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Synthesis of β-Alanine From Isoleucine and Propionate Catabolism via Aminotransferases

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ABSTRACT

In plants, the nonproteinogenic amino acid β-alanine plays a role in response to hypoxia, flooding, drought, heat, and heavy metal stress conditions. It is also a key intermediate in the synthesis of essential molecules including vitamin B5 and coenzyme A (CoA) through the condensation reaction with pantoate. While the syntheses of pantoate, vitamin B5, and CoA appear to be conserved across plants and bacteria, the synthesis of β-alanine is not. Bacteria and fungi use aspartate, whereas plants can use uracil, spermidine, or propionate to synthesize β-alanine. Given that these three precursors can be formed from the metabolism of glutamine, arginine, isoleucine, and valine, the synthesis of β-alanine could be linked to numerous pathways. Studies of valine catabolism in *Arabidopsis* suggested that some branched-chain amino acids could in fact serve as precursors for the synthesis of β-alanine. Using GC–MS and isotopically labeled isoleucine and propionate, we linked their metabolism to the synthesis of β-alanine via a proposed transamination of malonate semialdehyde. We then identified three aminotransferases that each catalyzed this final reversible transamination reaction. These results affirm our hypothesis that isoleucine metabolism is also linked to the synthesis of β-alanine via the transamination of metabolic intermediates.

1 | Introduction

β-alanine is a naturally occurring, nonproteinogenic, amino acid synthesized by plants (Parthasarathy, Savka, and Hudson 2019), bacteria (Cronan 1980), and fungi (White, Gunyuzlu, and Toyn 2001). Through condensation with the valine-derived molecule pantoate, β-alanine serves as a precursor for the synthesis of coenzyme A, a key component of many metabolic processes, including cellular respiration and fatty acid metabolism. In bacteria and fungi, β-alanine is primarily synthesized from aspartate (Cronan 1980; White, Gunyuzlu, and Toyn 2001). In plants, β-alanine is synthesized via a variety of pathways as recently reviewed by Parthasarathy, Savka, and Hudson (2019) in response to hypoxia, flooding, drought, heat, and heavy metal stress conditions (Kaplan et al. 2004; Rizhsky et al. 2004; Sun et al. 2010; Drakeford, Mukherjee, and Reid 1985; Ganie 2021). Specifically, there is evidence of β-alanine being produced from uracil in higher plants such as *Brassicaceae*, *Plumbaginaceae*, and *Poaceae* families (Tsai and Axelrod 1965; Walsh et al. 2001; Duhazé et al. 2003; Zrenner et al. 2009). Others have shown that β-alanine can be generated from spermidine and spermine, and possibly other polyamines, in maize shoots (Terano and Suzuki 1978), tomato pericarp discs (Rastogi and Davies 1990), and wheat (Gondor et al. 2021). β-alanine can also be synthesized from propionate in wheat, safflower, and pea tissues (Hatch and Stumpf 1962), as well as *Limonium latifolium* leaf discs (Rathinasabapathi 2002). However, uracil, spermidine or

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spermine, and propionate are synthesized themselves from various amino acids such as glutamine, arginine, isoleucine, and valine (Witte and Herde 2020; Chen et al. 2019), suggesting that the production of β-alanine could be linked to many possible precursors. Given that the branched-chain amino acid valine already plays a critical role in serving as the precursor for pantoate, we investigated the role of other branched-chain amino acids as sources of β-alanine, specifically isoleucine, because of its direct metabolic link to the formation of propionyl-CoA, the biological derivative of propionate.

Isoleucine is catabolized initially, similar to the other branched-chain amino acids, valine, and leucine, to an acyl-CoA thioester. At that point, the catabolic pathways segregate and each amino acid derivative is uniquely converted to the energy-rich "end-products"-propionyl-CoA, in the case of valine and isoleucine or acetyl-CoA, in the case of isoleucine and leucine. Because much of branched-chain amino acid degradation occurs in the mitochondria (Binder 2010), it is presumed that acetyl-CoA is used directly in the citrate cycle and propionyl-CoA is further metabolized via a pathway similar to β-oxidation and valine degradation to ultimately produce acetyl-CoA (Lucas et al. 2007). This occurs through the proposed reaction of converting malonate semialdehyde to acetyl-CoA via the enzyme (methyl)malonate semialdehyde dehydrogenase [EC 1.2.1.27] (Figure 1). Instead, here we present data showing that isoleucine can be converted to β-alanine in both *Arabidopsis thaliana* and wheat seedlings via aminotransferase-catalyzed reactions, as proposed in previous plant studies (Parthasarathy, Savka, and Hudson 2019; Hatch and Stumpf 1962; Parthasarathy et al. 2019).

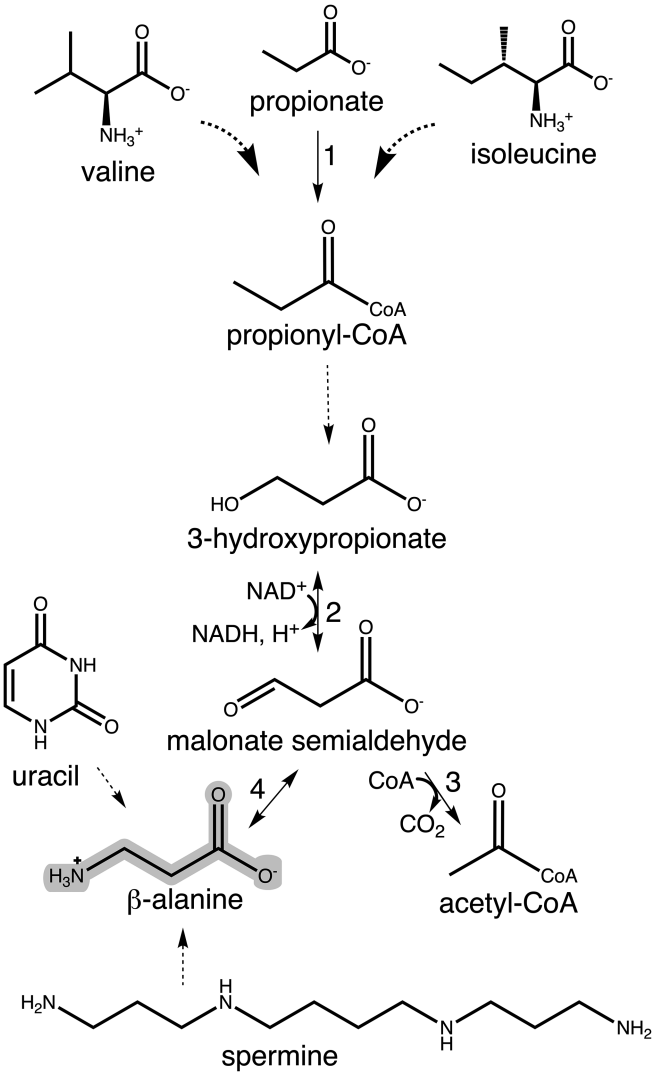

FIGURE 1 | Known and proposed pathways for the synthesis of β-alanine in plants. Previous studies show that β-alanine can be made from uracil, propionate, and spermine. Here, we propose extending the pathway by starting with valine and isoleucine. Propionate, valine, and isoleucine can be converted to propionyl-CoA either through degradative pathways of BCAA degradation or via (1) propionate-CoA ligase [EC 6.2.1.13]. Propionyl-CoA is then converted through a series of reactions to 3-hydroxypropionate and then to malonate semialdehyde via (2) 3-hydroxyisobutyrate dehydrogenase [HDH1, EC 1.1.1.31] and finally to acetyl-CoA via (3) methylmalonate semialdehyde dehydrogenase [MMSD, EC 1.2.1.27], where it then enters the citrate cycle. Alternatively, malonate semialdehyde can be converted to β-alanine via an (4) aminotransferase as proposed in this study. Dashed arrows represent additional catalyzed reactions not central to this study.

