

TECHNICAL ADVANCE

Application of a high-throughput HPLC-MS/MS assay to *Arabidopsis* mutant screening; evidence that threonine aldolase plays a role in seed nutritional quality

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Received 13 March 2004; revised 10 May 2004; accepted 14 May 2004.

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Summary

Beyond their essential function as the building blocks of proteins, amino acids contribute to many aspects of plant biochemistry and physiology. Despite this, there are relatively large gaps in our understanding of the biochemical pathways and regulation of amino acid synthesis in plants. A rapid (1.5 min versus 20–90 min for standard methods) HPLC-MS/MS assay for separating 19 amino acids was developed for quantifying levels of free amino acids in plant tissue. This assay was used to determine the free amino acid content in the seeds of 10,000 randomly mutagenized *Arabidopsis* lines, and 322 *Arabidopsis* lines with increased levels of one or more amino acids were identified. The heritability of the mutant phenotype was confirmed for 43 lines with increased seed levels of the aspartate-derived amino acids Ile, Lys, Thr, or Met. Genetic mapping and DNA sequencing identified a mutation in an *Arabidopsis* threonine aldolase (AT1G08630, EC 4.1.2.5) as the cause of increased seed Thr levels in one mutant. The assay that was developed for this project has broad applicability to *Arabidopsis* and other plant species.

Keywords: amino acid, threonine, *Arabidopsis*, HPLC-MS/MS, isoleucine, mutant screen.

Introduction

Amino acids play many important roles beyond incorporation into proteins in flowering plants (Coruzzi and Last, 2000). Glu, Gln, Asp and Asn are sources of nitrogen and precursors for a wide variety of metabolic pathways. These are also major nitrogen transport molecules that are regulated in response to environmental signals such as light, and may in fact act as signaling molecules in the plant (Brenner *et al.*, 2000; Lam *et al.*, 1998). In addition, the role of amino acids in plant morphogenesis and stress adaptation is widespread, with a variety of amino acids acting as precursors for the synthesis of hormones and secondary metabolites.

The synthesis of amino acids by plants is also agriculturally important. Animals, including humans, rely on plant foods as dietary sources of essential amino acids that they cannot synthesize themselves. However, Lys, Thr, Met, Trp, and Ile are present in lower than optimal amounts in seeds of plants such as corn, soy and rice. These crops are widely used as feed for farm animals and also make up the majority of the diet of some human populations (Coruzzi and Last, 2000). Currently, animal feed is supplemented with chemically synthesized versions of these amino acids and, as such, they are

attractive targets for nutritional improvement through biotechnology.

Transgenic approaches have been successfully employed to improve amino acid balance in plant seeds (Anderson *et al.*, 2003; Galili and Hofgen, 2002). These strategies have targeted allosterically regulated enzymes and activities that compete for metabolic flux into the pathway of interest. Genetic inactivation of the catabolic enzyme Lys ketoglutarate reductase was also effectively employed to increase Lys levels in *Arabidopsis* seeds (Zhu *et al.*, 2001). Although these directed transgenic approaches have been successful, they do not identify hitherto unknown targets for engineering improved amino acid content.

Genetic screens in *Arabidopsis* have been used to discover new approaches for enhancing accumulation of nutritionally limiting amino acids. For example, dominant high Trp lines with deregulated anthranilate synthase alpha subunit activity were obtained by selection for *Arabidopsis* seedlings resistant to 6-methylanthranilate (Li and Last, 1996) or α -methyltryptophan (Kreps *et al.*, 1996). In another screen, three different mechanisms for increasing methionine levels were identified by selection for ethionine resistance in *Arabidopsis* (Bartlem *et al.*, 2000; Chiba *et al.*, 1999; Inaba *et al.*, 1994; Shen *et al.*, 2002). Although these screens have clearly been successful, they are limited by the fact that separate assays have to be performed for each biosynthetic pathway being targeted. In addition, screens have typically been performed on seedlings or young plants, and thus may miss regulation specific to older plants or seeds, long distance transport of amino acids, the loading of amino acids or their precursors into developing seeds, and altered catabolism.

A direct biochemical screen for altered amino acid accumulation in older plants or seeds would be the ideal approach to identify mutants affected in these processes. Similar mutant screens by high-performance liquid chromatography (HPLC) or gas chromatography (GC) enabled the identification of *Arabidopsis* mutants affecting a variety of metabolic pathways, including glucosinolates (Haughn *et al.*, 1991), cell wall sugars (Reiter *et al.*, 1997), and fatty acids (Browse and Somerville, 1991; Ohlrogge and Browse, 1995). However, until recently, direct screening for plant mutants with altered seed or leaf amino acid levels was hampered by the lack of a suitably fast assay. Commonly used methods for the analysis of amino acids involve derivatization and separation by HPLC coupled with a UV or fluorescence detector (FD) (Malmer and Schroeder, 1990; Schuster, 1988). The derivatization procedures tend to be tedious, and it takes a relatively long time (typically 20–90 min) to separate derivatized amino acids by HPLC. In contrast, the reverse-phase HPLC coupled to the tandem mass spectrometry detection method (HPLC-MS/MS) described here does not involve derivatization of the amino acids and the total run time is only 1.5 min.

We describe the use of this rapid and robust assay to screen seeds from a population of approximately 10,000 randomly mutagenized *Arabidopsis* lines, and to identify the 43 mutants that have heritable increases in one or more of the Asp-derived amino acids (Ile, Lys, Met, and Thr). Map-based cloning demonstrated that one mutant has increased seed Thr content as a result of a mutation in Thr aldolase. The methodology described in this report will allow similar mutant screens to be performed in other genetically tractable plants, and direct monitoring of nutritionally valuable molecules in conventional and molecular plant breeding.

Results

Assay development and validation

As the goal of this project was to measure the amino acid content in thousands of plant samples, it was necessary to develop a rapid method to separate and detect amino acids from plant tissue. The resulting HPLC-MS/MS method, described in detail in Experimental procedures, was developed using 20 commercially available L-amino acids (Asp, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val), and reliably detects 19 of the 20 amino acids. As it was important to minimize the run time, it was not possible to differentiate Ile and Leu, which have similar HPLC retention times, as well as molecular and daughter ions with the same mass to charge ratio.

