate downstream products. For instance, Heremans and Jacobs (1995 and 1997) reported that a single mutant with decreased AK sensitivity to Lys over-accumulates Lys, and a single mutant with decreased AK sensitivity to Thr over-accumulates Thr. Mourad and King (1995) reported that feedback-insensitive mutants of an Ile biosynthetic enzyme (Thr deaminase) over-accumulate Ile in leaves. Furthermore, it is not unusual for loss-of-function mutants of a branch-point
enzyme to shunt substrates into other branches. For example, multiple studies have shown that loss-of-
function mutants of the DHDPS2 protein, a branch-point enzyme leading to Lys biosynthesis, over-
accumulate Thr (Craciun et al., 2000; Sarrobert et al., 2000; Jones-Held et al., 2012). A second example is
the Thr increase in transgenic plants with reduced expression of CGS1, the branch-point enzyme towards
Met biosynthesis (Kim and Leustek, 2000).

However, it is rare for loss-of-function mutants of an upstream enzyme to accumulate downstream
products. In this study, we showed that loss-of-function mutations in the AK-HSDH2 gene cause
increased contents of free Asp-derived amino acids, especially Thr, in the leaves of four-week-old
Arabidopsis plants (Figure 4). The increases in free Asp, Lys, and Met were also observed in the ak1-1,
ak2-1, ak3-1, and ak-hsdh1-1 single mutants (Figure 4). However, the increase in free Thr was not seen in
the ak1-1, ak2-1, and ak3-1 mutants. These results indicated that loss-of-function mutations in the AK and
AK-HSDH genes have different effects on different Asp-derived amino acids. The contents of many other
amino acids, such as Asn, Gln, Glu, His, and Leu, showed coordinated increases in four-week-old single
mutants (Supplemental Figure S4), which demonstrates that perturbation in Asp-derived amino acid
biosynthesis in the single mutants may cause changes in the contents of other amino acids. These
coordinated changes were not observed in the double mutants at the same age.

Relative Contributions of Different Isoforms to the Activities

Dissecting the individual contributions of different AK and AK-HSDH isoforms will help determine the
target isoform for genetic engineering in crop species. We first attempted to use publically available
transcriptome data to retrieve the relative transcript intensities for the five AK and AK-HSDH genes.
Because the probes for AK2 and AK3 genes on the Arabidopsis ATH1 GeneChip are identical (Schmid et
al., 2005), the individual signal intensities for AK2 and AK3 cannot be extrapolated. Therefore, we used
quantitative RT-PCR to determine transcript abundances. The transcript abundances of AK1, AK-HSDH1,
and AK-HSDH2 in the wild type determined with quantitative RT-PCR were comparable to their relative
signal intensities in wild-type Arabidopsis rosette leaves in the AtGenExpress developmental baseline experiment, determined by microarray analysis (Schmid et al., 2005). The Pearson’s correlation coefficient between the two sets of data was 0.9105, which provides a strong and independent validation of our quantitative RT-PCR data. The RT-PCR data demonstrated that AK2 is the most abundant transcript among the three AKs and AK-HSDH1 is more abundant than AK-HSDH2.

Our AK activity data showed that mono-functional AKs collectively contribute more to the overall AK activity than the dual-functional AK-HSDHs, which is consistent with the estimated values reported by Curien et al. (2009). Curien et al. (2009) proposed that AK1 is the primary contributor to the overall AK activity because among the three AKs, AK1 is least sensitive to Lys inhibition (Curien et al., 2007). The estimation of Curien et al. (2009) was based on the relative protein abundances in chloroplast stroma and the sensitivities of different AK and AK-HSDH isoforms to physiological concentrations of allosteric effectors. However, due to the cross reactions of antibodies between isoforms, the authors were only able to determine the total amount of AK isoforms and the total amount of AK-HSDH isoforms; they were unable to determine the relative abundances of each isoform (Curien et al., 2009). Thus, they estimated the individual contributions of AK and AK-HSDH isoforms based on the hypothetic assumptions that AK1 and AK2 are equally abundant and that AK-HSDH1 and AK-HSDH2 are equally abundant (Curien et al., 2009). We had three lines of evidence suggesting that AK2 is the primary contributor to the overall AK activity and that AK-HSDH1 is the primary contributor to the overall HSDH activity: transcript abundances measured with quantitative RT-PCR; the activities measured in the absence of allosteric effectors; and activities measured in near-physiological concentrations of Lys, Thr, Ala, and SAM.