2 | Methods

2.1 | Amino Acid Analysis

2.1.1 | Seedling Growth

Approximately 350–450 wild-type or transgenic *A. thaliana* seeds or Hard Red Winter Wheat seeds (Sustainable Seed Company) were surface sterilized for 30 s to 1 min with 70% EtOH, then with 10% bleach for 30 min, before rinsing four to six times with sterile water in laminar flow hood. *A. thaliana* seeds were resuspended in sterile water and pipetted onto plates of one-half strength Murashige–Skoog medium (MS) and 1% sucrose solidified with 1% agar. They were incubated at 4°C for 48 h to synchronize growth and then moved to the long-day growth room (21°C–23°C; 16 h light, 8 h dark). Wheat seeds were placed on water-soaked filter paper in sterile trays before moving to the dark for 48 h. After synchronization, trays were moved to the long-day growth room (21°C–23°C; 16 h light, 8 h dark) for 4 days.

2.1.2 | ^{13}C Treatment and Amino Acid Extraction

Seedlings were divided evenly and treated with isotopically labeled compounds in MS liquid media similar to Lucas et al. (2007). Four-day-old seedlings were placed in 50 mL of media containing one-half strength MS media, pH 5.7 with or without 2 mM 2-^{13}C propionate or U-^{13}C isoleucine (Cambridge Isotopes) in a 250-mL conical flask. Flasks were wrapped in aluminum foil to limit the available light and gently shaken at 50 rpm (Thermo MaxQ 200) for 24 h (propionate-treated) or 72 h (isoleucine-treated) at 21°C°C–23°C. Following treatment, seedlings were collected using a Buchner funnel and rinsed three to five times with sterile water. Seedlings were quickly weighed before being flash frozen and manually ground to a fine powder in liquid nitrogen in a pre-chilled mortar and pestle. Powdered samples were either stored at −80°C, or amino acids were extracted as described below.

2.1.3 | Extraction of Amino Acids

For amino acid profiling of *A.thaliana* seeds, amino acids were extracted according to Gipson *et al. (*Gipson et al. 2017*)* Briefly, 5–10 mg of seeds was soaked in minimal hot water before homogenization. Frozen powdered plant material and the untreated seeds were homogenized in sterile, hot water (100°C) using glass Dounce homogenizers (for Arabidopsis seeds and seedlings) or the SPEX Mini-G 1600 at 1500 rpm for 3 min with 16 beads per sample (for wheat). Water was added at a ratio of 1 mL water to 100-mg plant material, with maximum 6-mL water per sample. Samples were homogenized thoroughly and transferred to a 15 -mL centrifuge tube (homogenizer was rinsed with 0.5- to 1-mL hot water which was added to the sample tube). Samples were then placed in a dry bath heated to 100°C for 10 min and then placed in an ice bath for at least 10 min. The samples were then centrifuged at 14000 g for 5 min at 4°C. The supernatant was either stored at −20°C or immediately prepared for lyophilization to concentrate the amino acids before analysis. Supernatants were transferred to 50-mL round-bottom flasks, flash-frozen in liquid nitrogen, and lyophilized for 24–36 h.

2.1.4 | Analysis of Amino Acids and Enzyme Reaction Products by GC–MS

Lyophilized material was suspended in 300 μL of sterile DI water, transferred to microcentrifuge tubes, and centrifuged at 4°C at 14,000 g for 5 min. The supernatant was transferred to a new tube, and the volume was recorded. Derivatization was carried out using the EZ:faast Amino Acid Analysis kit for GC–MS (Phenomenex). Samples were analyzed using the Agilent 7890A GC with 5975C VL MSD equipped with a Zebron ZB-AAA 10 m × 0.25 mm column (Phenomenex) with helium as the carrier gas. Norvaline (200 μM) was added to each sample as the internal standard, and the presence and quantity of free amino acids were determined by injecting 2 μL of sample at 250°C with split 15:1 injection. The oven was heated from 110°C to 300°C at 10°C/min with MS limits between 45 and 450 m/z. Reaction products were compared to standards provided in the Amino Acid Analysis kit for quantification or standards prepared at 1 mM each of alanine, β-alanine, GABA, and/or glycine for enzymes assays. Data were analyzed based on two injections of each sample for a minimum of three replicates. Quantity of β-alanine in seed samples was analyzed via one-way ANOVA with Tukey–Kramer post hoc test using GraphPad Prism version 10.2.3 for macOS, GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com.

2.2 | Aminotransferase Analysis

2.2.1 | Cloning of POP2, AGT3, PYD4

The full-length protein sequences encoded by the POP2, AGT3, PYD4 genes are predicted to be 513, 477, and 481 amino acids in length, respectively. MitoFates (Fukasawa et al. 2015) subcellular localization tool was used to predict the mitochondrial leader sequences for each protein. MitoFates predicted that the first 44, 21, and 31 residues encoded possible mitochondrial leader sequences for POP2, AGT3, and PYD4, respectively. Therefore, the corresponding nucleotides were excluded when cloning the cDNA and were then designated as POP2L, AGT3L, and PYD4L (Figure S1, blue highlight). An alternative start site was also selected based on protein sequence alignment with β-alanine aminotransferase characterized in mammalian and bacterial species using Clustal Omega sequence alignment program (Madeira et al. 2022). This second start site excluded the first 53, 54, and 60 residues and were designated as POP2S, AGT3S, and PYD4S (Figure S1, red highlight). 3′ primers were designed based on the predicted translated sequence to include the encoded stop site. The primers used for each construct can be found in Table S1. cDNA was obtained from Arabidopsis Biological Resource Center (POP2: clone ID U09523, GenBank accession AY142571 and AGT3: clone ID C104743, GenBank accession BT001918) or Riken BRC (PYD4a, accession: AY099816 and PYD4b, accession: AK226401). cDNAs were amplified via PCR using gene-specific primers (Table S1) and Q5® High-Fidelity DNA Polymerase (New England Biolabs) under the following conditions: 98°C for 30 s, followed by 34 cycles of 98°C for 10 s, 52°C for 30 s, and 72°C for 45 s, then a final extension time of 2 min at 72°C followed by a 4°C hold. The cDNA amplicon was gel purified and ligated into the N-term 6x His-tag, pET28a(+) vector (Novagen) using the NEBuilder® HiFi DNA Assembly kit and transformed into NEB 5-alpha competent *Escherichia coli* (*E. coli*) following the manufacturer's instructions (New England Biolabs). Plasmids were purified (QIAprep spin miniprep kit, Qiagen) from positive colonies and correct sequences were confirmed via sequencing (Eurofins USA).