In further work, *Arabidopsis* leaves and seeds were used for assay development and validation. The goal was to develop a simple and fast extraction method that was amenable to automation and high throughput. Extraction efficiency was determined by re-extracting plant residue remaining after removal of the extraction buffer supernatant. A series of three extractions of the same samples showed that approximately 60% of the extractable amino acids are recovered in the first supernatant. Approximately 10% of the amino acids detected in the second and third extraction are the result of the residual extraction buffer left in the tube. As it provided excellent reproducibility and throughput, only one extraction of seed or leaf tissue was used for all subsequent experiments.

To test recovery in this extraction procedure, representative acidic, basic, and neutral deuterium-labeled amino acids (Asp-d3, Lys-d4 and Met-d3; acidic, basic, and neutral amino acids, respectively) were spiked into *Arabidopsis* leaves at a concentration of 60 $\mu\text{g ml}^{-1}$ prior to the extraction. In six independent experiments, approximately 90% of the deuterium-labeled amino acids were recovered in the first extraction. The same three deuterated amino acids were spiked into *Arabidopsis* leaves prior to extraction at concentrations ranging from 0.01 to 75 $\mu\text{g ml}^{-1}$ to create calibration curves, which were linear in this concentration

range. Based on the mass chromatographic peak signal to noise ratio, the limit of detection for the three deuterated amino acids was determined to be approximately 10 ng ml^{-1} .

We compared the characteristics of this assay to the standard HPLC with the fluorescence detection (HPLC-FD) assay (Malmer and Schroeder, 1990; Schuster, 1988) for the detection of amino acids in plant tissue. Three different samples were analyzed by HPLC-MS/MS and HPLC-FD: a standard solution containing purified amino acids (Asn, Asp, Gln, Ile, Leu, Thr, Lys, and Met), *Arabidopsis* leaf extracts, and extracts made from *Arabidopsis* leaves spiked with the purified L-amino acids. Assay accuracy was assessed by quantifying each amino acid as a percentage of the total amino acids detected. As shown in Figure 1, the results of the two assays were similar. Asn, Lys, and Met are not

included in Figure 1b because their levels in leaves were too low for reliable detection by the HPLC/FD assay.

Representative chromatograms of amino acids detected from *Arabidopsis* seed extracts are shown in Figure 2 and the masses measured for each amino acid are listed in Table 1. The levels of free Cys and Gly in *Arabidopsis* seeds are too low to be quantified reliably by HPLC-MS/MS. Lys and Gln are separated by about 0.14 min in the same mass chromatogram, and Gln is considerably more abundant than Lys in *Arabidopsis* seeds. Thus, the tail of the Gln peak can adversely affect the quantification of Lys in this assay. As mentioned previously, Ile and Leu are not separated by this method.

Mutant screen

The 1.5-min HPLC-MS/MS assay described above was sufficiently fast and reliable to use for a large-scale screen for *Arabidopsis* mutants with altered amino acid content. Although the assay detects both leaf and seed amino acids with high efficiency, we chose to perform a mutant screen for alteration of free amino acids in seeds. Two major factors were considered in this decision: the level of biological variability was slightly lower in the seeds than the leaves, presumably reflecting the well-characterized diurnal regulation of amino acid levels in green tissue (Coruzzi and Last, 2000), and seeds of crop plants are the main targets of efforts to alter amino acid levels.

Approximately 10 000 M_3 and M_4 seed samples were included in a mutant screen, in which seed amino acid content was measured by HPLC-MS/MS. Mutants were identified based on the significant deviation of the seed content of one or more amino acids from the average of the population as a whole. Given the possibility of the growth chamber variation and differences between the Columbia (Col-0) and Landsberg *erecta* (*Ler*) land races, analysis was carried out on groups of 96 plants (three flats). Each group was derived from one mutant pool and was grown together at the same time on the same growth chamber shelf. The mean and standard deviation of the levels of free amino acids in each group of 96 plants were determined, both as a percentage of total amino acids and as a peak area ratio relative to the peak area of a Val-d8 standard added to each sample. A total of 322 lines showed significantly increased amino acid levels (three standard deviations above the mean) in two different extractions of the same seed samples. Among these, at least one of the Asp-derived amino acids (Ile, Lys, Met, and Thr) was increased in 103 mutant lines. These mutants were of particular interest because of the essential and sometimes limiting role of Asp-derived amino acids in human and animal diets.

To confirm that the amino acid phenotypes are heritable, all of the 103 mutants affected in Asp-derived amino acids were grown together with wild-type plants in a replicated

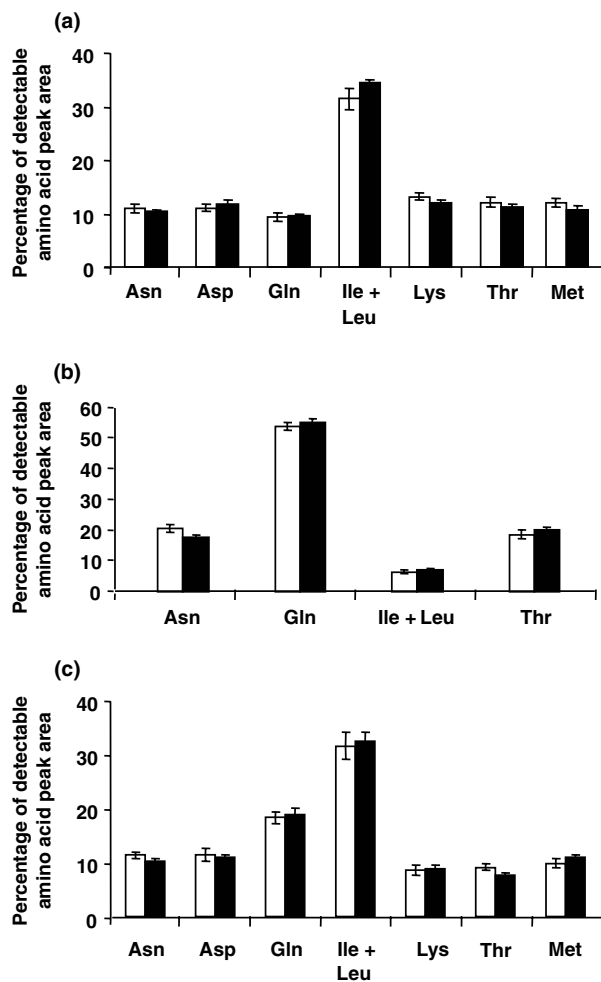


Figure 1. Comparisons of amino acid measurement by HPLC-MS/MS (white bars) and HPLC/FD (black bars). (a) Standard amino acids in water ($4 \mu\text{g ml}^{-1}$). (b) *Arabidopsis* leaf extracts. (c) *Arabidopsis* leaf extracts spiked with standard amino acids ($4 \mu\text{g ml}^{-1}$). Each bar represents the mean and standard deviation of 10 samples.

