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Two *Arabidopsis* Threonine Aldolases Are Nonredundant and Compete with Threonine Deaminase for a Common Substrate Pool [W]

Vijay Joshi,[a] Karen M. Laubengayer,[a,1] Nicolas Schauer,[b] Alisdair R. Fernie,[b] and Georg Jander[a,2]

[a] Boyce Thompson Institute for Plant Research, Ithaca, New York 14853
[b] Max Planck Institute for Molecular Plant Physiology, Potsdam-Golm 14766, Germany

Amino acids are not only fundamental protein constituents but also serve as precursors for many essential plant metabolites. Although amino acid biosynthetic pathways in plants have been identified, pathway regulation, catabolism, and downstream metabolite partitioning remain relatively uninvestigated. Conversion of Thr to Gly and acetaldehyde by Thr aldolase (EC 4.1.2.5) was only recently shown to play a role in plant amino acid metabolism. Whereas one *Arabidopsis thaliana* Thr aldolase (*THA1*) is expressed primarily in seeds and seedlings, the other (*THA2*) is expressed in vascular tissue throughout the plant. Metabolite profiling of *tha1* mutants identified a >50-fold increase in the seed Thr content, a 50% decrease in seedling Gly content, and few other significant metabolic changes. By contrast, homozygous *tha2* mutations cause a lethal albino phenotype. Rescue of *tha2* mutants and *tha1 tha2* double mutants by overproduction of feedback-insensitive Thr deaminase (*OMR1*) shows that Gly formation by THA1 and THA2 is not essential in *Arabidopsis*. Seed-specific expression of feedback-insensitive Thr deaminase in both *tha1* and *tha2* Thr aldolase mutants greatly increases seed Ile content, suggesting that these two Thr catabolic enzymes compete for a common substrate pool.

INTRODUCTION

Animals rely on plants as dietary sources of amino acids that they cannot synthesize themselves. However, some essential amino acids are present at growth-limiting levels in the world's major field crops, including maize (*Zea mays*) (Lys, Trp, and Met), wheat (*Triticum aestivum*) (Lys), rice (*Oryza sativa*) (Lys, Ile, and Thr), soybean (*Glycine max*) (Met and Thr), and potato (*Solanum tuberosum*) (Ile, Met, and Cys). Four of these amino acids (Thr, Ile, Lys, and Met) are synthesized from Asp via a branched pathway with well-studied regulation by feedback inhibition and substrate competition (Coruzzi and Last, 2000). Targeted manipulation of this biosynthetic pathway has been used to increase the levels of Asp-derived amino acids (Galili et al., 2005) and may represent a way to improve the nutritional quality of crop plants. Although amino acid catabolism in plants remains relatively uninvestigated, altered regulation of amino acid breakdown is also an attractive target for plant metabolic engineering. For instance, inhibition of Lys ketoglutarate reductase, combined with upregulation of biosynthesis, greatly increases seed Lys levels (Zhu et al., 2001; Zhu and Galili, 2003).

Three pathways for Thr catabolism are illustrated in Figure 1. Thr deaminase has been studied in plants as the committing enzyme leading to Ile synthesis (Wessel et al., 2000; Halgand et al., 2002; Garcia and Mourad, 2004). Thr dehydrogenase catalyzes Thr breakdown in animals and microbes (Epperly and Dekker, 1991; Edgar, 2002), but this enzymatic activity has not yet been confirmed in plants. Thr aldolase (EC 4.1.2.5), which catalyzes the reversible reaction Thr ↔ Gly + acetaldehyde, has been investigated in rats, yeast, and bacteria, where the reaction proceeds toward Gly + acetaldehyde (Monschau et al., 1997; Liu et al., 1998a, 1998b; House et al., 2001).

Relatively little research has been done on Thr aldolase in plants, but it is likely that this plant enzyme also functions in Thr catabolism. In [α-^{13}C]Gly labeling experiments of whole *Arabidopsis thaliana*, only labeled Gly and Ser accumulated, indicating that Gly is not converted directly to Thr by Thr aldolase in vivo (Prabhu et al., 1996, 1998). Metabolite profiling of *Medicago truncatula* cell cultures showed that the production of Gly from Thr, likely by Thr aldolase, is induced by treatment with methyl jasmonate (Broeckling et al., 2005). Seed Thr content was increased by the *Arabidopsis tha1-1* Thr aldolase missense mutation (Jander et al., 2004). By contrast, overproduction of yeast Thr aldolase in *Arabidopsis* decreased Ile levels and increased Met levels, suggesting not only substrate competition between Thr aldolase and Thr deaminase but also upregulation of the Asp-derived amino acid pathway (Fernie et al., 2004).

L-*allo*-Thr, an isomer of L-Thr that has been detected in *Arabidopsis* metabolite profiling experiments (Fiehn et al., 2000), could also be a substrate for Thr aldolase. Both L-Thr and L-*allo*-Thr are substrates for low-specificity Thr aldolases found in yeast and bacteria (Liu et al., 1997, 1998a). Proteins purified from maize and mung bean (*Vigna radiata*) produced Gly from L-*allo*-Thr and L-Ser but not L-Thr (Masuda et al., 1980, 1982). However, this may indicate Ser hydroxymethyltransferase (SHMT), rather than Thr

[1] Current address: Florida International University, 11200 S.W. 8th Street, Miami, FL 33199.
[2] To whom correspondence should be addressed. E-mail gj32@cornell.edu; fax 607-254-2958.
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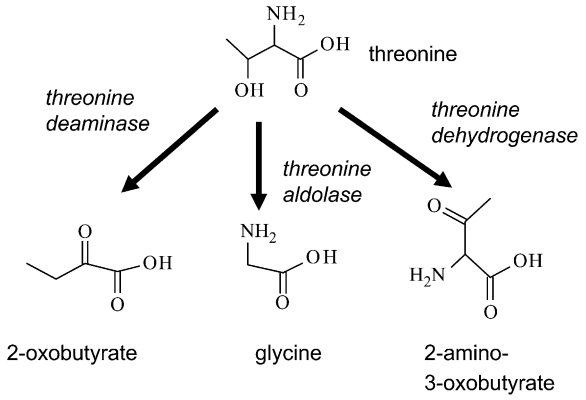

Figure 1. Three Pathways for Thr Degradation.

aldolase, activity. Similarly, purified SHMTs from rat, rabbit, human, and *Eschericia coli* cleave not only L-Ser but also L-*allo*-Thr in preference to L-Thr (Ogawa et al., 2000; Contestabile et al., 2001).

Bioinformatic analysis of the *Arabidopsis* genome shows two likely Thr aldolase genes with 67% amino acid sequence identity: *THA1* and *THA2*. In previous work, we demonstrated that THA1 has Thr aldolase activity in vitro (Jander et al., 2004). Here, we confirm that THA2 is also a Thr aldolase. Furthermore, we use insertional mutations and promoter fusions to study the function of both *Arabidopsis* Thr aldolases and investigate their possible use in plant metabolic engineering.

RESULTS

Arabidopsis THA2 Is a Thr Aldolase

To confirm that *THA2* encodes a Thr aldolase, a full-length cDNA was cloned into the tetracycline-repressible yeast vector pCM185. This plasmid was used to transform haploid yeast strains W3031B (control) and YM13 (*gly1 shm1 shm2*). YM13 is a Gly auxotroph due to mutations in Thr aldolase and both yeast SHMT genes (McNeil et al., 1994; Monschau et al., 1997). As in the case of *THA1* (Jander et al., 2004), transformation with *THA2* rescued the YM13 Gly auxotrophy. However, this does not occur when transgene expression was repressed with tetracycline (Figure 2).