2.2.2 | Expression and Purification of POP2, AGT3, PYD4

The plasmids pET28a(+)::At3g22200.2 (POP2), pET28a(+)::At2g38400.1 (AGT3), pET28a(+)::At3g08860.1 (PYD4) were transformed into *E. coli* BL21-DE3 cells (New England Biolabs). For protein expression and purification, cells were grown in 500-mL LB broth containing 50 μg/mL kanamycin at 37°C, 225 rpm to an OD600 nm of 0.4–0.6. Protein expression was induced by adding 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) with shaking at 225 rpm for an additional 18 h at 16°C. Cells were pelleted by centrifugation at 3500 g at 4°C for 20 min and stored at −20°C or purified. Pellets containing recombinant POP2 and AGT3 were suspended in 15 mL of 50-mM potassium phosphate (pH 7.5), 0.3 M KCl, 1 mM dithiothreitol (DTT), and 10% glycerol. Pellets containing recombinant PYD4 were resolubilized under the same conditions with 0.45% Triton-X added for increased protein solubility. Lysozyme (1 mg/mL) was added to the mixture and incubated on ice with gentle shaking. After 30 min, the cells were sonicated (UP400S, Hielscher Ultrasonics) at 100% amplitude for 6 min at 30 s intervals with 30 s rest periods. The mixture was centrifuged (Avanti J-26 XPI) at 35000 g for 25 min at 4°C; the supernatant was collected, and recombinant protein was purified using either 3 mL HisPur Ni-NTA spin column or 1-mL HisPur cartridge (both Thermo Fisher Scientific) attached to a peristaltic pump according to manufacturer's recommendations using the following buffers: Equilibration buffer (50 mM potassium phosphate, 0.3 M of KCl, pH 8.0), Wash buffer (50 mM of potassium phosphate, 0.3 M of KCl, 20 mM of imidazole, pH 8.0), and Elution buffer (50 mM of potassium phosphate, 0.3 M of KCl, 300 mM of

imidazole, pH 8.0). Aliquots of the recombinant proteins were visualized on a 4%–20% tris-glycine gel (Bio-Rad) stained with PageBlue (Thermo Fisher Scientific). Fractions containing purified protein were pooled together and simultaneously concentrated and desalted via ultrafiltration using Amicon® Ultra-15 centrifugal filters (Merck Millipore) with a molecular weight cutoff of 30 kDa. Concentration was later determined against BSA protein standards using SDS-PAGE gel electrophoresis or a 660-nm protein assay (Pierce). Purified recombinant protein was stored in 50-mM potassium phosphate (pH 7.5) with 20% glycerol at −80°C.

2.2.3 | Aminotransferase Reactions

2.2.3.1 | *'Native'* Reactions: Glycine/GABA + Pyruvate ➔ Glyoxylate/Succinate Semialdehyde + L-Alanine. The activity of each purified recombinant protein was determined using known substrates identified in the literature (Parthasarathy et al. 2019; Clark et al. 2009; Liepman and Olsen 2003). The AGT3L, PYD4L, POP2L, and POP2S activity assays contained 50 mM TAPS (pH 9), 0.1 mM PLP, 2 mM pyruvate (or water as a "no substrate" control), and 40 μg purified recombinant protein (or water as a "no enzyme control") in 200 μL. The reaction was initiated with 2 mM glycine for AGT3L and PYD4L or GABA for POP2L and POP2S reacted for 30 min at 30°C. The reactions were terminated by the addition of 100 μL cold n-propanol to the reaction mixture, and the products were derivatized via the EZ:faast Amino Acid Analysis kit (Phenomenex). The reaction product, L-alanine, was measured by GC–MS.

2.2.3.2 | *'Reverse'* Reactions: β-Alanine + Pyruvate ➔ Malonate Semialdehyde + L-Alanine. The standard activity assay for the reverse reaction contained 50 mM TAPS (pH 9), 2 mM pyruvate or water ("no substrate" control), 0.1 mM PLP, 40 μg purified recombinant AGT3L, PYD4L, POP2L, and POP2S and was conducted at 30°C. The final reaction volume was 200 μL. The reaction was initiated by the addition of 2 mM β-alanine. A "no enzyme" control was also included with each reaction in which water was added in place of the respective aminotransferase. After 30 min, the reaction was terminated by the addition of 100 μL of cold n-propanol, and the reaction components were derivatized via the EZ:faast Amino Acid Analysis kit (Phenomenex) for analysis by GC–MS with focus on product formation, alanine.

2.2.3.3 | *'Forward'* Reactions: 3-Hydroxypropionate + NAD ➔ Malonate Semialdehyde + L-Alanine ➔ Pyruvate + β-Alanine. The standard activity assay for the forward reaction contained 50 mM TAPS (pH 9), 5 mM 3-hydroxypropionate, 0.1 mM PLP, 3 mM NAD^+, 40 μg purified recombinant POP2S, POP2L, AGT3L, or PYD4L, and 500 ng 3-hydroxyisobutyrate dehydrogenase from *Arabidopsis thaliana* and was conducted at 30°C. Coupled assays used a final volume of 300 μL; the reaction was initiated by the addition of 1 mM alanine or water ("no substrate" control) and incubated for 30 min and then terminated by the addition of cold n-propanol to a 100-μL aliquot of reaction mixture. A "no enzyme" control was also included with each reaction in which water was added in place of the respective aminotransferase. The reaction components were derivatized via the EZ: faast Amino Acid Analysis kit (Phenomenex) and analyzed by GC–MS with a focus on the formation of the product β-alanine.

All aminotransferase reaction spectra shown here are from one representative replicate of at least three separate experiments (using different enzyme preparations) prepared in GraphPad Prism version 10.2.3 for macOS, GraphPad Software, Boston, Massachusetts, USA, www.graphpad.com.

2.3 | Accession Numbers

The accession numbers for each aminotransferase are included in Table S1.

3 | Results

3.1 | *β-Alanine in Transgenic Seeds*

Disruptions in valine degradation can lead to detrimental consequences such as embryo lethality, decreased germination rates, and alterations to branched-chain amino acids (Gipson et al. 2017). For example, two alleles of homozygous, transgenic methylmalonate semialdehyde dehydrogenase mutants resulted in seeds with smaller embryos, decreased storage reserves, reduced germination rates, and increased levels of valine and leucine as determined by GC–MS. Given MMSD's additional proposed role in propionyl-CoA metabolism (Figure 1), we expanded the amino acid analysis and discovered that seeds of T-DNA insertion mutants of *mmsd* (loss-of-function *mmsd-1* and reduced expression *mmsd-2*) also showed higher levels of β-alanine compared to other amino acids (Figure 2).

Almost no β-alanine was detected in wild-type *Arabidopsis* seeds and none in mutant seeds with decreased 3-hydroxyisobutyrate dehydrogenase expression (*hdh1–2*, reaction (2) in Figure 1). HDH1 is responsible for catalyzing the conversion of 3-hydroxypropionate to malonate semialdehyde, prior to the production of acetyl-CoA or β-alanine. However, β-alanine was observed in complemented *mmsd-1* seeds (*mmsd-1 35S::MMSD)*. Previous data showed that all other phenotypes (seed weight, storage reserves, and germination rates) of the mutant *mmsd-1* seeds were restored in the complemented plant line (Gipson et al. 2017). It is important to note that the complemented *mmsd-1* plants were generated using a constitutively expressed promotor (not the native promoter) and a truncated gene sequence, suggesting that MMSD activity was only partially restored, such that, in seeds, β-alanine levels were not near zero as expected.