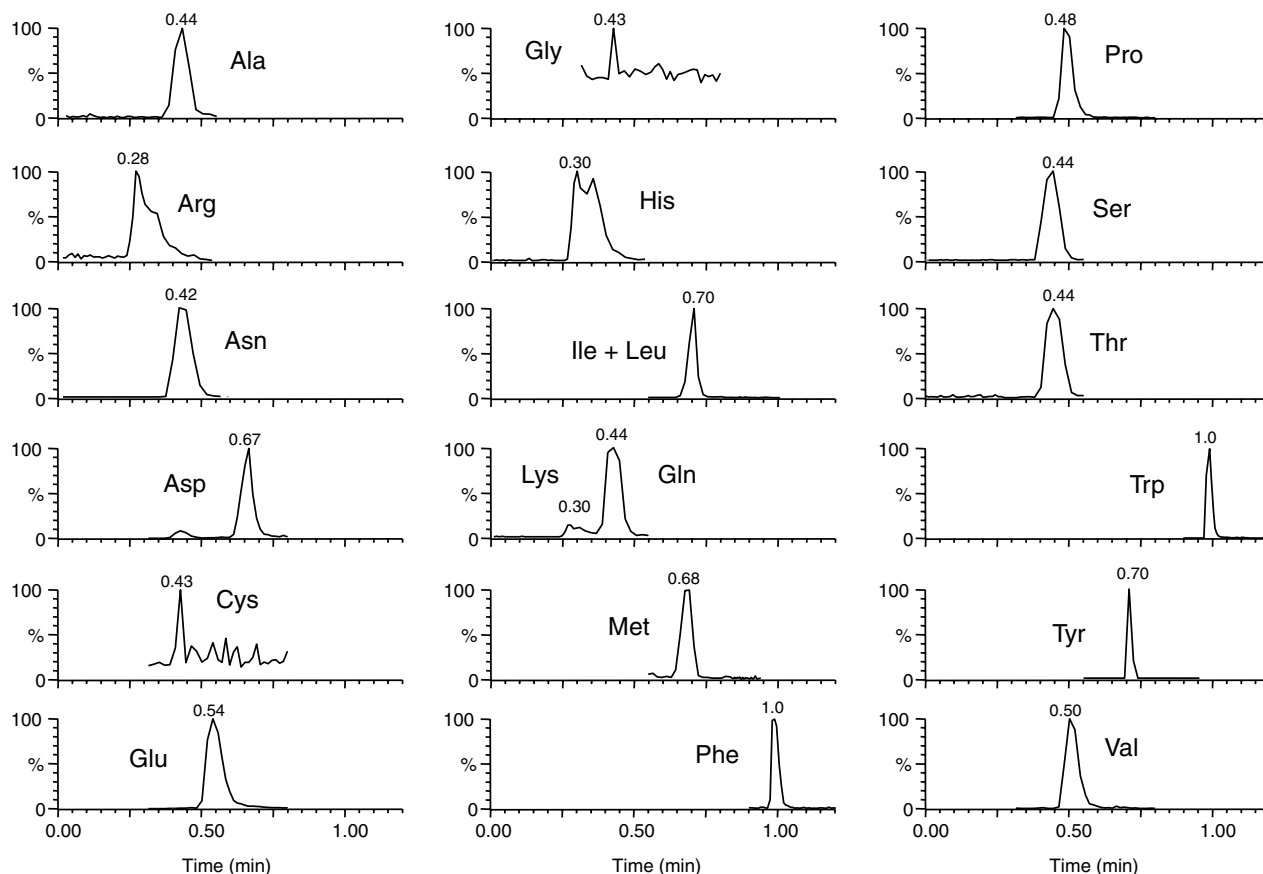


Figure 2. Sample mass chromatograms of free amino acids in *Arabidopsis* seeds.

Masses measured for each amino acid are as described in Table 1. Y-axis units are arbitrary, with the height of the highest peak of each measurement being set as 100%. Numbers above the peaks represent the approximate elution times.

planting. Seeds were harvested and subjected to amino acid analysis by HPLC-MS/MS. As there was some uncertainty in the Lys measurement using the 1.5-min HPLC-MS/MS assay, these mutants were confirmed using a modified assay that allowed better separation of Lys and Gln by HPLC and subsequent detection by MS/MS (see Experimental procedures). Forty-three mutant lines have significantly increased seed amino acid levels (Figure 3) and were named HSI, HSL, HSM, or HST (for high seed Ile, Lys, Met, or Thr), with the numbers in order of decreasing amino acid levels. Several of the mutants show increases in more than one of the Asp-derived amino acids, with concomitant increases in Thr and Met being the most common. However, each mutant is represented only once in Figure 3 by the amino acid with the greatest fold increase over the wild type.

All 43 of the mutants were crossed to the opposite ecotype (Col-0 or Ler) and the F₁ seeds were planted next to wild-type plants. The seed amino acid content of pooled F₂ seeds from individual F₁ plants was measured using the 1.5-min HPLC-MS/MS assay. For a large fraction of the mutants (19 out of 43, marked with an '*' in Figure 3) the level of these amino acids in the pooled F₂ seeds was significantly higher than