Calculating from the values in Figure 6, the ratio of the AK activity to the HSDH activity measured in wild-type Arabidopsis leaves in the absence of allosteric effectors was 0.35:1.00. This ratio was close to the ratio (0.4:1.00) of the total AK protein abundance to the total AK-HSDH protein abundance, determined by ELISA with antibodies against purified recombinant proteins (Curien et al., 2009). In the presence of near-physiological concentrations of Lys, Thr, Ala, and SAM, the ratio of the AK activity to...
the HSDH activity in wild-type Arabidopsis leaves decreased to 0.20:1.00. This suggested that the total
AK activity is more inhibited than the total HSDH activity in near-physiological concentrations of Lys,
Thr, Ala, and SAM.

Our AK activity data showed that simultaneous loss of AK-HSDH2 and mono-functional AK1 or AK2
has an additive effect on the reduction of the overall AK activity. However, the *ak-hsdh2-1 ak3-1* double
mutant demonstrated an unexpectedly higher AK activity than the single mutants. This result could be the
combined effect of several factors. First, among three *AK* genes, the *AK3* transcript is least abundant and
it is preferentially expressed in the xylem of leaves (Yoshioka et al., 2001). Second, among the three AK
proteins, AK3 is most sensitive to Lys inhibition (Curien et al., 2007). Third, although AK-HSDH1 is not
the primary contributor to the overall AK activity, the *AK-HSDH1* transcript in the *ak-hsdh2-1 ak3-1*
double mutant was 67% higher than that in the *ak-hsdh2-1* single mutant (Figure 5).

Loss-of-function mutations in one *AK* or *AK-HSDH* gene did cause some changes in the expression of
other *AK* or *AK-HSDH* gene(s) but none of these changes were systematic up- or down-regulations
(Figure 5). Loss-of-function mutations in an *AK* or *AK-HSDH* gene also resulted in changes in the
transcript levels of other biosynthetic and catabolic genes and some of the changes were shared among
different mutants (Supplemental Figures S5 – S6). For example, the *TD1* transcript level was
unanimously increased in the six single mutants. Other examples include the increase of the *SAMS1*
transcript and the decrease of the *BCAT1* transcript in most of the single and double mutants.

**Correlations among Transcript Abundance, Enzyme Activities, and Free Amino Acids**
To identify systematic patterns among transcript abundance, enzyme activities, and free amino acids, we carried out pairwise Pearson’s correlation analysis (see Supplemental Table S5 for the complete list of correlations and Figure 7 for representative correlations). First, the total amount of $AK$ transcripts
positively correlated with the Lys-sensitive AK activity; and the total amount of AK-HSDH transcripts positively correlated with both the Thr-sensitive AK activity and the total HSDH activity. These data suggested that transcriptional regulations play an important role in controlling the activities of the

Figure 7. Correlations among the ratio of the total AK transcripts to the total AK-HSDH transcripts, the ratio of the total AK activity to the total HSDH activity, and the ratio of free Lys to the total amount of free Met, Thr, and Ile.

(a) Correlation between the ratio of the total AK transcripts to the total AK-HSDH transcripts and the Lys/(Met+Thr+Ile) ratio in four-week-old plants.
(b) Correlation between the ratio of the total AK activity to the total HSDH activity and the ratio of the total AK transcripts to the total AK-HSDH transcripts in four-week-old plants.
(c) Correlation between the Lys/(Met+Thr+Ile) ratio and the ratio of the total AK activity to the total HSDH activity in four-week-old plants.