3.2 | Carbon Tracing via GC–MS

To identify a metabolic link between the function of MMSD in branched-chain amino acid metabolism and the increased β-alanine levels measured in the transgenic seeds, we conducted ^{13}C labeling studies using GC–MS. We treated 4-day-old wild-type *Arabidopsis* seedlings and 6-day-old wheat seedlings

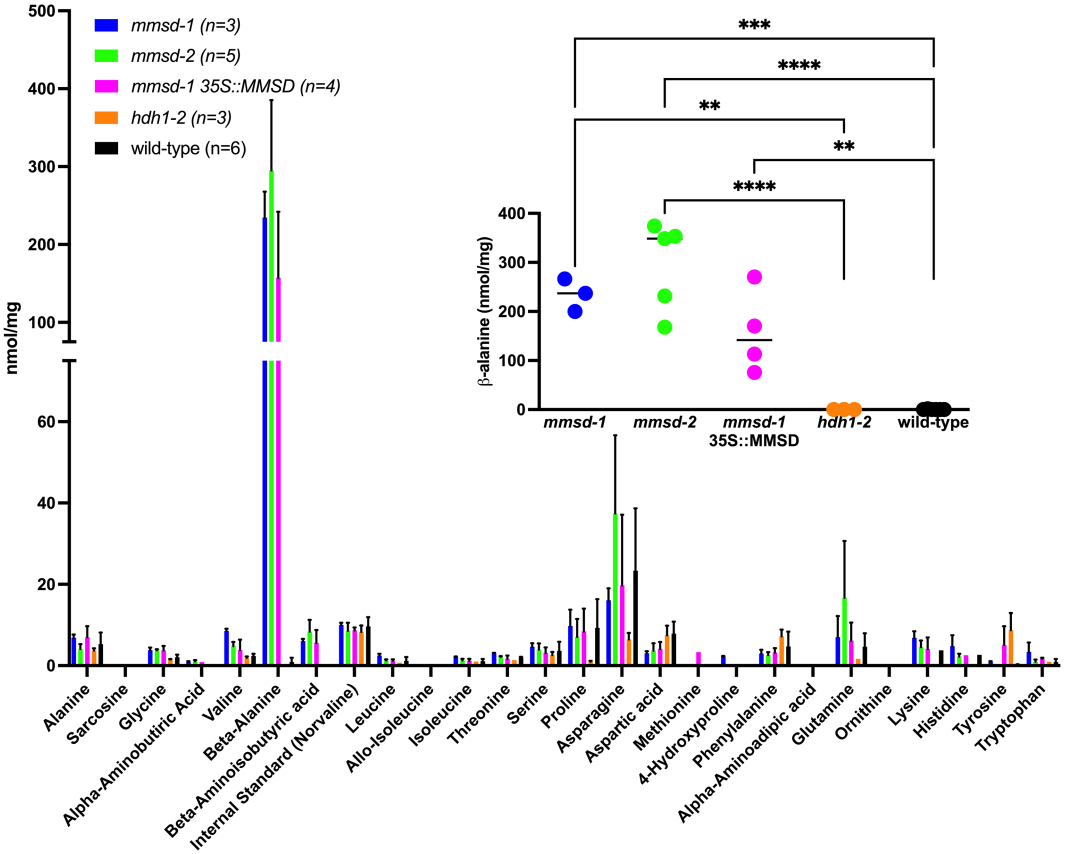

FIGURE 2 | The amino acid profiles of wild-type and transgenic *Arabidopsis thaliana* seeds. Shown are amino acid profiles for seeds with no or reduced *MMSD* expression (*mmsd-1* and *mmsd-2*, respectively), complemented *MMSD* expression (*mmsd-1 35S::MMSD*), and reduced *HDH1* expression compared to wild type. $n \geq 3$ of 30- to 40-mg sample of desiccated mature seeds. Error bars represent SD. Inset, β-alanine only data for the five seed lines where bar represents median quantity. $^{**}p < 0.007$, $^{***}p < 0.0004$, $^{****}p < 0.0001$.

with either 2–^{13}C propionate or U-^{13}C isoleucine as described by Lucas *et al. (*Lucas et al. 2007*)* Metabolites were identified based on the presence of the ^{13}C label on associated carbons according to the proposed pathway (Figure 3A). Using both internal and external standards, we identified the β-alanine peak and examined the MS profile for the presence of ^{13}C, which resulted in an increased abundance of the corresponding isotope peaks. For both *Arabidopsis* and wheat samples treated with 2–^{13}C-propionate, many of the ionized fragments of β-alanine showed increased abundance of the mass peak at +1 mass units, as predicted from the proposed metabolic pathway. The same was observed for plant seedlings treated with U-^{13}C isoleucine, resulting in fragments with increased abundance at +4 mass units (Figure 3B,C).

3.3 | Aminotransferase Activity

The evidence from the labeling studies showed that isoleucine could serve as a precursor for β-alanine; therefore, we worked to identify possible enzymes that might catalyze the reaction from malonate semialdehyde to β-alanine as no other studies had shown a direct link. Early work in wax bean cotyledons suggested that an aminotransferase could catalyze this proposed reaction (Stinson and Spencer 1969). Subsequent studies on aminotransferases in yeast, human, and *Pseudomonas aeruginosa* specifically showed β-alanine aminotransferase activity (Andersen et al. 2007; Blancquaert et al. 2016; Yao, He, and Lu 2011). Recent work in *Arabidopsis* showed that a putative enzyme (PYD4, At3g08860) could serve as a β-alanine aminotransferase (Parthasarathy et al. 2019). Based on sequence homology to both eukaryotic and prokaryotic homologs, we identified two other possible aminotransferases in *Arabidopsis*: alanine:glyoxylate aminotransferase 3 (AGT3, At2g38400) and the **γ**-aminobutyrate aminotransferase pollen-pistil incompatibility 2 (POP2, At3g22200) (Figure S1A). AGT3 shares 71% identity with PYD4 and POP2 shows at least 22% identity with AGT3 and PYD4 as measured by ClustalOmega (Madeira et al. 2022) (Figure S1B). These *Arabidopsis* sequences show, on average, 23% identity (41% similarity) with the nonplant β-alanine aminotransferases (from *H. sapiens*, *P. aeruginosa*, and *L. kluyveri*).

To characterize enzymatic activity, we selected two possible start sites for each aminotransferase and labeled as PYD4S and PDY4L, AGT3S and AGT3L, and POP2S and POP2L based on predicted mitochondrial leader sequences (via MitoFates; Fukasawa et al. 2015) and sequence similarity to aminotransferases with demonstrated β-alanine transaminase activity. Protein preparations of PYD4S and AGTS3 either did not show expression or were not easily soluble and thus were not used for further analysis. The other four samples expressed well in *E.coli* and were tested for PLP-dependent enzyme activity based on other studies with these proteins (Parthasarathy et al. 2019;