In vitro assays with THA2 Thr aldolase that was partially purified from the transformed YM13 yeast strain were used to confirm the enzymatic activity. THA2-containing yeast extract was incubated with concentrations of L-Thr ranging from 1 to 50 mM, and the Thr aldolase activity was assayed as the production of acetaldehyde over time (Paz et al., 1965; Liu et al., 1998a). From these data we calculated that the apparent K_m of THA2 for L-Thr is 3.8 ± 0.8 mM (mean ± SD of three independent measurements; see Supplemental Figure 1 online). This value is similar to those calculated for *Arabidopsis* THA1 (7.1 mM) and yeast Thr aldolase (4.3 mM) (Monschau et al., 1997; Jander et al., 2004).

Mutations in *THA1* Have Recessive, Seed-Specific Effects on Thr

Although we were not able to detect tha1-1 enzymatic activity in vitro (Jander et al., 2004), partial activity in this missense mutant is suggested by the fact that yeast strain YM13 expressing tha1-1 grows at low (50 μM) Gly concentration, whereas untransformed YM13 does not (see Supplemental Figure 2 online). Therefore, we obtained line GK-767E02 (*tha1-2*) (Rosso et al., 2003), which carries a T-DNA insertion that truncates THA1 at amino acid 66 (Figure 3; see Supplemental Table 1 and Supplemental Figure 3A online). There was no *THA1* mRNA detectable by RT-PCR of total RNA isolated from leaves and flowers of homozygous *tha1-2* mutant lines, indicating a likely knockout mutation. *THA2* transcription in these tissues was unaffected by the *tha1-2* mutation (see Supplemental Figure 3A online). Like homozygous *tha1-1* (see Supplemental Figure 3B online), the *tha1-2* mutation causes a large increase in seed Thr content and relatively small decreases in most other seed amino acids (Figure 4A). Both complementation crosses between *tha1-1* and *tha1-2* and transformation with a genomic *THA1* construct (see Supplemental Figure 4 online) showed that increased seed Thr levels are caused by mutations in this gene. Using a single-seed Thr assay, we determined that four out of 15 seeds from *THA1-1/tha1-1* heterozygotes had elevated Thr content (see Supplemental Figure 5 online), close to the 25% that would be expected if the *tha1-1* mutation had recessive, seed-specific effects.

Since Thr aldolases from other organisms are able to act on L-*allo*-Thr, we determined whether the concentration of this nonprotein amino acid is also increased in *tha1* mutants. In *tha1-1* and *tha1-2* seeds, the level of L-*allo*-Thr was increased from nearly undetectable levels to 30 and 10 pmol/mg, respectively (Figure 4B). However, from these results, it is not possible to determine whether L-*allo*-Thr is produced by isomerization of the overabundant L-Thr in these mutants or by other cellular processes. In the latter case, THA1 may play a role in the removal of L-*allo*-Thr.

Elevated Thr was not detected in vegetative tissue of 10-d-old *tha1* seedlings with two true leaves. However, the Gly content was significantly lower in *tha1-1* and *tha1-2* mutant seedlings than in the Columbia (Col-0) wild type (Figure 4C).

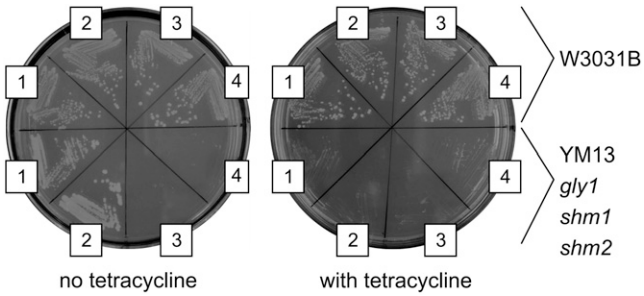

Figure 2. Cloned *Arabidopsis THA1* and *THA2* Thr Aldolases Relieve the Auxotrophy of Yeast Strain YM13 on Plates without Gly.

The top half of each plate is the W3031B wild-type *GLY1*;*SHM1*;*SHM2* haploid strain; the bottom half is the isogenic Gly auxotrophic strain YM13 *gly1*;*shm1*;*shm2*. At the right, promoter expression is repressed with 10 μg/mL tetracycline. 1, *THA1* clone; 2, *THA2* clone; 3, pCM185 vector control; 4, no plasmid control.

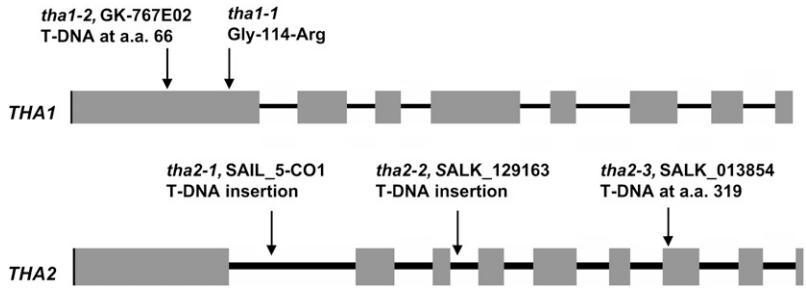

Figure 3. Location of Mutations in *THA1* and *THA2*.

Introns are indicated by thin bars and exons by fat bars. DNA sequences of the T-DNA insertion junction sites are presented in Supplemental Table 1 online.

This suggests that there is a Gly deficit in the absence of synthesis by THA1. The enhanced Thr sensitivity of *tha1-1* and *tha1-2* is further evidence that THA1 is active in seedlings. The Thr concentration that inhibits root growth 50% (IC_{50}) for wild-type seedlings (0.53 ± 0.02 mM) is significantly higher than the IC_{50} for *tha1-1* (0.23 ± 0.01 mM) and *tha1-2* (0.24 ± 0.01 mM) seedlings (see Supplemental Figure 6A online). The significantly decreased Ser levels in *tha1* and *tha2* seeds (Figure 4A) may indicate that this amino acid is used for seed Gly biosynthesis in the absence of THA1.

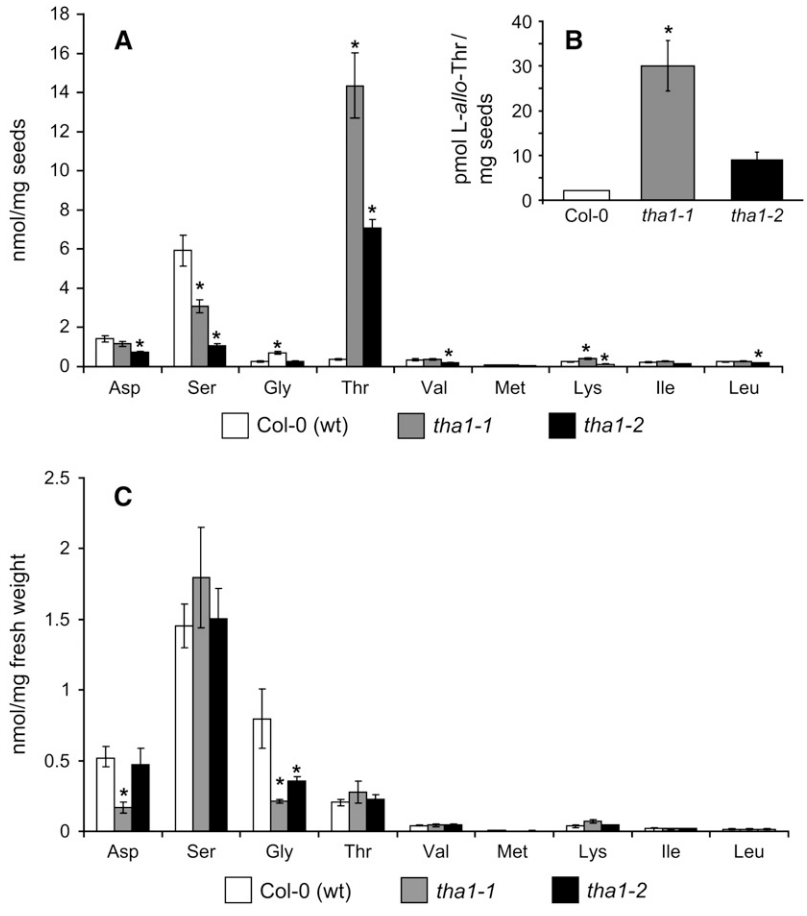

Figure 4. Changes in the Free Seed Amino Acids Resulting from the *tha1-1* and *tha1-2* Mutations.