AKs/AK-HSDHs stands for the ratio of the total AK1, AK2, and AK3 transcripts to the total AK-HSDH1 and AK-HSDH2 transcripts. Lys/(Met+Thr+Ile) stands for the ratio of free Lys to the total amount of free Met, Thr, and Ile. The total AK and HSDH activities shown in this figure were measured in the absence of Lys, Thr, Ala, and SAM. Each point (diamond) represents one genotype. Error bars represent SE of the mean (n = 3 - 5). Dotted line is the linear regression between free and protein-bound Asp-derived amino acids. The correlation coefficient (r) and the p-value are shown for each plot on the edges of the triangle: positive correlations are in red; negative correlations are in blue.
corresponding AK and AK-HSDH proteins. Second, there were correlations between enzyme activity and free amino acid content: the total AK activity positively correlated with the free (Lys+Met+Thr+Ile)/Asp ratio; the Lys-sensitive and Thr-sensitive AK activities negatively correlated with free Lys and free Thr, respectively; the total HSDH activity (i.e., the HSDH activity of AK-HSDHs) negatively correlated with free Thr; and the ratio of the AK activity to the HSDH activity negatively correlated with the free Lys/(Met+Thr+Ile) ratio. These data led to the hypothesis that the balance between Lys-sensitive AKs and Thr-sensitive AK-HSDHs is important for determining the amounts and ratios of Asp-derived amino acids, and vice versa. This hypothesis is also supported by previous studies. Heremans and Jacobs (1997) reported that a mutant with decreased AK sensitivity to Lys had an increased amount of Thr, a second mutant with decreased AK sensitivity to Thr had an increased amount of Lys, and the double mutant had wild-type levels of Lys and Thr. Third, the total amount of Asp-derived amino acids positively correlated with the total AK transcripts, and free Lys, Met, and Ile positively correlated with the DHDPS, CGS1, and TD1 transcripts, respectively. These data are consistent with AKs being the committing enzymes for Asp-derived amino acid biosynthesis and DHDPSs, CGS1, and TD1 being the committing enzyme for Lys, Met, and Ile biosynthesis, respectively (Figure 1). Fourth, free Met positively correlated with the transcripts of Met catabolic genes SAMS1 and SAMS2; and free Thr negatively correlated with the total AK-HSDH transcripts and positively correlated with the transcript of Thr catabolic gene THA2. These correlations suggested the possibilities that Thr may inhibit the expression of its biosynthetic genes and that Met and Thr may induce the expression of their catabolic genes. This is not uncommon: Zhu-Shimoni and Galili (1998) reported that the regulation of AK-HSDH could occur at the transcriptional level and Rebeille et al. (2006) found that Met strongly induced the expression of MGL, which encodes a Met catabolic enzyme Met gamma-lyase.

The influence of changes in Asp-derived amino acid biosynthesis on the contents of other amino acids highlights the idea that metabolic regulation is far more complex than illustrated in the textbook. Some of the other amino acids, such as Asn, Glu, and Gln, were either involved in or related to Asp-derived amino
acid biosynthesis. Glu is directly involved in the biosynthesis of Lys and Ile (Jander and Joshi, 2009). In the presence of ATP, Asp and Gln can be converted to Asn and Glu by Asn synthetases (Coruzzi and Last, 2000). Because Asp, Asn, Glu, and Gln are inter-related, it is somewhat expected that the four amino acids demonstrated coordinated increases in the six four-week-old single mutants (Figure 4 and Supplemental Figure S4). In addition to Asn, Glu, and Gln, the contents of His and Leu, which are not related to Asp-derived amino acid biosynthesis, showed increased accumulation in the six single mutants and no change in the three double mutants (Supplemental Figure S4). One possible reason is that the biosynthetic pathways of His and Leu involve Glu and Gln. His biosynthesis uses Gln and Glu as substrates (Stepansky and Leustek, 2006); free His had a very strong positive correlation with free Gln ($r = 0.9028, p = 0.0003$) and a moderate positive correlation with free Glu ($r = 0.6327, p = 0.0497$). Leu can be synthesized from the transamination between Glu and 4-methyl-2-oxopentanoate (Binder, 2010); free Leu had a moderate positive correlation with free Glu ($r = 0.5385, p = 0.1083$). It is not uncommon that perturbation in the biosynthesis of Asp-derived amino acids results in changes in other amino acids. For instance, van Bochaute et al. (2013) reported that long-term inhibition of the DHDPS activity caused increased levels of His, Leu, Phe, and Val. Further studies are needed to understand why changes in Asp-derived amino acids have such wide-ranging impacts on leaf amino acid accumulation in general.