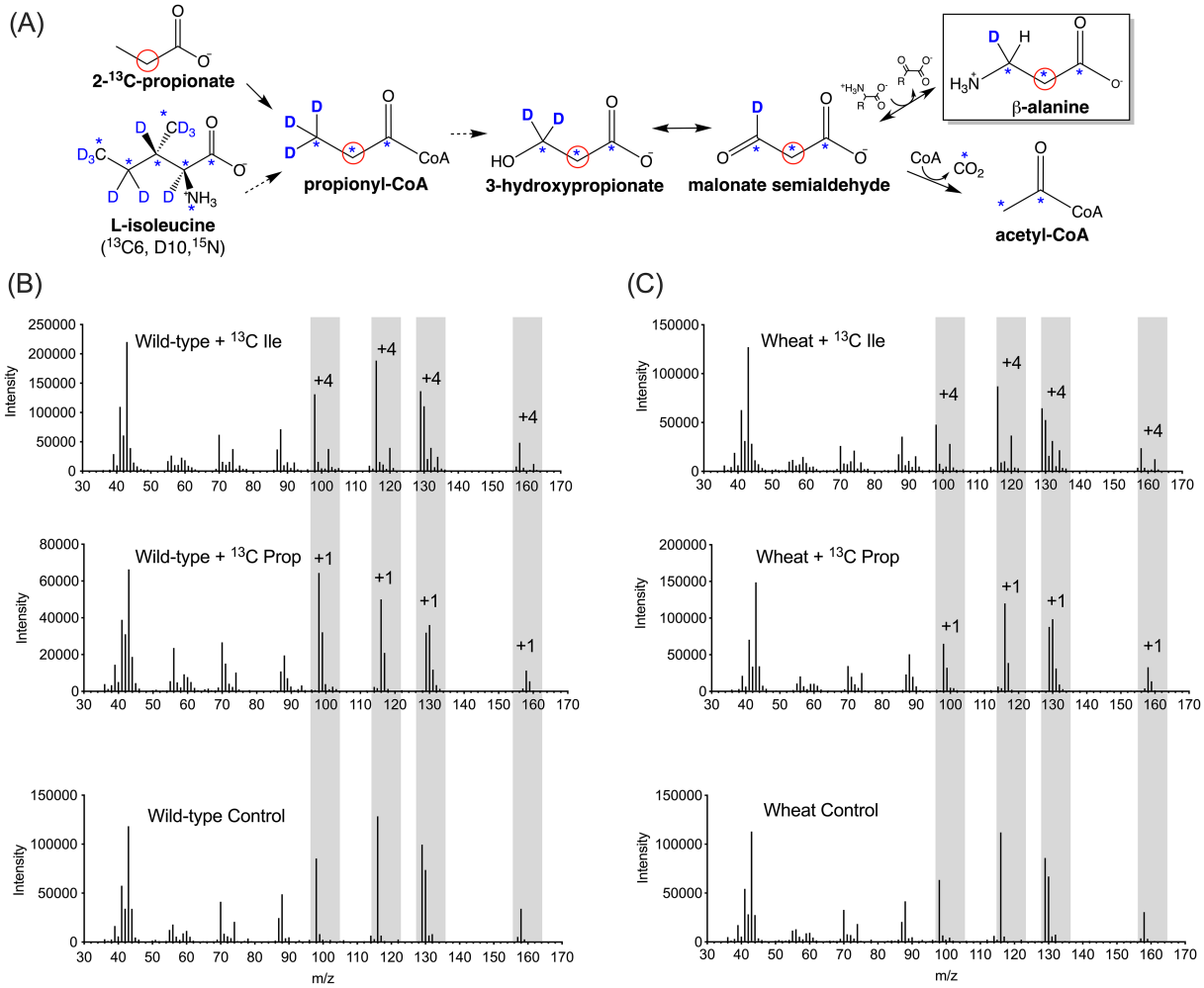

FIGURE 3 | (A) Predicted labeling patterns starting with 2–^{13}C propionate and U-^{13}C L-isoleucine. The red circle follows ^{13}C2 of propionate to ^{13}C2 of β-alanine. Blue asterisks and "D" follow the ^{13}C and deuterated hydrogen atoms of isoleucine, resulting in ^{13}C1-C3 and ^{2}H of C3 on β-alanine. The representative mass spectra of wild-type *Arabidopsis* (B) and wheat (C) seedlings treated with 2-^{13}C propionate and U-^{13}C isoleucine. Peaks shown are mass fragments of derivatized β-alanine as described in the Methods section. Data shown are representative spectra of separate experiments with $n = 6$ (*Arabidopsis thaliana*) and $n = 4$ (wheat).

Clark et al. 2009; Liepman and Olsen 2003) (Figure S2). Given the predicted reversibility of most aminotransferases, enzyme preparations were tested for activity with β-alanine in both directions. "Forward" reactions measured the enzyme's ability to synthesize β-alanine using a "one-pot", coupled reaction. Reaction components included 3-hydroxypropionate, NAD^+, and the enzyme 3-hydroxyisobutyrate dehydrogenase to synthesize malonate semialdehyde, which in the presence of the predicted β-alanine aminotransferase, PLP, and L-alanine then produced β-alanine (Figure 4). We also confirmed that reaction components for the coupled reaction did not interfere with the β-alanine aminotransferase reaction (Figure S3). The "Reverse" reactions included each respective enzyme preparation along with β-alanine, pyruvate, and PLP and were monitored for the production of L-alanine (Figure 5). All four aminotransferase preparations (AGT3L, PYD4L, POP2S, and POP2L) catalyzed both the "forward" and "reverse" directions as shown via the production of β-alanine and L-alanine, respectively. Peaks appearing at 2.14, 3.04, and 3.25 min represent L-alanine, β-alanine, and norvaline (ISTD), respectively in Figures 4 and 5.

4 | Discussion

Methylmalonate semialdehyde dehydrogenase is responsible for catalyzing two similar irreversible reactions: methylmalonate semialdehyde to propionyl-CoA in valine degradation and malonate semialdehyde to acetyl-CoA in propionyl-CoA metabolism. Both reaction products, propionyl-CoA and acetyl-CoA, feed into the citrate cycle, making it possible for molecules such as valine, isoleucine, and odd-chain fatty acids serve as alternative carbon sources in plants, especially during germination before the seedling has established photosynthetic processes.

Mutations that affect the function of MMSD resulted in seeds with decreased levels of soluble protein and free fatty acids, poor germination, and increased levels of valine, leucine, and β-alanine (Gipson et al. 2017). Increased levels of valine and leucine are a common phenotype in plants with disruptions to BCAA degradation (Gu, Jones, and Last 2010; Peng et al. 2015; Angelovici et al. 2013; Araújo et al. 2010); however, the significant accumulation of β-alanine appeared to be unique to *mmsd* mutants (i.e., *hdh-1* seeds do not accumulate β-alanine). The high levels of β-alanine