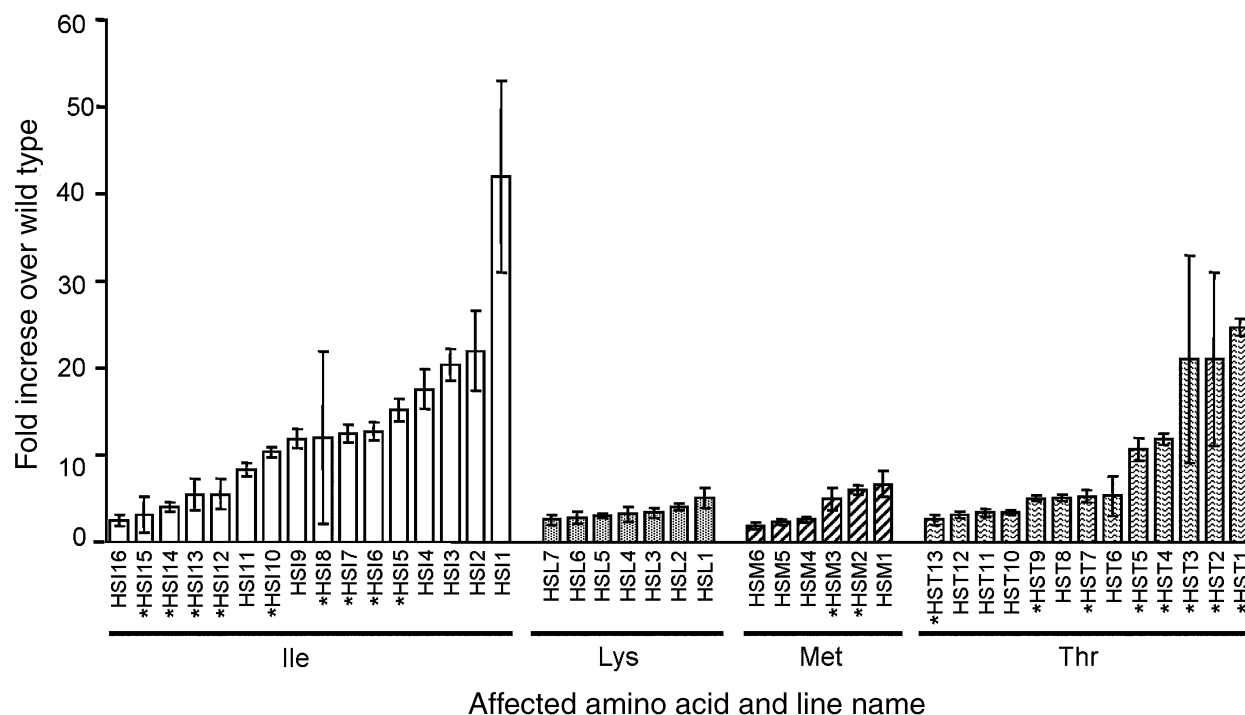
that in wild-type plants, suggesting that these mutations could be dominant or haplo-insufficient. Some dominant mutations were still segregating in the generation used for mutant confirmation, resulting in a relatively large standard error of the difference of the means and a likely underestimate of the true amino acid increase (e.g. lines HSI8, HST2, and HST3 in Figure 3). Sequencing of known genes related to Asp-derived amino acid biosynthesis (Amir *et al.*, 2002; Galili, 2002; Singh and Shaner, 1995) and genetic mapping in the F₂ generation revealed no mutations that were genetically linked to the high Ile, Lys, Met or Thr mutant phenotypes.

Branched-chain amino acid levels are correlated in mutants and mapping populations

The 1.5-min HPLC-MS/MS assay did not permit separate detection of Ile and Leu. To determine whether the 16 mutants that showed increased levels of Ile + Leu were affected in one or both of these amino acids, a variation of the original assay was developed (see Experimental procedures). All mutants that showed increased levels in the combined

Table 1 Collection times, collision energy and masses of observed ions

Time (min)	Amino acid	Collision energy (eV)	Mass of parent >daughter ions
Function 1 (0–0.55)	Ala	13	90.0 > 44.6
	Ser	11	106.1 > 60.2
	Thr	10	120.1 > 74.2
	Asn	13	132.97 > 74.3
	Lys/Gln	11	147.17 > 130.11
	His	15	156.0 > 110.2
	Arg	25	175.26 > 116.18
Function 2 (0.3–0.8)	Gly	10	76.4 > 30.5
	Pro	13	116.1 > 70.2
	Val	13	118.12 > 72.35
	Cys	13	122.2 > 76.24
	Val-d8 ^a	11	126.08 > 80.3
	Asp	15	134.07 > 74.2
Function 3 (0.55–0.95)	Ile + Leu	11	132.11 > 86.26
		11	132.12 > 86.26
		11	132.13 > 69.29
	Met	11	149.97 > 104.16
	Tyr	13	182.1 > 136.1
Function 4 (0.9–1.5)	Phe	13	166.1 > 120.1
	Phe-d8 ^b	13	174.31 > 128.3
	Trp	10	205.08 > 188.09

^aLoading control.^bExtraction control.**Figure 3.** Fold increase over wild type in seed amino acid content for 43 mutants affected in the production of Asp-derived amino acids. Each bar represents the average of three to eight mutant measurements compared with the average amino acid content of seven to 24 wild-type plants that were grown together and assayed by HPLC-MS/MS in the same microtiter plate. Error bars represent the standard error of the difference of the mutant and wild-type means for each line. An * next to the line name indicates a mutation that had an apparently dominant or semi-dominant phenotype in the F₁ generation.

Ile + Leu peak in the original assay had increased levels of both Ile and Leu (Figure 4), with an excellent linear correlation found between these two biosynthetically related amino acids ($r^2 = 0.92$). A strong correlation ($r^2 = 0.94$) was also observed between Ile and the other branched-chain amino acid Val (data not shown).

Only the three branched-chain amino acids showed significant differences in seed levels between the Col-0 and Ler wild-type lines, with Ler levels being two to threefold higher (Figure 4 shows Ile and Leu). In addition, unlike the other 17 amino acids, the branched-chain amino acid levels showed a high degree of transgressive segregation in mapping populations, with a 70-fold range of Ile + Leu levels in seeds from 460 Col-0 \times Ler F₂ plants, and a 20-fold range of Ile + Leu concentrations in seeds from 96 Col-0/Ler recombinant inbred (RI) lines (Lister and Dean, 1993). Seed Val concentration was significantly correlated with that of Ile + Leu in both the F₂ population ($r^2 = 0.91$) and the RI lines ($r^2 = 0.94$) (data not shown).

Line HST2 has a mutation in threonine aldolase

The HST2 mutation was mapped using a previously outlined protocol (Jander *et al.*, 2002). A mapping population of approximately 250 F₂ lines was planted, and the amino acid phenotype of pooled F₃ seeds from each F₂ plant was determined by HPLC-MS/MS (Figure 5). The 25% of F₂ lines

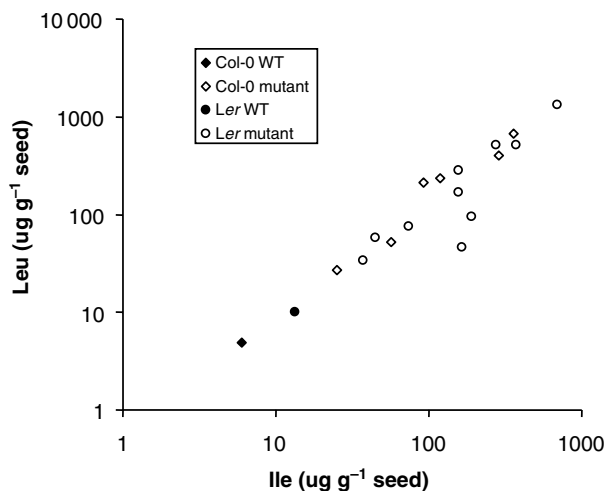


Figure 4. Correlation of seed Ile and Leu contents of Col-0, *Ler*, and mutants. Mutant data points are the average of three to eight measurements; wild-type Col-0 and *Ler* the average of 32 measurements. Mutants are the same as the Ile + Leu mutants in Figure 4.