Overall, this study showed that loss-of-function mutations in the $AK$ and/or $AK-HSDH$ genes result in reduced transcript levels and gene product activities of the corresponding genes, alterations in the transcript levels of other biosynthetic and catabolic genes, and increases in the contents of Asp-derived (and some other) amino acids. Pairwise Pearson’s correlation analysis revealed that the ratio of total AK activity to total HSDH activity negatively correlates with the free Lys/(Met+Thr+Ile) ratio. Although the amounts and ratios of Asp-derived amino acids were also found to correlate with the transcript levels of a few other biosynthetic and catabolic genes, these patterns were identified from analyzing the responses of amino acid contents and transcript levels to loss-of-function mutations in the $AK$ and/or $AK-HSDH$ genes. Therefore, we hypothesize that the balance between Lys-sensitive AKs and Thr-sensitive AK-HSDHs is
important in controlling the amounts and ratios of Asp-derived amino acids. The results from this research revealed some complexity in the regulation of aspartate-derived amino acid contents, for example, the impacts of changes in Asp-derived amino acid biosynthesis on amino acids that are not directly related to this pathway.

MATERIALS AND METHODS

Plant Materials, Array Mapping, Sequencing, Genotyping, and Growth Conditions

*Arabidopsis thaliana* T-DNA lines (SALK_003685, SALK_019023, SALK_043533, SALK_059678, SALK_082155, SALK_125957, and WiscDsLox461-464J6) used in this study were obtained from the Arabidopsis Biological Resource Center. All of them are in the Col ecotype (Alonso et al., 2003; Woody et al., 2007). Homozygosity was confirmed by PCR, as described in Ajjawi et al. (2010).

To locate the mutation in SALK_082155 that causes the Thr increase, a SALK_082155 plant was crossed to a Landsberg *erecta* (Ler) wild-type plant. The F2 plants were tested for their leaf free amino acid contents as described below. For array mapping, leaf tissues were harvested (one leaf/plant) from 13 F2 plants with the highest Thr concentrations and 27 F2 plants with the lowest Thr concentrations. Leaf tissues from high- and low-Thr plants were pooled and ground separately, and ~100 mg of powder were used for DNA extraction using the DNeasy plant mini kit (Qiagen, http://www.qiagen.com). The two resulting DNA samples were labeled with biotin using the BioPrime DNA labeling system (Life Technologies, http://www.lifetechnologies.com), according to manufacturer’s instructions. Labeled DNAs were hybridized to two Arabidopsis ATH1 gene chips (Affymetrix, http://www.affymetrix.com). As described by Hazen et al. (2005), the raw data were analyzed with R scripts, which is available at http://naturalvariation.org/methods/. Hybridization and targeted gene sequencing (see below) were done at the MSU Research Technology Support Facility.
After the mapping internal was narrowed to a 1.75 Mb region on chromosome 4, the candidate gene, AK-HSDH2 (At4g19710), of SALK_082155 was sequenced. Primers AK-HSDH2_5’L and AK-HSDH2_5’R were used to amplify with PCR the 5’ half of the gene, and primers AK-HSDH2_3’L and AK-HSDH2_3’R were used to amplify the 3’ half of the gene (Supplemental Table S6).