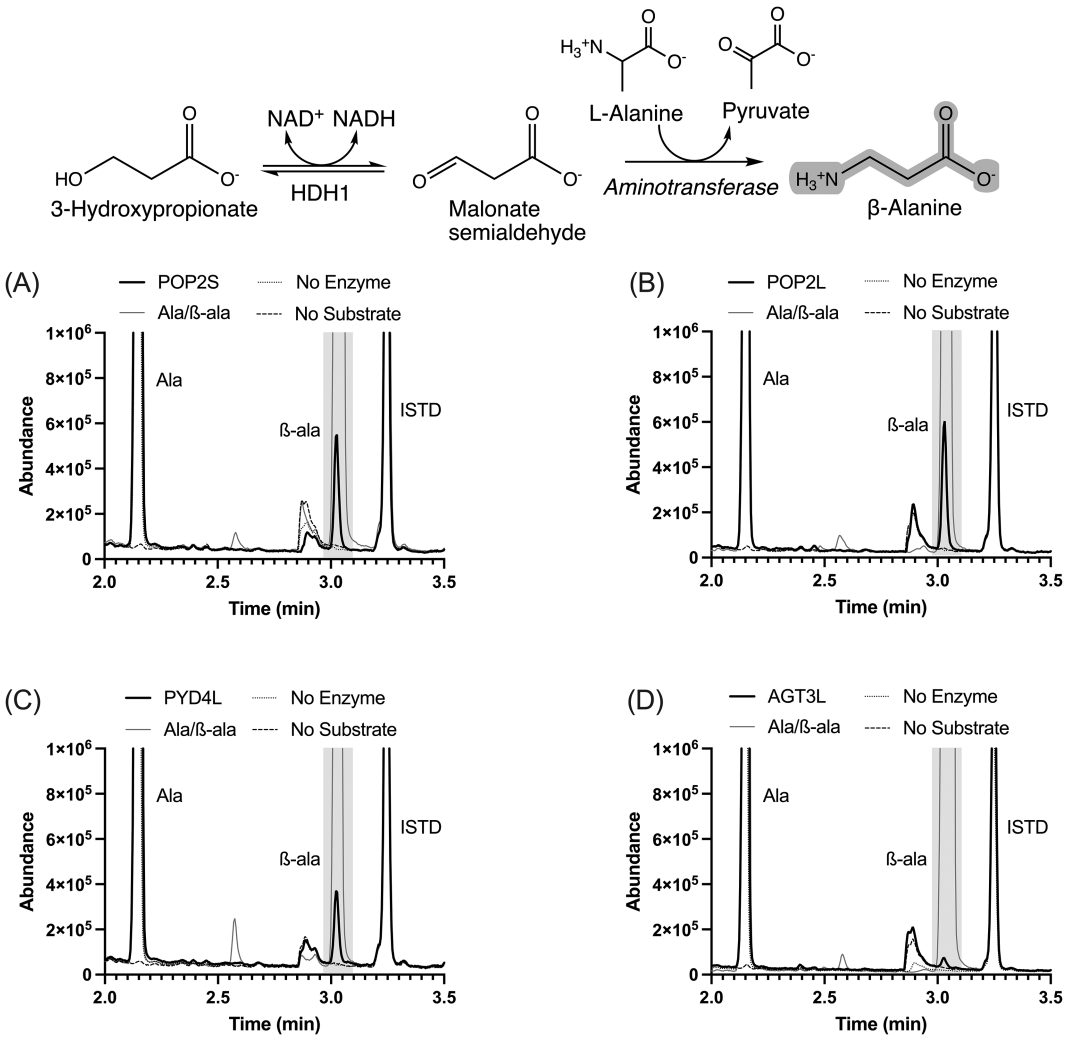

FIGURE 4 | "Forward" enzyme-catalyzed reactions. Ion chromatograph of reaction components from enzyme-catalyzed reactions with (A) POP2S, (B) POP2L, (C) PYD4L, and (D) AGT3L assayed using the 'forward' conditions (L-alanine as a substrate) compared to a no enzyme and no substrate control. The peak matching the product, β-alanine, is highlighted in gray. Norvaline was used as an internal standard (ISTD). Chromatograms shown are from one of three enzyme preparations where each reaction was run in duplicate.

could be due to direct metabolic impact, such that the inability to form the product acetyl-CoA resulted in a build-up of malonate semialdehyde (from isoleucine or odd-chain fatty acids), which was then converted to the more stable β-alanine via the proposed aminotransferase reaction. Further metabolism of accumulated β-alanine is dependent on the availability of pantoate, whose synthesis may be inhibited due to the disruption to BCAA degradation. However, the disruptions to these pathways likely do not account for the observed levels of β-alanine in the *mmsd* mutants. It is also possible that the *mmsd* mutation signaled for the accumulation of β-alanine as part of a general stress response. A broader analysis of the expression of impacted genes and metabolites found in *mmsd* mutants may provide insight into whether the observed phenotype is the result of metabolic disruptions, a stress response, or a combination of the two.

Regardless, the phenotypes of the *mmsd* mutants did suggest that there was a link between β-alanine and valine or isoleucine catabolism via propionyl-CoA. Treatment of *Arabidopsis* and wheat seedlings with ^{13}C-labeled propionate and isoleucine resulted in the ^{13}C-label appearing in β-alanine, supporting our hypothesis. It would be expected then that valine, whose catabolic product is propionyl-CoA, could also metabolize to make β-alanine via the same reactions used by isoleucine. However, previous work using similar conditions for isotope-labeling showed that when seedlings are treated with valine, they convert it to leucine instead of catabolizing it to propionyl-CoA (Lucas et al. 2007). β-alanine is known to be synthesized from a variety of precursors beyond just propionyl-CoA, such as from uracil and spermine. Therefore, the primary precursor metabolite is yet to be determined, as it may be condition dependent. Therefore, subsequent experiments should compare these β-alanine synthetic routes under various conditions, such as during germination, hypoxia, flooding, or drought.

The ^{13}C-labeling and *mmsd* seed data suggested that there could be an enzyme that catalyzes the conversion of the isoleucine and propionate intermediate malonate semialdehyde to β-alanine. Given that it would be an exchange between an amino acid and oxoacid, a likely candidate would be from the aminotransferase family of enzymes. These enzymes use the coenzyme pyridoxal-5′-phosphate (PLP) to catalyze