with the highest Thr content (presumably homozygous mutant) have an average 25-fold increase over wild type, similar to the 20-fold seed Thr increase seen during mutant confirmation (Figure 3). Unlike with many of the other mutants, no significant changes in other amino acids were identified for the HST2 mutant. The 25% of F_2 lines with the lowest levels of seed Thr (presumably homozygous wild type at the site of the mutation) were genotyped with five markers on each of the five chromosomes, and genetic linkage of the HST2 mutation was found to two markers on chromosome 1. Genotyping with additional markers narrowed the position of the HST2 mutation to a region of chromosome 1 between markers CER474622 on BAC T21E18 and CER432610 on BAC F7G19 (<http://www.arabidopsis.org/Cereon>). Analysis of the sequence in this region identified a Thr aldolase homologue (AT1G08630; EC 4.1.2.5) as a

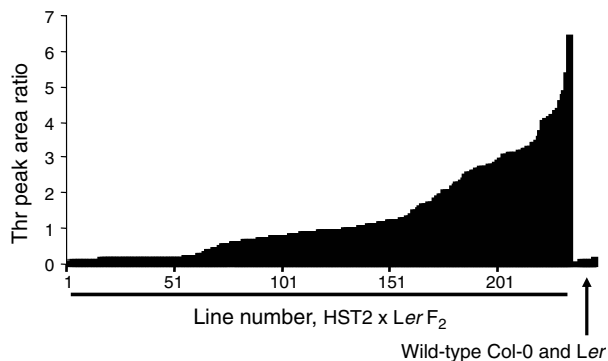


Figure 5. Distribution of seed Thr content in segregating F_2 lines derived from a cross between mutant line HST2 and wild-type *Ler*. Bars representing seed Thr content of four Col-0 and four *Ler* plants are indicated on the right. The Y-axis scale represents Thr peak areas divided by the peak area of a Val-d8 standard added to each sample.

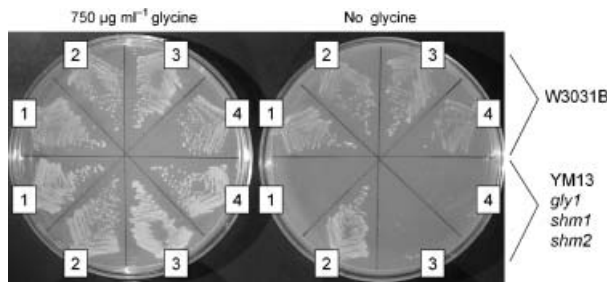


Figure 6. Cloned *Arabidopsis* Thr aldolase relieves the Gly auxotrophy of yeast strain YM13.

The top half of each plate is the W3031B wild-type *GLY1*; *SHM1*; *SHM2* haploid strain; the bottom half is the isogenic Gly auxotroph strain YM13 *gly1*; *shm1*; *shm2*. 1 = no plasmid control; 2 = wild-type chromosome 1 Thr aldolase; 3 = HST2 mutant Thr aldolase; 4 = pCM185 vector control.

candidate gene. Thr aldolase catalyzes the reversible reaction $\text{Thr} \rightarrow \text{Gly} + \text{acetaldehyde}$ in mammals and microbes (Liu *et al.*, 1997, 1998a,b; Monschau *et al.*, 1997). In all previously studied biological systems this reaction proceeds toward Gly, and loss of function mutations in a Thr catabolic enzyme might be expected to increase seed Thr levels in *Arabidopsis*. Sequencing of AT1G08630 from line HST2 identified a G340A base change (as expected for EMS mutagenesis) resulting in the missense mutation Gly-114-Arg.

The assertion that AT1G08630 encodes an *Arabidopsis* Thr aldolase was strengthened by the transformation of cDNA clones into the yeast strain YM13 (*gly1*; *shm1*; *shm2*), which is a Gly auxotroph caused by mutations in Thr aldolase and both yeast Ser hydroxymethyltransferases (SHMT, EC 2.1.2.1) (McNeil *et al.*, 1994; Monschau *et al.*, 1997). Whereas transformation with the wild-type allele allowed YM13 to grow in the absence of Gly, the Gly-114-Arg mutant allele from HST2 and the empty pCM185 vector did not (Figure 6). To verify that lack of complementation was the result of the starting G340A base change, the cloned mutant cDNA was re-amplified from the transformed yeast strain and the absence of additional mutations was confirmed by DNA sequencing. As expected, none of the constructs had a discernable effect on the growth of either YM13 or the isogenic wild-type strain W3031B on plates containing Gly (Figure 6).

In vitro biochemical assays showed that the cloned *Arabidopsis* gene encodes Thr aldolase activity. Crude extracts prepared from yeast strain YM13 transformed with wild type *Arabidopsis* Thr aldolase efficiently catalyzed acetaldehyde release from Thr, with an apparent K_m of 7.1 ± 0.9 mM for Thr (mean and standard deviation of three independent measurements). There was no detectable Thr aldolase activity in untransformed YM13 or in YM13 transformed with mutant *Arabidopsis* Thr aldolase, indicating that the Gly-114-Arg missense mutation severely compromises enzyme function. Using the same acetaldehyde assay, we determined an apparent K_m for Thr of 4.8 mM for Thr

aldolase activity from the wild-type yeast strain W3031B, similar to the published apparent K_m of 4.3 mM (Monschau *et al.*, 1997) for this enzyme, which was determined by measuring glycine release.

Analysis of the *Arabidopsis* genomic sequence (The Arabidopsis Genome Initiative, 2000) identified another likely Thr aldolase (AT3G04520) on chromosome 3, with 70% amino acid sequence identity to the chromosome 1 gene (AT1G08630). We propose naming the AT1G08630 and AT3G04520 genes *THA1* and *THA2* (Threonine Aldolase), respectively. The mutation that was identified in line HST2 is designated the *tha1-1* allele.