SALK_082155 was backcrossed to the Col wild type to segregate the single-base insertion in the AK-HSDH2 gene and the T-DNA insertion in the At1g55805 locus. The segregating F2 plants were genotyped for both loci. Primers SALK_082155LP and SALK_082155RP were used to test for the presence of the T-DNA insertion in the At1g55805 locus (Supplemental Table S6). Primers Exon2_L and Exon4_R were used to amplify a 716 bp region flanking the single-base thymine insertion in the fourth exon (Supplemental Table S6). In the absence of the single-base thymine insertion, the PCR product can be digested into two fragments (197 bp and 519 bp) by restriction enzyme Bsp1286I. In the presence of the single-base thymine insertion, the PCR product cannot be digested by Bsp1286I. Seeds from F2 plants with the single-base thymine insertion in both copies of the AK-HSDH2 gene and with no T-DNA insertion in either copy of the At1g55805 gene were harvested and grown for downstream analyses, including quantitative Reverse Transcription (RT)-PCR, immunoblots, activity assays, and free amino acid measurements.

Mutants and wild-type (Col and Ler) Arabidopsis plants were grown in a growth chamber on a 12-h-light/12-h-dark photoperiod. The light intensity was 150 μmol photons m^{-2} s^{-1}, the temperature was 20°C and the relative humidity was 50%. Unless otherwise stated, plants used for quantitative RT-PCR, immunoblots, activity assays, and free amino acid measurements were four weeks old. To minimize the impacts of growth conditions on transcript levels, enzyme activities, and amino acid contents, the wild-type and nine mutant plants were grown in the same growth chamber and harvested one hour after the lights came on.

Quantitative RT-PCR
Quantitative RT-PCR was performed as described by Lu et al. (2011c). For consistency purposes, the 8th - 10th oldest true leaves from four-week-old plants were harvested (Supplemental Figure S7), frozen, ground to a fine powder, and used for quantitative RT-PCR analysis. Total RNA was extracted from using the RNeasy plant mini kit (Qiagen), digested with RNase-free DNase I (Qiagen), and reverse-transcribed with oligo dT(15) primer and Moloney murine leukemia virus reverse transcriptase (Promega, http://www.promega.com). Quantitative PCR was performed on a StepOnePlus™ Real-Time PCR system (Life Technologies). Primers used for quantitative RT-PCR are listed in Supplemental Table S6. Quantification of transcript levels was achieved by comparing the threshold cycle numbers of unknown samples against a standard curve with known copy numbers.

Immunoblots

For consistency purposes, the 8th - 10th oldest true leaves from four-week-old plants were harvested (Supplemental Figure S7), frozen, ground to a fine powder, and used for soluble protein extraction and immunodetection of the AK-HSDH2 protein. Total soluble proteins were extracted in a buffer containing 100 mM HEPES (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 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, 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 (Life Technologies) according to the manufacturer’s instructions. After electrophoresis, the proteins were electroblotted onto a polyvinylidene difluoride membrane as described by Lu et al (2011c). Immunodetection of the AK-HSDH2 protein on the polyvinylidene difluoride membrane was performed using the SuperSignal west pico rabbit immunoglobulin G detecting kit (Thermo Fisher Scientific, www.piercenet.com) and analyzed by the Gel Logic 1500 imaging system (Kodak, www.kodak.com). The anti-AK-HSDH2 polyclonal antibody was made by Open Baysystems (now part of Thermo Scientific, http://www.thermoscientificbio.com/openbiosystems). A 14-amino acid peptide (corresponding to amino acids 820 to 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.

**Enzyme Extraction for AK and HSDH Activity Assays**

Protein samples for AK and HSDH activity assays were prepared as described by Di Marco and Grego
(1975) and Matthews et al. (1975). Due to the large amount of tissues required for enzyme extraction, the
entire above-ground portion of four-week-old plants was harvested (Supplemental Figure S7), 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 EDTA, 1.4 mM 2-mercaptoethanol, 5 mM Thr, and
30% [v/v] glycerol) in a ratio of 2.5 mL/g fresh weight. Unground residues were removed by filtering
through two layers of cheesecloth and centrifuging at 6°C for 30 min at 20,000 g. To remove endogenous
ions or metabolites that would potentially influence the downstream activity assays, leaf protein extracts
were purified by adding ammonium sulfate to 50% saturation (0.27 g/mL) and shaking on ice for 30 to 60
min. Proteins were collected by centrifuging at 6°C for 10 min at 3000 rpm and re-dissolved in the
resuspension buffer (50 mM potassium phosphate buffer [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 contains 0.5 mM Thr (Bryan, 1969). The protein
concentration was determined using the Bradford method, as described above.