(A) Thr and metabolically related amino acids in seeds.
(B) L-*allo*-Thr in seeds.
(C) Free amino acid content in seedlings. Mean ± SD of five samples. * $P < 0.01$, Student's *t* test, comparing mutant and wild-type samples.

THA2 Function Is Essential for *Arabidopsis*

To study the role of THA2 in plant metabolism, we obtained three independent T-DNA insertions in this gene. PCR amplification and DNA sequencing confirmed insertions in the first intron (*tha2-1*), the third intron (*tha2-2*), and after amino acid 319 (*tha2-3*) (Figure 3; see Supplemental Table 1 online) in lines SAIL_5_C01, SALK_129163, and SALK_013854, respectively (Sessions et al., 2002; Alonso et al., 2003). It was not possible to isolate homozygous *tha2-1* and *tha2-2* mutants (see Supplemental Figure 3C online), suggesting that the gene is essential for the life of the plant. Examination of siliques on *THA2*/*tha2-2* plants showed that 98 out of 399 developing seeds had an albino phenotype (Figure 5A), consistent with *tha2-2* being a seed-specific recessive mutation. Crosses between *tha2-1* and *tha2-2* failed to complement, confirming that the phenotype results from mutations in the *THA2* gene. Mature seeds from *THA2*/*tha2-1* and *THA2*/*tha2-2* plants all look phenotypically normal (Figure 5B), but 25% of the developing seedlings are albino, fail to mature beyond the cotyledon stage (Figure 5C), and do not accumulate any chlorophyll pigments (see Supplemental Figure 7 online). PCR-based genotyping showed that both albino seeds and albino seedlings are homozygous for T-DNA insertions in *THA2*, whereas their green siblings are either heterozygous or homozygous wild type (see Supplemental Figure 3D online). It was not possible to rescue the *tha2* albino phenotype by adding the metabolically related amino acids Met, Lys, Lys + Met, Thr, Ile, or Gly to the agar plates on which the seeds were germinated. Similarly, seeds germinating in liquid culture were not rescued by the addition of acetaldehyde. Spraying flowering-stage *THA2*/*tha2* heterozygotes with Thr, Ile, Met, Gly, or acetaldehyde failed to prevent the formation of albino seeds.

Analysis of seed amino acid content in pooled seeds from *THA2*/*tha2-1* and *THA2*/*tha2-2* heterozygotes showed moderate increases in the levels of Thr and branched-chain amino acids (Figure 6A), perhaps indicating haplo-insufficiency for Thr aldolase in these lines. The levels of all amino acids except Pro were decreased in homozygous *tha2-2* albino seeds and 10-d-old seedlings (Figures 6B and 6C). However, this general decrease in free amino acids could be a secondary effect resulting from the absence of photosynthesis. Unlike the *tha2-1* and *tha2-2* mutations, the *tha2-3* T-DNA insertion, which truncates the final 36 amino acids of THA2, is not lethal. Homozygous *tha2-3* mutants (see Supplemental Figure 3E online) do not show any visible phenotypic differences compared with wild-type plants. Furthermore, analysis of the seed amino acid content of *tha2-3* homozygotes showed no significant Thr increase compared with wild-type Col-0 (Figure 6A). In the crystal structure of *Thermotoga maritima* Thr aldolase, the C terminus is free in solution (Kielkopf and Burley, 2002). Therefore, it is possible that 36–amino acid truncation of THA2 does not have a significant effect on the overall structure and function of the protein.

A *THA2*/*tha2-2* heterozygote was used as the female parent in crosses to both *tha1* alleles. In the F2 generation, *tha1 THA2*/*tha2-2* progeny were identified by herbicide resistance (sulfadiazine and kanamycin) and PCR-based genotyping. No F2 progeny homozygous for both *tha1* and *tha2* mutations were identified. The seed Thr content of the *tha1-1 THA2*/*tha2-2* and *tha1-2 THA2*/*tha2-2* plants was increased 55- and 25-fold relative to Col-0 (see Supplemental Figure 8 online). This increase is similar to that observed in the *tha1-1* and *tha1-2* parent lines in the same experiment. There were no consistent, significant changes in the abundance of other seed amino acids.

Metabolic Profiling of Thr Aldolase Mutants

To investigate the broader metabolic changes that are induced by Thr aldolase mutations, we measured amino acids, sugars, and organic acids by gas chromatography–mass spectrometry (GC-MS) of flowers, leaves, stems, siliques, and seeds from Col-0 wild-type, *tha1-1*, *tha1-2*, *THA2-1*/*tha2-1*, and *THA2-2*/*tha2-2* plants. For the amino acids that were detected, the GC-MS data (see Supplemental Table 3 online) confirmed the results obtained previously by HPLC. The *tha1-1* and *tha1-2* mutations caused 120- and 50-fold increases in seed Thr, respectively. Free Gly content of most vegetative tissue in *tha1* mutants showed a moderate decrease, generally <50%, despite no significant changes in the Thr levels. Both *THA2-1*/*tha2-1* and *THA2-2*/*tha2-2* heterozygotes had significant alteration in the amino acid profiles of most tissue types. Haplo-insufficiency is suggested by the fact that lower amounts of Gly, a product of Thr aldolase reaction, were detected in all tissues of *THA2*/*tha2* heterozygotes. In vegetative tissue, branched-chain amino acids (Ile, Leu, and Val)

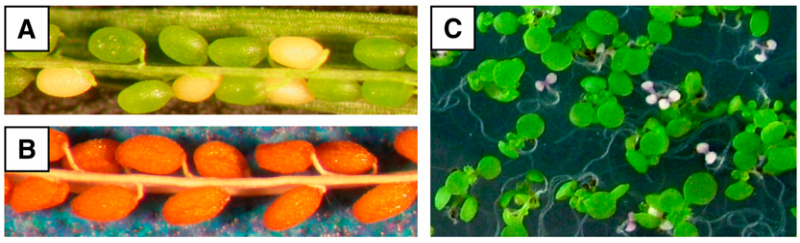

Figure 5. Seed and Seedling Phenotypes of *tha2* Mutants.

(A) Homozygous *tha2-2* seeds on heterozygous *THA2*/*tha2-2* plants are albino, whereas *THA2*/*tha2-2* heterozygous and *THA2* wild-type siblings are green.

(B) Mature mutant and wild-type seeds look identical.

(C) Homozygous *tha2-2* seedlings are albino and do not progress beyond the cotyledon stage. *THA2*/*tha2-2* heterozygous and *THA2* wild-type siblings develop normally.