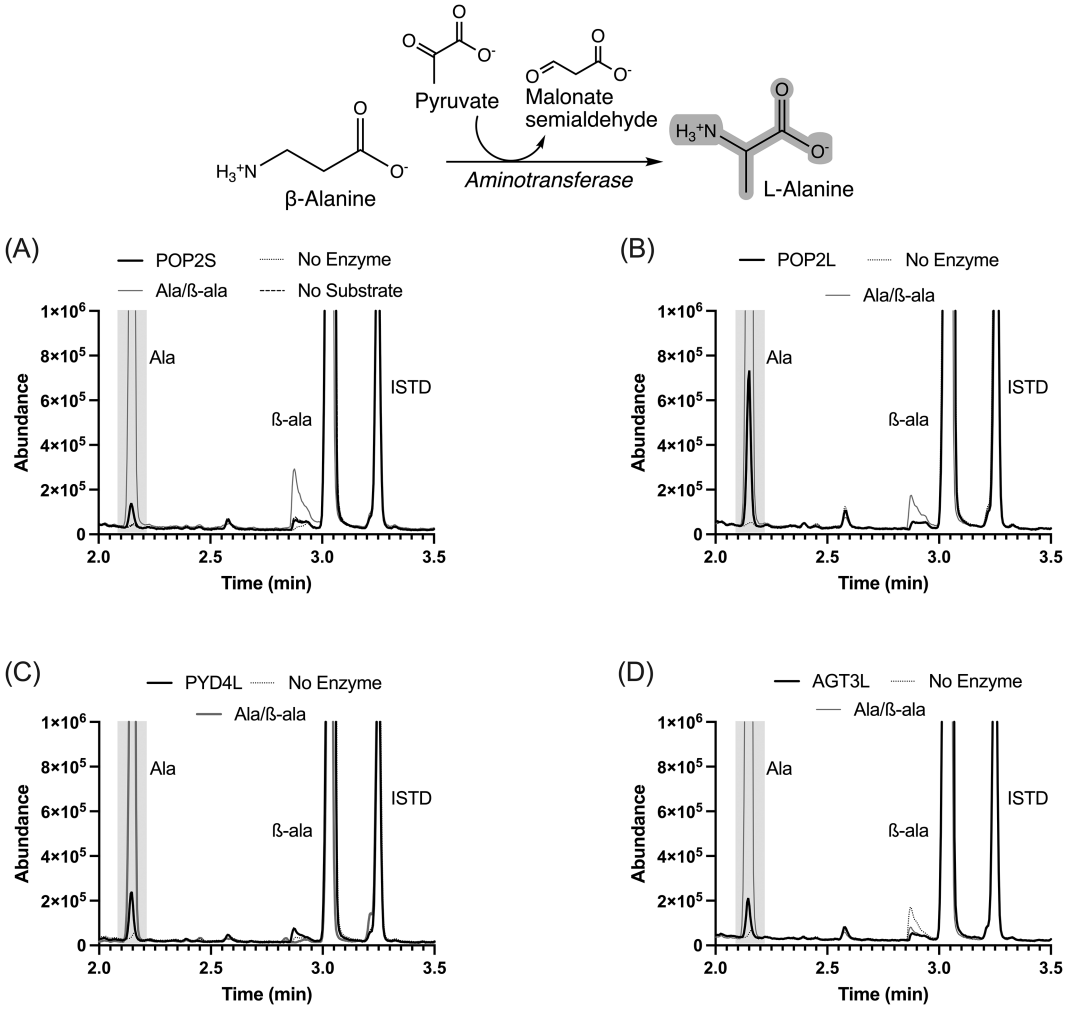

FIGURE 5 | "Reverse" enzyme-catalyzed reactions. Ion chromatograph of reaction components from enzyme-catalyzed reactions with (A) POP2S, (B) POP2L, (C) PYD4L, and (D) AGT3L assayed using "reverse" conditions (β-alanine and pyruvate as substrates) compared to a no enzyme and no substrate control. The peak matching the product, L-alanine, is highlighted in gray. Norvaline was used as an internal standard (ISTD). Chromatograms shown are from one of three enzyme preparations where each reaction was run in duplicate. No substrate controls for PYD4L, AGT3L, and POP2L showed no production of L-alanine (data not shown).

the interconversion of amino acids and oxoacids by amino group transfer. Therefore, we chose three prospective aminotransferases and tested their ability to convert malonate semialdehyde to β-alanine. Previous data implied that PYD4 could function as a β-alanine aminotransferase; however, the researchers were unable to measure direct enzyme activity (Parthasarathy et al. 2019). AGT3 was selected based on its high sequence similarity to PYD4 and to mammalian AGT2 (ABAT) enzymes (Figure S1B). We also selected a less similar enzyme, POP2, previously functionally characterized as an aminotransferase (Clark et al. 2009), but with no evidence for its ability to catalyze a reaction with β-alanine. AGT3 and POP2 were identified in mitochondria extracts via mass spec (Senkler et al. 2017; Niehaus et al. 2020); however, AGT3 contains a type 1 peroxisomal targeting sequence, so it is possible that AGT3 is not only localized to the mitochondria. The subcellular localization of PYD4 is unclear with localization predicted to be in the mitochondria (Hooper et al. 2014) and the peroxisome (Niessen et al. 2012). Therefore, two different constructs were designed for each candidate given the uncertainty of the location of potential mitochondrial leader sequences. AGT3L, PYD4L, POP2S, and POP2L all showed strong protein expression (Figure S1C) and enzyme activity (Figure S2). When tested for the ability to catalyze reactions related to the synthesis or use of β-alanine, AGT3L appeared to favor the formation of L-alanine, even when enzyme concentration was doubled (data not shown), while PYD4L showed no strong directional preference (Figures 4 and 5).

Unlike AGT3 and PYD4, both constructs of POP2 showed enzyme activity. This is likely because the two start sites differ by only 10 amino acids compared to AGT3 and PYD4, where start sites differ by more than 25 amino acids (Figure S1). While POP2L and POP2S have nearly the same amino acid sequence (Figure S1A), POP2S favored the synthesis of β-alanine and POP2L showed no directional preference, similar to PYD4L. It is possible that the N-terminus of POP2 is important for binding β-alanine or releasing L-alanine in the reverse direction, leading to the decreased production of L-alanine. Metabolite data associated with the roots and shoots of loss-of-function *pop2–1* mutants showed increased levels of both L-alanine and β-alanine compared to wild-type (Renault

et al. 2010), supporting POP2's reversibility. Even though gene expression data suggested that during early days in germination, *AGT3* and *PYD4* had higher expression than *POP2* (Winter et al. 2007), we cannot say for certain which of these enzymes is more likely to synthesize β-alanine in *Arabidopsis* based on the in vitro reaction conditions. However, BLAST searches using PYD4 from *Arabidopsis* also revealed homologous L-alanine:glyoxylate aminotranserases (AGTs) in wheat, suggesting that β-alanine synthesis may be catalyzed by similar reactions as those observed in *Arabidopsis* (Figure S4). Additional experiments that functionally characterize these proteins is needed to determine which, if any, is the primary aminotransferase in plants. Overall, the ability of all three enzymes to convert malonate semialdehyde to β-alanine speaks to the versatility of these aminotransferases, as these were not the enzymes' predicted or previously characterized substrates.

5 | Conclusion

β-alanine is a key precursor in the natural synthesis of molecules such as pantothenate (vitamin B5), coenzyme A, β-alanine betaine, and carnosine. It is also used industrially in feed and food supplements, in the synthesis of sweeteners, and as a polymer for water purification systems (Wang et al. 2021). Given its widespread use, an understanding of all the possible ways β-alanine can be synthesized could lead to more environmentally friendly industrial processes, and greater understanding of how and when organisms use β-alanine under normal and stress conditions. Here we showed that both isoleucine and propionyl-CoA can be converted into β-alanine via several different aminotransferase enzymes. ^{13}C-labeling experiments by GC–MS in *Arabidopsis* and wheat showed the production of β-alanine, while AGT3, PYD4, and POP2 all catalyzed in vitro enzyme assays using or producing β-alanine as is typical of many PLP-dependent aminotransferase enzymes. Together, these data extend earlier work that connected propionate with β-alanine synthesis (Rathinasabapathi 2002; Parthasarathy et al. 2019) and clarified another possible pathway for β-alanine synthesis in plants. It is also important to consider that because branched-chain amino acid metabolism is tightly controlled in plants, valine is the sole precursor for pantoate, and that our data show isoleucine (and theoretically, valine) can produce β-alanine, branched-chain amino acids may play a significant role in vitamin B5 and coenzyme A synthesis generally.

Author Contributions

K.A.R. designed the experiments. K.A.R., M.H.G., and J.B. wrote the manuscript and analyzed the data. M.H.G., J.B., K.C.W.S, P.R., T.S., C. W, and M. N performed the experiments. All authors approved the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data available upon request from the corresponding author.

References

Andersen, G., B. Andersen, D. Dobritzsch, K. D. Schnackerz, and J. Piškur. 2007. "A Gene Duplication Led to Specialized γ-Aminobutyrate and β-Alanine Aminotransferase in Yeast." *FEBS Journal* 274, no. 7: 1804–1817. https://doi.org/10.1111/j.1742-4658.2007.05729.x.

Angelovici, R., A. E. Lipka, N. Deason, et al. 2013. "Genome-Wide Analysis of Branched-Chain Amino Acid Levels in Arabidopsis Seeds." *Plant Cell* 25, no. 12: 4827–4843. https://doi.org/10.1105/tpc.113.119370.

Araújo, W. L., K. Ishizaki, A. Nunes-Nesi, et al. 2010. "Identification of the 2-Hydroxyglutarate and Isovaleryl-CoA Dehydrogenases as Alternative Electron Donors Linking Lysine Catabolism to the Electron Transport Chain of Arabidopsis Mitochondria." *Plant Cell* 22, no. 5: 1549–1563. https://doi.org/10.1105/tpc.110.075630.

Binder, S. 2010. "Branched-Chain Amino Acid Metabolism in Arabidopsis Thaliana." In *Arabidopsis Book*, vol. 8, e0137. Rockville, MD: American Society of Plant Biologists. https://doi.org/10.1199/tab.0137.