Discussion

Dramatically improved methods in analytical chemistry, which speed up and lower the cost per sample of small molecule measurement, are changing the way that we study metabolism. The HPLC-MS/MS assay described in this report allows determination of 18 amino acids from plant tissue in 1.5 min, which is 15–60-fold faster than traditional derivatization and reverse-phase HPLC. With the previous method, it would have been very time-consuming to screen 10 000 mutant families for altered seed amino acid content. In contrast, using an autosampler and HPLC-MS/MS, one individual was able to perform this primary screen in 6 months, and 43 confirmed mutants were identified with increased levels of Ile, Lys, Met or Thr.

In principle, the direct screening method employed could yield mutants affected in a variety of mechanisms, including altered biochemical or genetic regulation of pathway enzymes (including allosteric effects), increased amino acid transport, loading into the developing seeds, or reduced catabolism. DNA sequencing of *Arabidopsis* genes known to affect regulation of biosynthesis of Asp-derived amino acids, including feedback sensitive and branch point enzymes, revealed no mutations that accounted for the observed increased seed amino acid levels. Thus, these 43 mutations include previously unidentified loci involved in plant amino acid metabolism. This is presumably because the mutant screen described here is fundamentally different from previous approaches that have been used to identify plant amino acid genes. Whereas most previously known plant amino acid mutants were identified by selections using toxic amino acid analogues in seedling screens, toxic mixtures of amino acids on seedlings, or transgenic expression of enzymes with altered regulation, we screened directly for free amino acid levels in seeds.

Consistent with our expectation that the screen had yielded mutations in previously unknown genes, the mapping and sequencing of the AT1G08630 Thr aldolase gene from the high seed Thr mutant HST2 (*tha1-1*) identified a Gly114Arg missense mutation. Although the wild-type gene reversed the Gly auxotrophy of the yeast strain YM13, the

Gly114Arg mutant allele did not (Figure 6), an indication that this residue is essential for the function of the protein. Similarly, *in vitro* assays with crude yeast extracts showed Thr aldolase activity only for the wild type and not the mutant enzyme. An important role for the Gly114 residue in enzyme function is also suggested by its conservation in Thr aldolase from species as diverse as the animals *Anopheles gambiae*, *Drosophila melanogaster*, and *Caenorhabditis elegans* as well as *Escherichia coli*, *Thermotoga maritima*, and other bacteria (Kielkopf and Burley, 2002). In the crystal structure of *T. maritima* Thr aldolase (Kielkopf and Burley, 2002), the homologous Gly-108 residue does not make contact with the enzyme substrates or the pyridoxal phosphate cofactor. However, the position of this Gly residue in the interior of the protein might suggest that substitution with the larger, charged Arg residue would disrupt protein structure.

It was not possible to measure the decrease in seed Gly levels predicted for a Thr aldolase mutation, because the levels of free Gly are already undetectably low in wild-type seeds (Figure 2). In contrast to pleiotropy observed for mutations affecting other amino acid biosynthetic genes (Zhu and Galili, 2003), the *tha1-1* mutation causes no significant changes in the levels of any other free amino acids. However, it is possible that assays of additional *Arabidopsis* tissue types would show changes in the levels of Gly or other amino acids.

Surprisingly, in F_2 populations derived from a Col-0 *tha1-1* × wild-type *Ler* cross, there is a roughly 3:1 high:low seed Thr ratio (Figure 5), which is typical of dominant gain of function mutations. A dominant genetic effect is unexpected, given the lack of *in vitro* activity of the Gly114Arg mutant protein. There are several possible explanations for the 3:1 segregation ratio: (i) the mutant allele may act in a dominant negative manner; (ii) the observed 3:1 segregation may reflect gene dosage effects (haplo-insufficiency); or (iii) a recessive *tha1-1* mutation might produce a 3:1 high:low threonine segregation ratio, because we assayed pooled F_3 seeds from individual F_2 plants. In this latter case, a heterozygous F_2 parent would produce seeds that individually segregate in a 1:2:1 Mendelian manner, resulting in pooled F_3 seeds that have elevated Thr levels compared with wild type, even though only 25% of all seeds are homozygous mutant.

We are not aware of previous studies of Thr aldolase activity or Thr aldolase mutations in plants. In fact, in a recent review, it was argued that this enzyme is unlikely to play an important role in plant amino acid metabolism (Bourguignon *et al.*, 1999). Reports of 'Thr aldolases' purified from maize and mung beans more than 20 years ago are, in retrospect, likely to be improper classifications of SHMTs (Masuda *et al.*, 1980, 1982). The purified maize and mung bean enzymes were reported to cleave L-*allo*-Thr (a non-natural substrate) and L-Ser, but not L-Thr. Such

substrate specificity has been found for several purified SHMT enzymes including rat, rabbit, human, and *E. coli* (Ogawa *et al.*, 2000). In contrast, purified Thr aldolases do not have the capacity to bind tetrahydrofolate, which is necessary for the SHMT Ser cleavage reaction (Kielkopf and Burley, 2002), and the V_{\max}/K_m values for cleavage of Thr by Thr aldolases are much higher than those by SHMTs (Ogawa *et al.*, 2000). The absence of a tetrahydrofolate binding site in THA1 also makes it unlikely that this enzyme rescues the Gly auxotrophy of the yeast strain YM13 because of SHMT rather than Thr aldolase activity.

Although the exact function of the Thr aldolase in plants remains unknown, there are several possibilities that are not mutually exclusive. First, high levels of Thr can have toxic effects on plants (Sarrobot *et al.*, 2000), and Thr aldolase may serve to remove excess Thr. Secondly, Thr aldolase may function as an alternate pathway for Gly biosynthesis in plants, in addition to the Gly produced during photorespiration and from 3-phosphoglycerate as a branch off of glycolysis (Bourguignon *et al.*, 1999). A third possible role for plant Thr aldolase is that the reaction $\text{Thr} \rightarrow \text{Gly} + \text{acetaldehyde}$ functions in the production of acetyl-CoA, which can be produced from acetaldehyde by the sequential action of acetaldehyde dehydrogenase and acetyl-CoA synthase. Conversely, if this reversible reaction proceeds in the direction $\text{Gly} + \text{acetaldehyde} \rightarrow \text{Thr}$ under some physiological conditions, then Thr aldolase might help to catalyze Thr biosynthesis, or perhaps reduce the amount of acetaldehyde produced in plants emerging from anoxic stress (Kursteiner *et al.*, 2003; Tsuji *et al.*, 2003).