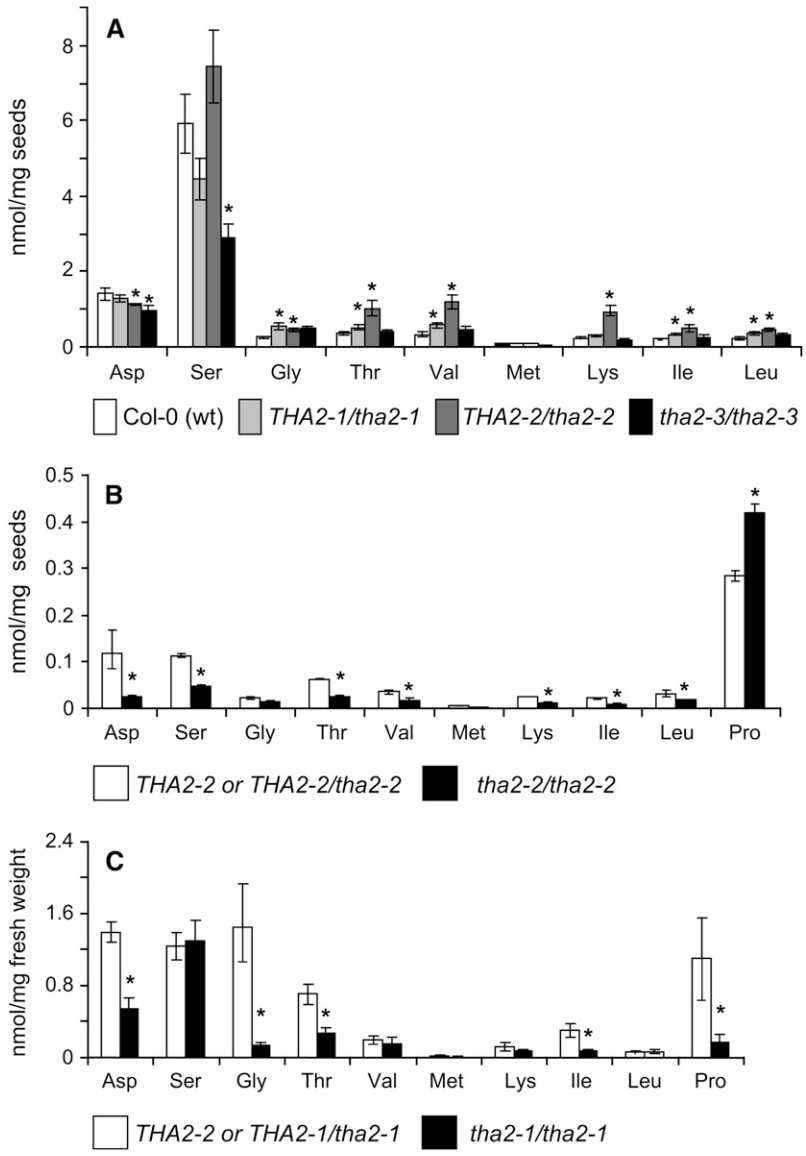

Figure 6. Amino Acid Content of *tha2* Seeds and Seedlings.

(A) *THA2/tha2-1* and *THA2/tha2-2* heterozygotes have slightly elevated levels of Thr and branched-chain amino acids in the seeds. Thr and Ile content in homozygous *tha2-3/tha2-3* plants is not significantly different from the wild type.
(B) and **(C)** Most free amino acids are decreased in albino seeds **(B)** and seedlings **(C)** relative to wild-type or heterozygous seedlings.
Mean ± SD of five samples. *P < 0.01, Student's *t* test.

showed increases in *THA2/tha2* compared with the wild type in all tissue types except in stems, where there was a decrease.

Consistent changes were also seen in the levels of organic acids and sugars in the *tha1* and *THA2/tha2* mutant lines (see Supplemental Table 3 online). Citrate and isocitrate levels in seeds and flowers of all four mutants were significantly lower than in the Col-0 wild type. Similarly, levels of most sugars were significantly decreased in flowers and siliques of *THA2/tha2* heterozygotes. The two *tha1* mutants exhibited strikingly different sugar profiles. Overall sugar levels were significantly decreased in *tha1-1* but not *tha1-2* flowers. Glucose, mannose, fructose, and galactose were significantly (threefold to sixfold) increased in *tha1-2* leaves but not in *tha1-1* leaves. These differences may result from partial function of the *tha1-1* missense mutation.

THA1 and *THA2* Have Different Expression Patterns

THA1 and *THA2* promoter fusions to β-glucuronidase (GUS) were used to study the expression patterns of the two genes (Figure 7). The *THA1* promoter shows expression primarily in seeds and young seedlings, which is consistent with the phenotypic effects that we observed in these growth stages. By contrast, the *THA2*

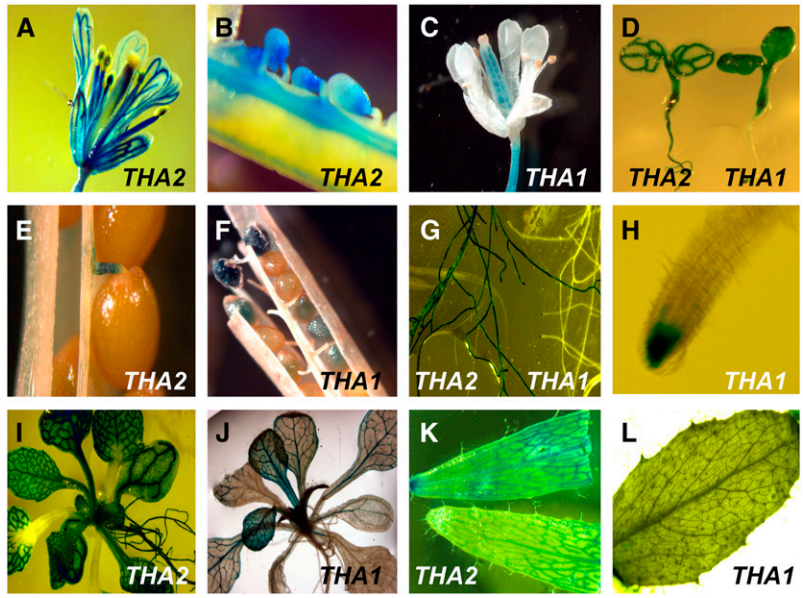

Figure 7. GUS Promoter Fusions Show *THA1* and *THA2* Expression Patterns.

Expression of THA1-GUS and THA2-GUS promoter fusions in flowers and developing seeds (**[A]** to **[C]**), seedlings **(D)**, almost-mature seeds (**[E]** and **[F]**), roots (**[G]** and **[H]**), 2-week-old rosettes (**[I]** and **[J]**), and cauline leaves (**[K]** and **[L]**).

promoter drives GUS expression in all vascular tissues of the plant, including the flowers and the funiculus. Unlike *THA1*, which is expressed in seeds almost all the way to maturity (Figure 7F), *THA2* is expressed only at an early developmental stage (Figure 7B). As the seeds mature, expression shifts to the attachment point of the funiculus and eventually disappears. In roots, *THA1* expression can only be detected at the root tips, but *THA2* expression occurs in the entire root (Figures 7G and 7H). Analysis of publicly available microarray data with Genevestigator (Zimmermann et al., 2004) showed an expression pattern that is consistent with the GUS staining pattern (Figure 7), with highest expression of *THA1* in the developing seeds and *THA2* in the flowers and shoot apex (see Supplemental Figure 9 online).