**AK Activity Assay**

The AK activity was quantified using the hydroxamate-ferric chloride method, as described by Dotson et
al. (1989) and Paris et al. (2002). The product of the AK reaction, L-aspartyl-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 NH₂OH-HCl, 515 mM KOH, 20 mM MgCl₂, 150 mM
KCl, 40 mM ATP, and 100 μL of enzyme extracts. 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% trichloroacetic acid, 370 mM FeCl₃, 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 mixed 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., 2002).

**HSDH Activity Assay**

The HSDH activity was quantified by measuring the rate of NADP⁺ reduction (Matthews et al., 1975; Paris et al., 2002). 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 preparation in a final volume of 0.75 mL. Reactions were initiated by the addition of enzyme preparation and 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., 2002).

**Leaf Free Amino Acid Assay by HPLC-Tandem Mass Spectrometry**

For one-week-old plants, the entire above-ground portion of plants was harvested and approximately 20 plants per replicate were used. For two-week-old plants, the first two true leaves were harvested and approximately 10 plants per replicate were used. For three-week-old plants, the youngest three mature rosette leaves (e.g., the 2nd to 4th oldest true leaves) were harvested and one plant per replicate was used. For four-week-old plants, the 9th oldest true leaf was harvested and one plant per replicate was used. Five replicates per genotype were used for all ages tested. Leaf numbers of one-, two-, three-, and four-week-old plants are shown in Supplemental Figure S7. Free amino acids were extracted and analyzed with the HPLC-Tandem Mass Spectrometry method (Jander et al., 2004; Lu et al., 2008; Gu et al., 2012).
quantification, mixtures of 20 protein amino acids plus L-Phe-α,β,β, 2,3,4,5,6-d₈ and L-Val-2,3,4,4,5,5,5-d₈ of 0.9 μM were analyzed along with the leaf samples.

Leaf Protein-bound Amino Acid Assay by Acid Hydrolysis and HPLC-Tandem Mass Spectrometry

For consistency purposes, the 9th oldest true leaf from four-week-old plants was harvested (Supplemental Figure S7), frozen, ground to a fine powder and used for leaf protein-bound amino acid assay. Ground tissues were hydrolyzed at 120°C for 24 h in 6 M HCl according to Nguyen et al. 2012. After the evaporation of 6 M HCl, free and protein-bound amino acids were extracted and analyzed with the HPLC-Tandem Mass Spectrometry method (Jander et al., 2004; Lu et al., 2008; Gu et al., 2012). For quantification of protein-bound amino acids, free amino acids were subtracted. During acid hydrolysis, Cys and Trp are destroyed, Met is partially oxidized, and Asn and Gln are converted to Asp and Glu, respectively (Anders, 2002). Therefore, Asn and Asp are reported together, as are Glu and Gln.

Data Analysis

All quantitative analyses (amino acid contents, transcript levels, protein abundances, and enzyme activities) were performed on three to five biological independent replicates, as described in the responding figure or table legends. All statistical analyses were performed with JMP 11 statistical software (SAS Institute). Student’s t-test was used to compare mean among each pair of plant groups. Pearson’s correlation was used to assess the relationships between transcript levels, enzyme activities, and amino acid contents and their ratios. The strength of correlations was categorized according to Fowler et al., (2013): 0 ≤ |r| < 0.20, very weak or negligible; 0.20 ≤ |r| < 0.40, weak; 0.40 ≤ |r| < 0.70, moderate; 0.70 ≤ |r| < 0.90, strong; 0.90 ≤ |r| ≤ 1.00, very strong (Fowler et al., 2013).