The mapping of branched-chain amino acid (Ile, Leu, and Val) mutations would be complicated by the fact that the effects of transgressive segregation in Col-0 \times Ler RI and F₂ lines are as large as the effects of most of the induced mutations (Figure 3). The correlation of branched-chain amino acid levels in mutants (Figure 4), F₂ lines, and RI populations, indicates that the induced mutations and natural variation have similar effects on all three branched-chain amino acids. This tight coregulation of branched-chain amino acid levels may be a more general phenomenon in plants. For instance, a similar effect is seen in a population tomato lines that have portions of the *Lycopersicon pennellii* genome introgressed into *L. esculentum* (D. Zamir, personal communication).

The high-speed HPLC-MS/MS assay described here can be easily applied to other plant species, animals, or microbes to determine amino acid content. Modifications of the tissue extraction and HPLC-MS/MS detection protocols would allow extension of this amino acid assay to a more general metabolite profiling method. By using this assay for an *Arabidopsis* mutant screen, we have identified a mutation that causes high seed Thr levels by reducing the activity of Thr aldolase. Further work with the mutants that were isolated is likely to lead to new insights into the

regulation and biosynthesis of Ile, Lys, Met, Thr, and other amino acids. Whereas most previous work on plant amino acid biosynthesis has been carried out as an extension of what is known from microorganisms, the approach described here will allow the identification of plant-specific regulatory and biosynthetic pathways. A better understanding of plant amino acid biosynthesis will aid in the improvement of crop plants through biotechnology or molecular breeding.

Experimental procedures

Growth of *Arabidopsis* and mutant screening

Col-0, Ler and RI lines (stock number CS1899) were obtained from the Arabidopsis Biological Resource Center (www.arabidopsis.org). Mutagenized Col-0 and Ler seeds were purchased from Lehle Seeds (Round Rock, TX, USA). Generation of mutant populations has been described previously (Van Eenennaam *et al.*, 2003). Plants were grown in Conviron (Winnipeg, Canada) growth chambers in standard nursery flats (approximately 20 cm \times 40 cm) using Metromix 200 potting soil (Scotts, Marysville, OH, USA) at 23°C under continuous cool white fluorescent light with an intensity of 150 $\mu\text{mol m}^{-2} \text{sec}^{-1}$ photosynthetic photon flux density (PPFD). Plants were fertilized twice weekly with a dilute solution of Peters 20-20-20 fertilizer (Scotts), adjusted to a 50 ppm nitrogen concentration. A maximum of 96 M₂ lines were planted from each of 110 independent seed pools. As each pool contained an average of eight seeds from each of 1000 M₁ lines, the chances of planting sibling M₂ seeds were low. Nine weeks after planting, watering was discontinued and plants were allowed to dry at room temperature for approximately 1 month. Once the plants were completely dry, seed pools were harvested individually from each plant and stored in 1.4 ml tubes in 96-cell microtiter plates (Matrix Hudson, NH, USA) at 4°C and 25% relative humidity. Either M₃ seeds from M₂ plants or M₄ seeds from the subsequent generation of the mutant population were used for amino acid analysis. *Arabidopsis* lines described in this work will be made available for non-profit research and can be obtained by contacting G. Jander.

Yeast strains and growth conditions

Yeast strains W3031B (*MAT α ura3-1 trp1-1 ade2-1 his3-11,-15 leu2-3,-112 can1-100*) and YM13 (*W3031B shm1::HIS3 shm2::LEU2 gly1::URA3*) were kindly supplied by A. Bognar (University of Toronto). Yeast strains were grown on YPD medium (1% yeast extract, 2% peptone, 2% dextrose) or on SD medium (0.67% Bacto-yeast nitrogen bas, 2% dextrose) supplemented, where appropriate, with 750 $\mu\text{g ml}^{-1}$ Gly, 30 $\mu\text{g ml}^{-1}$ Leu, and 20 $\mu\text{g ml}^{-1}$ each uracil, Trp, adenine, and His.

Sample preparation

To prepare *Arabidopsis* seed samples for amino acid analysis, approximately 15 mg of seed were aliquoted into 1.4 ml tubes in a 96-well microtiter plate (Matrix) using a calibrated air displacement pipette made by stuffing glass wool into the end of a Pasteur pipette. The actual weight of the seed sample in each tube was determined using a Bohdan Balance Automator 200 (Mettler, Columbus, OH, USA). A single 3 mm stainless steel ball bearing

was added to each tube, the tubes were capped, and the entire plate was frozen in liquid nitrogen. Seeds were ground to a fine powder for 30 sec on a platform shaker powered by a modified Sawzall (Milwaukee Electric Tool Corporation, Brookfield, WI, USA). Samples were suspended by vortexing for 1–2 min in a 600 μ l extraction solution consisting of 80% ethanol, 19.9% water, 0.1% formic acid, 4 μ g ml⁻¹ L-Phe- $\alpha,\beta,\beta,2,3,4,5,6$ -d8 (Aldrich, Milwaukee, WI, USA). The extracts were clarified by centrifugation for 15 min at 3000 g, and 450 μ l of the supernatant from each well was transferred to a new microtiter plate. Aliquots of 100 μ l were transferred to additional microtiter plates and were dried down under nitrogen using a Turbovap (Zymark, Hopkinton, MA, USA) with heat at 25°C and nitrogen at 1.5–3 m³ h⁻¹. Lyophilized samples were stored at -80°C for up to several months. For amino acid analysis, 100 μ l water with 4 μ g ml⁻¹ L-Val-2,3,4,4,4,5,5,5-d8 (Aldrich) was added to each tube and samples were reconstituted by letting them dissolve for 30 min at 4°C. To prepare leaf samples for analysis, approximately 100 mg of *Arabidopsis* leaf material was placed into 1.4 ml tubes in a 96-well microtiter plate (Matrix). The tissue was frozen in liquid nitrogen and lyophilized, resulting in approximately 10 mg of dried leaf material. Further processing of these samples was the same as described above for *Arabidopsis* seeds.

Determination of amino acids by HPLC-MS/MS

Seed extracts were separated using a Phenyl-Hexyl Luna column (3 μ , 4.6 \times 30 mm; Phenomenex, Torrance, CA, USA) on a Shimadzu LC-10AD HPLC (Shimadzu, Kyoto, Japan). The sample injection volume was 5 μ l, and a Gilson (Middleton, WI, USA) 215 liquid handler equipped with an 819 injector was used to load the samples. Mobile phases used were: (A) 0.05% acetic acid in water and (B) 90% acetonitrile, 0.05% acetic acid in water. The total HPLC run time was 1.5 min, using the following gradient: 0–0.6 min 100% A, 1 ml min⁻¹; 0.61–1.0 min, 90% B, 1–3 ml min⁻¹; 1.1–1.4 min, 100% A, 3 ml min⁻¹, 1.4–1.5 min, 100% A, 3–1 ml min⁻¹. Amino acids were detected using a Micromass Triple Quadrupole Quattro Ultima mass spectrometer (Waters, Milford, MA, USA) using an electrospray positive mode, 3.0 kV capillary voltage, 25 V cone voltage, 120°C source temperature, and 300°C desolvation temperature. Collection times, collision energies and masses of observed ions for each amino acid were as described in Table 1.