Thr Aldolase Mutations Increase Substrate Availability for Thr Deaminase

The inability to rescue *tha2-1* and *tha2-2* mutant growth with exogenous addition of amino acids suggested that lethality could be due to excess Thr rather than the absence of a downstream

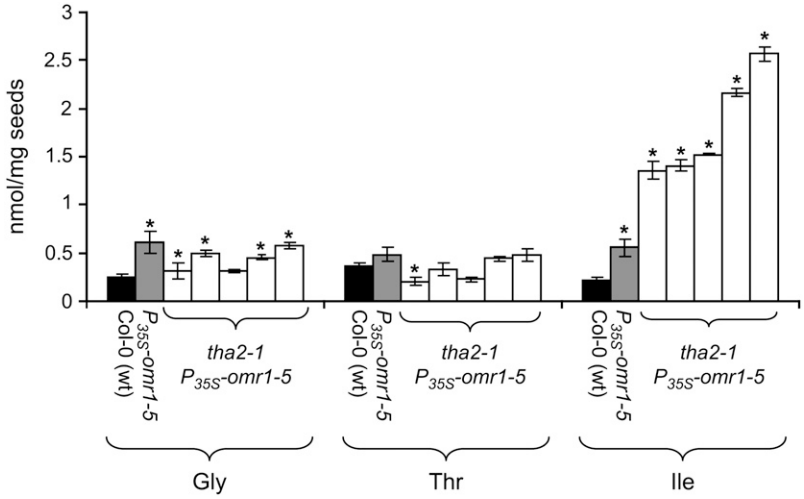

Figure 8. The Lethal Effects of *tha2-1* Thr Aldolase Mutations Are Rescued by Overexpression of Thr Deaminase (P_{35S}-*omr1-5*), Which Also Causes Significantly Elevated Seed Ile Content.

Progeny from five independent transformation events are shown. Mean ± SD of five samples. *$P < 0.01$, Student's *t* test compared with Col-0 (wild type).

metabolite. To test this hypothesis, we transformed *THA2*/*tha2-1* heterozygotes with T-DNA constructs that overproduce a feedback-insensitive form of Thr deaminase (the *omr1-5* allele of *OMR1*) using the cauliflower mosaic virus 35S promoter (Mourad and King, 1995; Garcia and Mourad, 2004). Thr deaminase catalyzes the formation of α-ketobutyrate from Thr (Figure 1) as the first step in the pathway leading to Ile. Therefore, overproduction would be expected to reduce Thr content. PCR-based genotyping of the T2 generation was used to identify *tha2-1*/*tha2-1* P_{35S}-*omr1-5* plants (see Supplemental Figure 3F online). Visual analysis of *tha2-1*/*tha2-1* P_{35S}-*omr1-5* homozygotes showed some chlorosis, leaf curling, and misshapen flowers (see Supplemental Figure 10 online) but otherwise viable plants with normal seed set. The P_{35S}-*omr1-5* transgene in the *THA2* background caused only a twofold increase in seed Ile content. By contrast, there was a 10-fold increase in seed Ile in the *tha2-1* background (Figure 8), suggesting that Thr availability is limiting the formation of Ile via feedback-insensitive Thr deaminase in wild-type seeds. It is possible that P_{35S}-*omr1-5* expression rescues *tha2-1*/*tha2-1* seeds and seedlings through the elimination of otherwise toxic amounts of Thr. However, experiments with P_{35S}-*omr1-5* and P_{35S}-*omr1-5* seedlings growing on agar showed only moderately increased resistance to exogenously added Thr (see Supplemental Figure 6B online). Since homozygous *tha2-1* and *tha2-2* mutations are lethal, we could not study transcript levels in the original T-DNA insertion lines. However, in the *tha2-1*/*tha2-1* P_{35S}-*omr1-5* double mutants, we were unable to detect any *THA2* transcript by RT-PCR (see Supplemental Figure 3F online), indicating that *tha2-1* is a knockout mutation.

Further genetic analysis was used to demonstrate that rescue of *tha2-1*/*tha2-1* albino seeds by P_{35S}-*omr1-5* depends entirely on the seed genotype. When pollen from *tha2-1*/*tha2-1* P_{35S}-*omr1*/P_{35S}-*omr1* plants was transferred onto *THA2*/*tha2-1* pistils, no albino seeds were observed. This is consistent with P_{35S}-*omr1* contributed by the pollen rescuing the 50% of progeny seeds from this cross that are homozygous for *tha2-1* mutation. Moreover, 50% of the individual seeds from the cross showed greatly increased Ile content (see Supplemental Figure 11A online), which is characteristic of *tha2-1*/*tha2-1* P_{35S}-*omr1-5* seeds (Figure 8). In the reciprocal experiment to study maternal effects of P_{35S}-*omr1-5*, *tha2*/*tha2* P_{35S}-*omr1-5*/+ plants were allowed to self-pollinate. Three progeny seed genotypes were expected: 25% *tha2*/*tha2*, 50% *tha2*/*tha2* P_{35S}-*omr1-5*/+, and 25% *tha2*/*tha2* P_{35S}-*omr1-5*/ P_{35S}-*omr1-5*. If maternal-only P_{35S}-*omr1-5* expression does not provide rescue of the mutation, then the 25% of the progeny seeds that are *tha2*/*tha2* should show an albino phenotype. Out of 193 progeny seeds that were examined, 46 were albino, not significantly different from the expected 25% ($P < 0.05$, χ^2 test). Therefore, maternal expression of P_{35S}-*omr1-5* is not sufficient to rescue *tha2*/*tha2* mutant seeds.

A different feedback-insensitive allele of Thr deaminase (P_{35S}-*omr1-7*; Garcia and Mourad, 2004) was used to transform homozygous *tha1-1* mutants. Greatly elevated Ile content was observed in *tha1-1* P_{35S}-*omr1-7* double mutants (Figure 9), even though the seed Ile changes induced by the *omr1-7* transgene itself are insignificant. The two double mutant lines with the highest seed Ile levels have the lowest Thr, suggesting competition for a common Thr pool between Thr aldolase and Thr deaminase. No visible defects beyond a mild chlorosis of the leaves were observed in *tha1-1* P_{35S}-*omr1-7* double mutants. Similar to the case of *tha2-1* P_{35S}-*omr1-5*, seed-specific expression of P_{35S}-*omr1-7* in the *tha1-1*/*tha1-1* genetic background resulted in greatly increased seed Ile content (see Supplemental Figure 11B online).

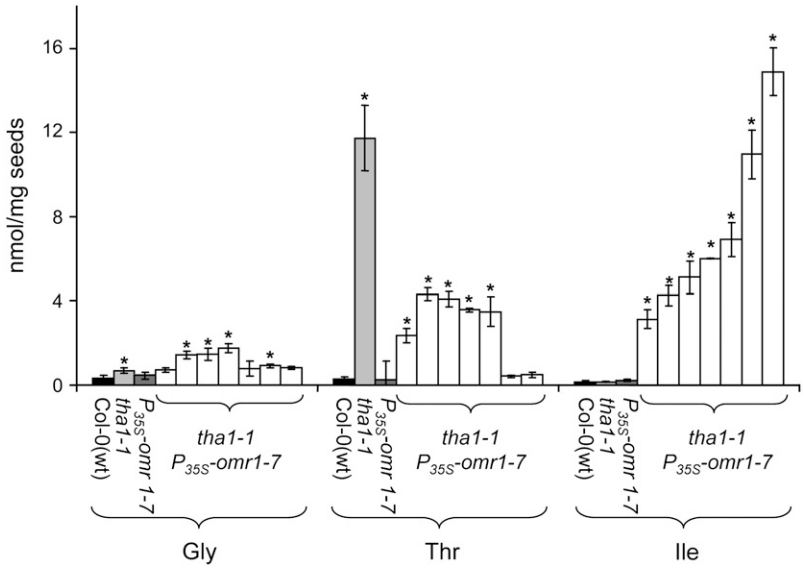

Figure 9. Elevated Thr Caused by the *tha1-1* Thr Aldolase Mutation Is Converted to Ile by Overexpression of Feedback-Insensitive Thr Deaminase (P_{35S}-*omr1-7*).