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; DHDPS1, At3g60880; DHDPS2, At2g45440; CGS1, At3g01120; TSI, At4g29840;
TD1, At3g10050; LKR-SDH1, At4g33150; SAMS1, At1g02500; SAMS2, At4g01850; SAMS3, At3g17390;
SAMS4, At2g36880; THAI, At1g08630; THA2, AT3G04520; BCAT1, At1g10060.

**Supplemental Data**

**Supplemental Figure S1.** Domains in full-length mono-functional AKs and dual-functional AK-HSDHs.

**Supplemental Figure S2.** The high Thr phenotype in SALK_082155 is caused by a second-site mutation.

**Supplemental Figure S3.** Representative siliques from ak-hsdh2-1/ak-hsdh2-1 AK-HSDH1-1/ak-hsdh1-l
and AK-HSDH2-1/AK-HSDH2-1 AK-HSDH1-1/AK-HSDH1-l plants.

**Supplemental Figure S4.** The contents of other free amino acids in leaves of four-week-old plants.

**Supplemental Figure S5.** Transcript levels of other genes involved in Asp-derived amino acid
biosynthesis in leaves of four-week-old plants.

**Supplemental Figure S6.** Transcript levels of select genes involved in Asp-derived amino acid
catabolism in leaves of four-week-old plants.

**Supplemental Figure S7.** Leaf numbers of one-, two-, three-, and four-week old Col wild-type
Arabidopsis plant grown under a 12-h-light /12-h-dark photoperiod.

**Supplemental Table S1.** The contents of protein-bound amino acids in leaves of four-week-old plants.

**Supplemental Table S2.** Leaf free amino acid contents in one-week-old plants.

**Supplemental Table S3.** Leaf free amino acid contents in two-week-old plants.

**Supplemental Table S4.** Leaf free amino acid contents in three-week-old plants.
Supplemental Table S5. Correlation coefficients and p values among transcript levels, AK activity, HSDH activity, free amino acid contents, and protein-bound amino acid contents and their ratios.

Supplemental Table S6. Primers used in this study.

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Figure 1. Biosynthesis and degradation of Asp-derived amino acids.

Feedback inhibition is shown as a dotted line with a bar. Activation is shown as a dotted line with an arrow head. AK, Asp kinase; AK-HSDH, Asp kinase-homoserine dehydrogenase; ALS, acetolactate synthase; ASD, Asp semialdehyde dehydrogenase; BCAT, branched-chain aminotransferase; CBL, cystathionine beta lyase; CGS, cystathionine gamma synthase; DAPAT, diaminopimelate aminotransferase; DAPDC, diaminopimelate decarboxylase; DAPE, diaminopimelate epimerase; DHAD, dihydroxyacid dehydratase; DHDPR, dihydronicotinamide reductase; DHDPS, dihydronicotinamide synthase; HMT, homocysteine S-methyltransferase; HSK, homoserine kinase; KARI, ketol-acid reductoisomerase; LKR-SDH, lysine-ketoglutarate reductase-saccharopine dehydrogenase; MS, Met synthase; SAM, S-adenosylmethionine; SAMS, S-adenosylmethionine Synthase; TD, Thr deaminase; THA, Thr aldolase; TS, Thr synthase.

Figure 2. Mutations in the AK-HSDH2 gene cause the Thr increase.

(a) 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.

(b) Fold change of Thr content in Col wild type and the two mutants. The values (mean ± SE, n = 5) are presented as the ratios to the Thr content in Col wild type. 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).
(c) Relative amounts of the \textit{AK-HSDH2} transcript. Total RNA was extracted from mature leaves and analyzed with quantitative RT-PCR. The level of the \textit{AK-HSDH2} transcript was normalized to that of the \textit{ACTIN2} transcript (AT3g18780). The values (mean ± SE, n = 3 - 6) are presented as the ratios to the transcript level in Col wild type. Asterisks indicate significant differences between the mutant and the Col wild type (Student’s \textit{t} test; *, \textit{p} < 0.05).