Ile/Leu separation method

For separation of Ile and Leu, the instrumentation described above for the 19 amino acid separation was used, but the HPLC-MS/MS protocol was changed. Mobile phases were: (A) 0.05% acetic acid in water and (B) 0.05% acetic acid in 100% acetonitrile. The total HPLC run time was 4 min using the following gradient: 0–2 min, 100% A, 1.5 ml min⁻¹; 2–3 min, 10% A, 90% B, 1.5 ml min⁻¹; 3–4 min, 100% A, 2 ml min⁻¹. Ile and Leu were detected using electrospray positive mode, 3.5 kV capillary voltage, 25 V cone voltage, 120°C source temperature, 250°C desolvation temperature, and 10 eV collision energy. The mass/charge ratio of the detected parent and daughter ions were 132.21 and 86.60, respectively.

Lys/Gln separation method

For separation of Lys and Gln, the instrumentation described above for the 19 amino acid separation was used, but the HPLC-MS/MS

protocol was changed. Mobile phases were: (A) 0.05% acetic acid in water and (B) 0.05% acetic acid in 100% acetonitrile. The total HPLC run time was 1.5 min using the following gradient: 0–0.6 min, 100% A, 1 ml min⁻¹; 0.6–1.2 min, 100% B, 2 ml min⁻¹; 1.2–1.5 min, 100% A, 2 ml min⁻¹. Ile and Leu were detected using electrospray positive mode, 3.5 kV capillary voltage, 25 V cone voltage, 120°C source temperature, 250°C desolvation temperature, and 15 eV collision energy. The mass/charge ratio of the detected parent and daughter ions were 147.11 and 84.6, respectively.

Genetic crosses, genotyping and DNA sequencing

Mutant plants were crossed to wild-type plants of the opposite ecotype (Col-0 or Ler) to generate F₁ seeds (Weigel and Glazebrook, 2002). The wild-type plant was used as the female parent in every cross. Chromosomal DNA was amplified by PCR and was sequenced using an ABI3700 sequencer (Applied Biosystems, Foster City, CA, USA) as described previously (Jander *et al.*, 2003; Van Eenennaam *et al.*, 2003). *Arabidopsis* single nucleotide polymorphisms (SNPs) were detected using the Taq-Man PCR assay (Jander *et al.*, 2002; Livak, 1999).

cDNA cloning

The wild-type allele of *Arabidopsis* chromosome 1 Thr aldolase (AT1G08630) was amplified from the full-length cDNA clone U148646 (ABRC) using the primers AGTCGGATCCATGGTG-ATGAGAAGTGTGGATCT and AGCTCTGCAGTTAGGTTCCGGCTT-GGTTCCCTG and was cloned into the tetracycline-repressible yeast vector pCM185 (Gari *et al.*, 1997) using the restriction enzymes BamHI and PstI. To clone the HST2 mutant allele of chromosome 1 Thr aldolase, mRNA was isolated from an HST2 flower stalk using the RNeasy plant RNA kit (Qiagen, Valencia, CA, USA) and was reverse transcribed using the Access RT-PCR kit (Promega, Madison, WI, USA). The mutant cDNA was amplified using the same primers as for the wild-type cDNA and was cloned into pCM185.

Yeast transformation

Yeast was transformed by the lithium acetate method (Ausubel *et al.*, 1998). Complementation of the Trp auxotrophy was used to select for transformation of strains W3031B and YM13 (McNeil *et al.*, 1994; Monschau *et al.*, 1997) with pCM185 and derived plasmid constructs carrying mutant and wild-type Thr aldolase. The correct HMT2 mutant and wild-type Thr aldolase DNA sequences were verified by PCR amplification and sequencing.

Thr aldolase assays

Yeast cultures (40 ml) were grown overnight in SD medium supplemented with 20 μ g ml⁻¹ adenine, 30 μ g ml⁻¹ leucine, 40 μ g ml⁻¹ tryptophan, 20 μ g ml⁻¹ uracil, 20 μ g ml⁻¹ histidine, and 750 μ g ml⁻¹ glycine. Crude yeast extracts were prepared by glass bead disruption (Ausubel *et al.*, 1998). Extracts were desalted using Econo-Pac 10DG columns (Bio-Rad, Hercules, CA, USA). Fractions were collected, protein concentration was measured by Bradford assay, and equal amounts of protein (50 μ g/reaction) were used for Thr aldolase assays. Thr aldolase reactions were carried out in 200 μ l total volume with 100 mM Hepes/NaOH, pH 8.0, 50 μ M pyridoxal-P, and 0, 1, 2, 5, 10, or 50 mM Thr as the substrate (Liu *et al.*, 1998a). Reactions were allowed to proceed for

30, 60, or 120 min at 30°C before termination and precipitation of proteins with 50 µl of 30% trichloroacetate. The pH of the reactions was adjusted to 4 with neutralization buffer (31.8 g K₂CO₃ in 100 ml 20 mM Tris-HCl, pH 8), and acetaldehyde was quantified by measuring the absorption of an azine derivative formed by the reaction of acetaldehyde with *N*-methyl benzothiazolone hydrazone (MBTH) (Paz *et al.*, 1965). Thr aldolase reaction samples were diluted in water, and 80 µl of the dilute solution was combined with 100 µl 0.1 M glycine buffer pH 4 and 20 µl 0.1% MBTH. The MBTH-acetaldehyde reaction was allowed to proceed for 45 min at 40°C, essentially to completion, and the amount of product formed was detected by absorption at 305 nm on a Spectramax Plus spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). A standard curve prepared with purified acetaldehyde was used to determine the amount of acetaldehyde produced in the reactions. The apparent *K_m* for Thr was calculated using a Lineweaver-Burke plot.

Acknowledgements

We thank Naren Kadaba for help in data analysis and the Monsanto Company sequencing group for DNA sequencing. This project was funded by the Monsanto Company, Renessen LLC, Atlantic Philanthropies, and the Boyce Thompson Institute.

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