Progeny from seven independent transformation events are shown. Mean ± SD of five samples. *$P < 0.01$, Student's *t* test compared with Col-0 (wild type).

The *tha1-2/tha1-2* and *tha2-1/tha2-1 P_{35S}-omr1-5* mutant lines were crossed, and homozygous *tha1-2/tha1-2 tha2-1/tha2-1 P_{35S}-omr1-5* triple mutant progeny were identified in the F2 generation (see Supplemental Figure 3G online). The homozygous triple mutants were viable, showing that Gly production from Thr by THA1 and THA2 is completely dispensable for *Arabidopsis*. The morphological defects of the triple mutants were similar to those of the *tha2-1/tha2-1 P_{35S}-omr1-5* double mutant shown in Supplemental Figure 10 online. At least in one case, seed Thr content of the triple mutant was significantly higher than that of the *tha2-1/tha2-1 P_{35S}-omr1-5* double mutant (Figure 10), indicating that the *tha1-2* and *tha2-1* knockout mutations have additive effects.

DISCUSSION

Promoter-GUS fusions indicate that the two *Arabidopsis* Thr aldolases have overlapping, though not identical expression patterns. The expression of *THA1* in developing seeds is consistent with the observation that Thr content is greatly elevated in *tha1* mutant seeds. *THA2* is also expressed in seeds and seedlings, but the most readily apparent phenotype is a complete lack of chlorophyll. Two possible explanations for the different phenotypes are (1) Thr is compartmentalized, and the two enzymes have different functions; or (2) *tha1* mutations cause a minor decrease in total Thr aldolase activity, whereas *tha2* mutations cause a larger decrease that is lethal. In the latter case, one would have to assume that synthesis of THA1 cannot be upregulated to compensate for the lack of THA2 in these tissues.

Both *THA1* and *THA2* are expressed in the vasculature of *Arabidopsis* leaves (Figures 7I and 7J). This expression pattern suggests a possible role in phloem loading or amino acid transport for Thr aldolase. The expression of *THA2* at the attachment point of the funiculus in developing seeds (Figure 7B) may indicate a gatekeeper function in the processing of metabolites as they are transported into the seeds. The more uniform expression of *THA1* throughout seed development (Figures 7C and 7F) indicates a broader, though nonessential, metabolic function during seed filling. Seed-specific expression of feedback-insensitive Thr deaminase contributed by the pollen in crosses counteracts the effects of both the *tha2-1* and the *tha1-2* mutations in a similar manner as whole-plant expression (see Supplemental Figure 11 online). Therefore, metabolic communication between maternal and seed tissue is not necessary to explain the observed rescue of *tha1* and *tha2* phenotypes by Thr deaminase.

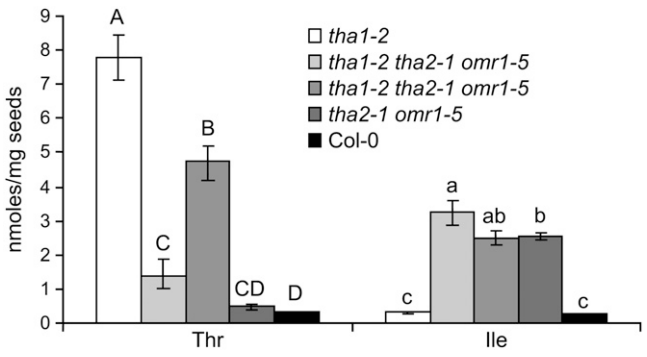

Figure 10. Seed Thr and Ile Content of *tha1-2/tha1-2 tha2-1/tha2-1 P35S-omr1-5* Triple Mutants (Two Independent Isolates) and the *tha1-2/tha1-2* and *tha2-1/tha2-1 P35S-omr1-5* Parent Lines.

Different letters indicate significant differences with Tukey-Kramer honestly significant difference test ($P < 0.05$). Mean ± SD of $n = 3$.

The rescue of *tha2-1* mutations by *P_{35S}-omr1-5* suggests that the absence of Gly is not the proximal cause of lethality. However, at this point, we cannot rule out the possibility that *tha2 P_{35S}-omr1-5* double mutants survive because the plants synthesize Gly by an as yet unknown pathway downstream of Thr deaminase. Production of Gly by THA1 is not necessary for the observed phenotypic rescue because *tha1-2/tha1-2 tha2-1/tha2-1 P_{35S}-omr1-5* triple mutants are viable and have a visible phenotype that is similar to *tha2-1/tha2-1 P_{35S}-omr1-5* double mutants. It is conceivable that acetaldehyde produced by Thr aldolase plays an essential role in plant metabolism. However, this seems unlikely due to the generally phytotoxic effects of acetaldehyde (Kursteiner et al., 2003; Tsuji et al., 2003; Fujishige et al., 2004; Hirayama et al., 2004).

It is tempting to speculate that the lethal albino phenotype of homozygous *tha2/tha2* seeds results from excess Thr in the affected tissues, perhaps via feedback inhibition of the Asp-derived amino acid pathway (Coruzzi and Last, 2000). However, our inability to rescue *tha2* mutants with exogenous Met, Lys, and other amino acids suggests otherwise. The 14 mM Thr observed in the perfectly normal-looking and viable *tha1-1* seeds (Figure 4) shows that mature seeds can tolerate a considerable amount of excess Thr without deleterious effects. By contrast, the IC_{50} for *Arabidopsis* root growth inhibition by exogenous Thr is 0.53 mM, though active transport may make the concentration in roots higher than in the surrounding medium. Similar to effects of *tha2* mutations described here, it has been suggested that knockout of the Lys biosynthetic enzyme DHDPS2 (dihydrodipicolinate synthase 2) causes the accumulation of toxic levels of Thr in *Arabidopsis* (Sarrobert et al., 2000). However, there is also as yet no proven mechanism by which this happens. It is also possible that a toxic metabolite derived from excess Thr contributes to plant lethality. One candidate compound might be L-*allo*-Thr, but exogenous L-*allo*-Thr is not significantly more toxic than L-Thr for root growth of *Arabidopsis* seedlings (V. Joshi, unpublished data).

In addition to Thr aldolase, at least two other plant enzymes, SHMT and glyoxylate aminotransferase, can produce Gly. There is quite considerable metabolic flux through Gly as a substrate for SHMT in the photorespiratory cycle. Therefore, one possible function of Thr aldolase is that it contributes to maintaining Gly homeostasis in plants at times of transition in the use of the photorespiratory cycle. Several lines of evidence suggest that Thr aldolase activity contributes to such Gly homeostasis in *Arabidopsis*: (1) Gly levels are decreased in *tha1* seedlings (Figures 4C); (2) Gly levels are decreased in vegetative tissue of *THA2/tha2* heterozygotes (see Supplemental Figure 7 online); (3) Ser, which can be interconverted with Gly by SHMT, is increased in *tha1* seed (Figure 4A); and (4) *THA1* mRNA levels are increased eightfold in seedlings of an *Arabidopsis* isocitrate lyase mutant (Cornah et al., 2004). Conversely, the reduced levels of isocitrate and citrate in seeds and flowers of both *tha1* and *THA2/tha2*

mutants (see Supplemental Table 3 online) may indicate increased production of Gly via isocitrate lyase and glyoxylate.