(d) Representative immunoblot of the AK-HSDH2 protein and Coomassie Brilliant Blue G250 stained gel showing the Rubisco large subunit (RbcL, loading control). The lanes were loaded on an equal protein basis.

(e) Images of four-week-old Col wild-type and \textit{ak-hsdh2} plants under a 12-h-light/12-h-dark photoperiod.

\textbf{Figure 3.} T-DNA locations and plant images of other single or double \textit{ak} and \textit{ak-hsdh} mutants.

(a) Schematic representation of the \textit{AK1, AK2, AK3}, and \textit{AK-HSDH1} genes and four 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. \textit{ak1-1}, WiscDsLox461-464J6; \textit{ak2-1}, SALK_003685; \textit{ak3-1}, SALK_043533; \textit{ak-hsdh1-1}, SALK_125957.

(b) Images of four-week-old Col wild-type and single or double mutant plants under a 12-h-light/12-h-dark photoperiod.

\textbf{Figure 4.} The contents of free Asp and Asp-derived amino acids in leaves of four-week-old plants.

(a) Asp; (b) Lys; (c) Met; (d) Thr; (e) Ile; (f) the total amount of Asp-derived amino acids Lys, Met, Thr and Ile. Values are presented as mean ± SE (n = 5). Values not connected by the same letter are significantly different (Student’s \textit{t}-test, \textit{p} < 0.05). Values connected by the same letter are not significantly different.

\textbf{Figure 5.} Transcript levels of \textit{AK} and \textit{AK-HSDH} genes in leaves of four-week-old plants.
(a) $AK1$; (b) $AK2$; (c) $AK3$; (d) $3AKs$; (e) $AK-HSDH1$; (f) $AK-HSDH2$; (g) $2AK-HSDHs$. Values are presented as mean ± SE ($n = 3 - 4$). Values not connected by the same letter are significantly different (Student’s $t$-test, $p < 0.05$). Values connected by the same letter are not significantly different.

**Figure 6.** AK and HSDH activities in leaf protein extracts.

(a) The AK activity measured with the hydroxamate-ferric chloride method.

(b) The HSDH activity measured with the NADP$^+$ reduction method. Values are represented as mean ± SE ($n = 3$). The AK and HSDH activities in physiological concentrations of effectors were measured in the presence of 70 μM Lys, 300 μM Thr, 400 μM Ala and 20 μM SAM. Values not connected by the same letter are significantly different (Student’s $t$-test, $p < 0.05$). Values connected by the same letter are not significantly different.

**Figure 7.** Correlations among the ratio of the total $AK$ transcripts to the total $AK-HSDH$ transcripts, the ratio of the total AK activity to the total HSDH activity, and the ratio of free Lys to the total amount of free Met, Thr, and Ile.

(a) Correlation between the ratio of the total $AK$ transcripts to the total $AK-HSDH$ transcripts and the Lys/(Met+Thr+Ile) ratio in four-week-old plants.

(b) Correlation between the ratio of the total AK activity to the total HSDH activity and the ratio of the total $AK$ transcripts to the total $AK-HSDH$ transcripts in four-week-old plants.

(c) Correlation between the Lys/(Met+Thr+Ile) ratio and the ratio of the total AK activity to the total HSDH activity in four-week-old plants.

$AKs/AK-HSDHs$ stands for the ratio of the total $AK1$, $AK2$, and $AK3$ transcripts to the total $AK-HSDH1$ and $AK-HSDH2$ transcripts. Lys/(Met+Thr+Ile) stands for the ratio of free Lys to the total amount of free Met, Thr, and Ile. The total AK and HSDH activities shown in this figure were measured in the absence of Lys, Thr, Ala, and SAM. Each point (diamond) represents one genotype. Error bars represent SE of the
mean (n = 3 - 5). Dotted line is the linear regression between free and protein-bound Asp-derived amino acids. The correlation coefficient (r) and the p-value are shown for each plot on the edges of the triangle: positive correlations are in red; negative correlations are in blue.