Although feedback-insensitive Thr deaminase alone significantly increases Ile in vegetative tissue (Garcia and Mourad, 2004), there is little or no effect on seed Ile content (Figures 8 and 9). However, if P_{35S}-*omr1* is combined with *tha1* or *tha2* mutations, seed Ile is greatly elevated (Figure 9). These results show that Thr aldolase and Thr deaminase compete for a common pool of Thr in developing seeds and that Thr availability is limiting for Ile formation. Similarly, Thr availability is rate-limiting for Ile formation in *Arabidopsis* leaves, as was demonstrated previously with metabolic precursor feeding studies (Lee et al., 2005). By reducing Thr aldolase and increasing Thr deaminase activity, we have redirected Thr flux from Gly toward α-ketobutyrate and Ile. Even in the absence of Thr deaminase overproduction, *THA2* haploinsufficiency increases the levels of branched-chain amino acids (see Supplemental Figure 7 online). Concomitant increases in Val and Leu are likely due to coregulation of the linked pathways leading to the formation of these amino acids. Similarly, production of Ile, Leu, and Val was coordinately regulated in interspecific tomato (*Solanum lycopersicum*) introgression lines (Schauer et al., 2006) and *Arabidopsis* mutants that were selected to have increased seed Ile content (Jander et al., 2004).

Thr, Lys, Met, and other limiting essential amino acids are commonly added as supplements to livestock feed. By providing an optimal ratio of amino acids, rather than increasing total protein content, it is possible to improve animal growth without having an excess of nonlimiting amino acids, which would be degraded to ammonia by livestock and excreted as an environmental pollutant (Pfefferle et al., 2003). Our results suggest that inhibiting Thr aldolase function in the seeds of crop plants could be used to increase Thr content. Indeed, comparison of EST data from several crops, including maize, rice, and soy (see Supplemental Figure 12 online), shows likely Thr aldolases whose expression could be manipulated to improve grain quality. The relatively specific effects of Thr aldolase mutations on seed Thr content make this enzyme an attractive target for plant genetic engineering. Increased production of Ile, another essential amino acid, can be limited by the availability of Thr as a substrate. By combining Thr aldolase mutations with overproduction of Thr deaminase, we demonstrated that seed Ile levels are greatly increased with few deleterious metabolic effects on the growth of the plant.

METHODS

Plant Material

Wild-type *Arabidopsis thaliana* ecotype Col-0 is from the ABRC (http://www.arabidopsis.org/abrc/). Thr aldolase T-DNA insertion mutants, all in the Col-0 background, were obtained from publicly available collections. Lines SALK_129163 (*tha2-2*) and SALK_013854 (*tha2-3*) are from the SIGnAL collection (http://signal.salk.edu/) (Alonso et al., 2003). Line SAIL_5_C01 (*tha2-1*) is from the Syngenta Arabidopsis Insertion Library (Sessions et al., 2002). Segregating SALK_129163 (CS6934), segregating SAIL_5_C01 (CS6960), and homozygous SALK_013854 (CS6935) insertion lines are available from the ABRC. GK-767E02 (*tha1-2*) is from the GABI-KAT collection (Rosso et al., 2003) and can be obtained from the Nottingham Arabidopsis Stock Centre (http://arabidopsis.info/). The Col-0 *tha1-1* mutant line (Jander et al., 2004) will be made available by the authors but requires a material transfer agreement from the Monsanto Company. Transgenic *Arabidopsis* lines and bacterial T-DNA vectors with P_{35S}-*omr1* constructs were kindly supplied by G. Mourad (Purdue University, Fort Wayne, IN) and require a material transfer agreement.

Growth Conditions

Plants were grown on Cornell Mix with Osmocoat fertilizer (Landry et al., 1995) in 20 × 40 cm nursery flats in Conviron growth chambers. Photosynthetic photon flux density was 200 μmol m^{-2} s^{-1}, the photoperiod was 16:8 day:night, the temperature was 23°C, and the relative humidity was 50%. For growth on agar, 1× Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with or without 1% sucrose was used, and the plates were placed in the same growth chambers as the soil-grown plants. *Arabidopsis* seeds were surface-sterilized by incubation for 1 min in 70% ethanol, followed by shaking in 50% commercial bleach (CleanAll Products; 5.25% sodium hypochlorite) with 0.01% Triton X-100 for 10 min. After five washes with sterile water, seeds were suspended in 0.1% Phytagar (Invitrogen). Seeds were cold-stratified by incubating at 4°C in the dark for 3 d.

To measure seedling Thr sensitivity, seeds were plated on MS agar with 0, 0.5, 1, 2, or 4 mM L-Thr. Root length was measured after 10 d. The IC_{50}, defined as the concentration at which the root length gets inhibited by 50%, was calculated by nonlinear regression using JMP 6 software (SAS Institute).

For experiments to rescue *tha2* homozygous seedlings, Met, Lys, Lys + Met, Thr, Ile, Gly, and Arg were added to MS agar plates at 100 μM and 1 mM concentrations. Acetaldehyde was added at 0, 10, 50, and 100 μM concentration to seeds germinating in 1× MS liquid cultures. For attempts to rescue the albino phenotype of developing seeds, flowering plants were sprayed and drenched with 100 μM and 1 mM Met, Lys, Lys + Met, Thr, Ile, and Gly at 2-d intervals for 10 d before and after flowering.

Genetic Crosses and Generation of Transgenic Plants

Crosses between *Arabidopsis* lines were performed as described (Weigel and Glazebrook, 2002). For complementation of the *tha1-1* mutation, the *THA1* gene, including the 5′ and 3′ untranslated regions, was amplified from genomic DNA of Col-0 using the primers gTHA1f and gTHA1r (see Supplemental Table 2 online) and cloned into pCAMBIA3301 (http://www.cambia.org/) and subsequently transferred to *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986). Plants were transformed using the floral dip method (Clough and Bent, 1998). T1 and T2 transformants were selected by spraying the seedlings twice with 300 μM glufosinate ammonium (1:200 dilution of Finale; Farnam Companies), and T3 seeds were used for amino acid analysis. For promoter-reporter fusions, the promoter regions, 2529 bp for *THA1* and 1818 bp for *THA2*, were amplified using the specific primers promTHA1F and promTHA1R, and promTHA2F and promTHA2R (see Supplemental Table 2 online), respectively. The resulting PCR products were moved into pDONR201 by BP clonase reactions (Invitrogen). The promoters were then transferred into the destination vector *pBGWFS7* (Karimi et al., 2002) using the LR clonase reaction. Binary vectors were introduced into *A. tumefaciens* strain GV3101 (Koncz and Schell, 1986) and subsequently used in plant transformation (Clough and Bent, 1998). Sequences of all the constructs were confirmed by PCR amplification and DNA sequencing before transformation using the Big Dye Terminator v.3.0 cycle sequencing kit (Applied Biosystems; ABI PRISM). T1 populations were selected by spraying with glufosinate ammonium (Finale; Farnam Companies), and the T2 generation was used to screen for GUS expression.

Yeast Experiments

Yeast strains W3031B (*MATα ura3-1 trp1-1 ade2-1 his3-11,-15 leu2-3,-112 can1-100*) and YM13 (W3031B *shm1::HIS3 shm2::LEU2 gly1::URA3*) (McNeil et al., 1994; Monschau et al., 1997) were kindly supplied by A.

Bognar (University of Toronto, Canada). Yeast strains were grown on YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) or on SD medium (0.67% Bacto-yeast nitrogen base and 2% dextrose) supplemented, where appropriate, with 750 μg/mL Gly, 30 μg/mL Leu, and 20 μg/mL each uracil, Trp, adenine, and His.

Primers *CDTHA2F* and *CDTHA2R* (see Supplemental Table 2 online) were used to amplify full-length *THA2* cDNA from ABRC clone S51 for cloning into pCM185 (Gari et al., 1997) using the *Bam*HI and *Pst*I restriction sites of the plasmid. 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 with pCM185 and a derived plasmid construct carrying the *THA2* Thr aldolase. The correct *THA2* cDNA sequence was verified by PCR amplification from yeast and DNA sequencing. Thr aldolase activity in yeast extracts was measured as described previously (Jander et al., 2004).

Verification of T-DNA Insertions in *THA1* and *THA2* by PCR

T-DNA insertion sites were confirmed using T-DNA insertion–specific primers (Lba1 for SALK and Lba3 for Syngenta lines) and flanking *THA1* or *THA2* sequence-specific primers. Amplified products were sequenced to determine actual site of insertion. For genotyping T-DNA insertions, gene-specific primers (*tha1-2* P1, *tha1-2* P2, *tha2-1* P1, *tha2-1* P2, *tha2-2* P1, *tha2-2* P2, *tha2-3* P1 and *tha2-3* P2; see Supplemental Table 2 online) that flank the insertion site were used to detect the wild-type allele, and *THA1* or *THA2*-specific primers in combination with a T-DNA primer (LBa1 or LBb1 for SALK, Lb3 for Syngenta, and GABI SEQ for GABI-KAT lines; see Supplemental Table 2 online) were used to identify the mutant alleles. T-DNA insertions were also selected by growth on herbicide-containing MS agar plates: SALK lines with 30 μg/mL kanamycin, GABI-KAT line with 11.25 mg/L sulfadiazine, and SAIL lines with 50 mg/L glufosinate. T-DNA insertions in SAIL lines growing on soil were selected by spraying with 300 μM glufosinate ammonium (Finale; Farnam Companies).

Amino Acid Assays

For analysis of amino acids, 10 mg of plant tissue was frozen in liquid nitrogen in 2-mL tubes and ground to fine powder with 3-mm steel beads using a Harbil model 5G-HD paint shaker. Ground tissue was taken up in 20 mM HCl (10 μL per mg of tissue), the extracts were centrifuged at 14,000 rpm for 20 min at 4°C, and the supernatant was derivatized with 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (Cohen and van Wandelen, 1997) using an AccQ-Fluor reagent kit (Waters). During derivatization, 5-μL extracts were mixed with 35 μL borate buffer, and the reaction was initiated by the addition of 10 μL 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate reagent followed by immediate mixing and incubation for 10 min at 55°C. Ten microliters of each sample were injected onto a 3.9 × 150-mm Nova-Pak C_{18} column (Waters) using a Waters 2690 pump system, and the data were recorded using Waters' Empower Software. L-Norleucine was used as an internal standard. Eluted amino acid derivatives were detected using a Waters model 2475 multi λ fluorescence detector with an excitation wavelength of 250 nm and an emission wavelength of 395 nm. Solvent A (containing sodium acetate and triethylamine at pH 5.05) was purchased premixed from Waters; Solvent B was acetonitrile:water (60:40). The gradient used was 0 to 0.01 min, 100% A; 0.01 to 0.5 min, linear gradient to 3% B; 0.5 to 12 min, linear gradient to 5% B; 12 to 15 min, linear gradient to 8% B; 15 to 45 min, 35% B; 45 to 49 min, linear gradient to 35% B; 50 to 60 min, 100% B. Flow rate was 1.0 mL min^{-1}. Standard curves were prepared using amino acids purchased from Sigma-Aldrich.

GUS Staining

Different plant tissues from transgenic plants carrying *THA1* promoter:GUS and *THA2* promoter:GUS were incubated in GUS assay solution (50 mM sodium phosphate, pH 7.2, 0.5 mM potassium ferrocyanide, 0.5 mM potassium ferricyanide, 20% methanol, and 2 mM 5-bromo-4-chloro-β-glucuronide) at 37°C for 12 to 16 h as described previously (Jefferson et al., 1987). Slight vacuum was applied to facilitate substrate infiltration. Chlorophyll-containing tissue was cleared in 70% ethanol for photographic analysis. Samples were examined and photographed using an SZX12 microscope (Olympus) equipped with a JENOTCX ProgRes C14 digital camera (Olympus).

Metabolite Profiling

Metabolite analysis by GC-MS was performed as described previously (Roessner-Tunali et al., 2003), with minor modifications. *Arabidopsis* tissue (50 mg of seeds and 100 mg of leaves, flowers, siliques, and stem) from Col-0 (wild type), *tha1-1*, *tha1-2*, *tha2-1*, and *tha2-2*, harvested at same developmental stages, was homogenized using a ball mill pre-cooled with liquid nitrogen. Chloroform extraction was done twice only in case of seeds. After centrifugation, 200 and 150 μL of the polar phase was reduced to dryness in vacuum for seeds and other tissue types, respectively. Reduced residues were redissolved in and derivatized for 120 min at 37°C (in 40 μL of 20 mg mL^{-1} methoxyamine hydrochloride in pyridine), followed by a 30-min treatment with 70 μL *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide at 37°C. Seven microliters of a retention time standard mixture (0.029% [v/v] *n*-dodecane, *n*-pentadecane, *n*-nonadecane, *n*-docosane, *n*-octacosane, *n*-dotracontane, and *n*-hexatriacontane dissolved in pyridine) was added prior to trimethylsilylation. The GC-MS system used comprised an AS 2000 autosampler, a GC 8000 gas chromatograph, and a Voyager quadrupole mass spectrometer (ThermoFinnigan). Both chromatograms and mass spectra were evaluated using the MASSLAB program (ThermoQuest), and normalization of metabolite levels with respect to wild-type levels was performed as described previously (Roessner et al., 2001).

Accession Numbers

Arabidopsis Genome Initiative locus identifiers for the genes mentioned in this article are *THA1* (At1g08630), *THA2* (At3g04520), and *OMR1* (At3g10050).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Sequences of the Junction Sites of *THA1* and *THA2* T-DNA Insertion Lines.

Supplemental Table 2. PCR Primers Used in the Experiments.

Supplemental Table 3. GC-MS Metabolite Profiling of Thr Aldolase Mutants.

Supplemental Figure 1. Lineweaver-Burke Plot Showing Apparent K_m for Thr of THA2.

Supplemental Figure 2. Growth of Yeast Expressing *Arabidopsis* tha1-1 on Medium Containing 50 μM Gly.

Supplemental Figure 3. PCR to Confirm Zygosity and RT-PCR to Confirm Gene Expression of Thr Aldolase Mutants.

Supplemental Figure 4. Complementation of the *tha1-1* Amino Acid Phenotype by Transformation.

Supplemental Figure 5. Thr Levels in Single Seeds of *THA1*/*tha1* Heterozygotes.

Supplemental Figure 6. Thr IC_{50} Determination for Wild-Type, *tha1*, and P_{35S}-*omr1* Seedlings.

Supplemental Figure 7. Chlorophyll Content of *tha2* Mutant Seedlings.

Supplemental Figure 8. Amino Acid Content of *tha1 THA2*/*tha2* Double Mutants.

Supplemental Figure 9. Digital RNA Gel Blot of *THA1* and *THA2* Expression in *Arabidopsis*.

Supplemental Figure 10. Photographs of *tha2-1*/*tha2-1* P_{35S}-*omr1-5* Rosette and Flowers.

Supplemental Figure 11. Seed Amino Acid Changes Resulting from Seed-Specific Expression of P_{35S}-*omr1-5* in the *tha2*/*tha2* and *tha1*/*tha1* Genetic Backgrounds.

Supplemental Figure 12. DNA Sequence Comparison of Thr Aldolases from *Arabidopsis* and Selected Crop Plants.

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