Blancquaert, L., S. P. Baba, S. Kwiatkowski, et al. 2016. "Carnosine and Anserine Homeostasis in Skeletal Muscle and Heart Is Controlled by β-Alanine Transamination." *Journal of Physiology* 594, no. 17: 4849–4863. https://doi.org/10.1113/JP272050.

Chen, D., Q. Shao, L. Yin, A. Younis, and B. Zheng. 2019. "Polyamine Function in Plants: Metabolism, Regulation on Development, and Roles in Abiotic Stress Responses." *Frontiers in Plant Science* 9: 1945. https://doi.org/10.3389/fpls.2018.01945.

Clark, S. M., R. Di Leo, P. K. Dhanoa, O. R. Van Cauwenberghe, R. T. Mullen, and B. J. Shelp. 2009. "Biochemical Characterization, Mitochondrial Localization, Expression, and Potential Functions for an Arabidopsis γ-Aminobutyrate Transaminase That Utilizes Both Pyruvate and Glyoxylate." *Journal of Experimental Botany* 60, no. 6: 1743–1757. https://doi.org/10.1093/jxb/erp044.

Cronan, J. E. 1980. "Beta-Alanine Synthesis in Escherichia Coli." *Journal of Bacteriology* 141, no. 3: 1291–1297. https://doi.org/10.1128/jb.141.3.1291-1297.1980.

Drakeford, D. R., I. Mukherjee, and D. M. Reid. 1985. "Some Early Responses of Helianthus Annuus L. to Flooding: I. The Effects of Flooding on the Uptake and Leakage of "Non-Electrolytes" by Roots." *Journal of Experimental Botany* 36, no. 11: 1705–1715. https://doi.org/10.1093/jxb/36.11.1705.

Duhazé, C., D. Gagneul, L. Leport, F. Robert Larher, and A. Bouchereau. 2003. "Uracil as One of the Multiple Sources of β-Alanine in Limonium Latifolium, a Halotolerant β-Alanine Betaine Accumulating Plumbaginaceae." *Plant Physiology and Biochemistry* 41, no. 11-12: 993–998. https://doi.org/10.1016/j.plaphy.2003.06.002.

Fukasawa, Y., J. Tsuji, S.-C. Fu, K. Tomii, P. Horton, and K. Imai. 2015. "MitoFates: Improved Prediction of Mitochondrial Targeting Sequences and Their Cleavage Sites." *Molecular & Cellular Proteomics* 14, no. 4: 1113–1126. https://doi.org/10.1074/mcp.M114.043083.

Ganie, S. A. 2021. "Amino Acids Other Than Proline and Their Participation in Abiotic Stress Tolerance." In *Compatible Solutes Engineering for Crop Plants Facing Climate Change*, edited by S. H. Wani, M. P. Gangola, and B. R. Ramadoss, 47–96. Cham: Springer International Publishing. https://doi.org/10.1007/978-3-030-80674-3_3.

Gipson, A. B., K. J. Morton, R. J. Rhee, et al. 2017. "Disruptions in Valine Degradation Affect Seed Development and Germination in Arabidopsis." *Plant Journal* 90, no. 6: 1029–1039. https://doi.org/10.1111/tpj.13538.

Gondor, O. K., J. Tajti, K. Á. Hamow, et al. 2021. "Polyamine Metabolism under Different Light Regimes in Wheat." *International Journal of*

Molecular Sciences 22, no. 21: 11717. https://doi.org/10.3390/ijms222111717.

Gu, L., A. D. Jones, and R. L. Last. 2010. "Broad Connections in the Arabidopsis Seed Metabolic Network Revealed by Metabolite Profiling of an Amino Acid Catabolism Mutant." *Plant Journal* 61, no. 4: 579–590. https://doi.org/10.1111/j.1365-313X.2009.04083.x.

Hatch, M. D., and P. K. Stumpf. 1962. "Fat Metabolism in Higher Plants. XVIII. Propionate Metabolism by Plant Tissues." *Archives of Biochemistry and Biophysics* 96: 193–198. https://doi.org/10.1016/0003-9861(62)90398-3.

Hooper, C. M., S. K. Tanz, I. R. Castleden, M. A. Vacher, I. D. Small, and A. H. Millar. 2014. "SUBAcon: A Consensus Algorithm for Unifying the Subcellular Localization Data of the Arabidopsis Proteome." *Bioinformatics* 30, no. 23: 3356–3364. https://doi.org/10.1093/bioinformatics/btu550.

Kaplan, F., J. Kopka, D. W. Haskell, et al. 2004. "Exploring the Temperature-Stress Metabolome of Arabidopsis." *Plant Physiology* 136, no. 4: 4159–4168. https://doi.org/10.1104/pp.104.052142.

Liepman, A. H., and L. J. Olsen. 2003. "Alanine Aminotransferase Homologs Catalyze the Glutamate:Glyoxylate Aminotransferase Reaction in Peroxisomes of Arabidopsis." *Plant Physiology* 131, no. 1: 215–227. https://doi.org/10.1104/pp.011460.

Lucas, K. A., J. R. Filley, J. M. Erb, E. R. Graybill, and J. W. Hawes. 2007. "Peroxisomal Metabolism of Propionic Acid and Isobutyric Acid in Plants." *Journal of Biological Chemistry* 282, no. 34: 24980–24989. https://doi.org/10.1074/jbc.M701028200.

Madeira, F., M. Pearce, A. R. N. Tivey, et al. 2022. "Search and Sequence Analysis Tools Services From EMBL-EBI in 2022." *Nucleic Acids Research* 50, no. W1: W276–W279. https://doi.org/10.1093/nar/gkac240.

Niehaus, M., H. Straube, P. Künzler, et al. 2020. "Rapid Affinity Purification of Tagged Plant Mitochondria (Mito-AP) for Metabolome and Proteome Analyses." *Plant Physiology* 182, no. 3: 1194–1210. https://doi.org/10.1104/pp.19.00736.

Niessen, M., K. Krause, I. Horst, et al. 2012. "Two Alanine Aminotranferases Link Mitochondrial Glycolate Oxidation to the Major Photorespiratory Pathway in Arabidopsis and Rice." *Journal of Experimental Botany* 63, no. 7: 2705–2716. https://doi.org/10.1093/jxb/err453.

Parthasarathy, A., L. E. Adams, F. C. Savka, and A. O. Hudson. 2019. "The Arabidopsis Thaliana Gene Annotated by the Locus Tag At3g08860 Encodes Alanine Aminotransferase." *Plant Direct* 3, no. 9: e00171. https://doi.org/10.1002/pld3.171.

Parthasarathy, A., M. A. Savka, and A. O. Hudson. 2019. "The Synthesis and Role of β-Alanine in Plants." *Frontiers in Plant Science* 10: 921. https://doi.org/10.3389/fpls.2019.00921.

Peng, C., S. Uygun, S.-H. Shiu, and R. L. Last. 2015. "The Impact of the Branched-Chain Ketoacid Dehydrogenase Complex on Amino Acid Homeostasis in Arabidopsis." *Plant Physiology* 169, no. 3: 1807–1820. https://doi.org/10.1104/pp.15.00461.

Rastogi, R., and P. J. Davies. 1990. "Polyamine Metabolism in Ripening Tomato Fruit: I. Identification of Metabolites of Putrescine and Spermidine." *Plant Physiology* 94, no. 3: 1449–1455. https://doi.org/10.1104/pp.94.3.1449.

Rathinasabapathi, B. 2002. "Propionate, a Source of β-Alanine, Is an Inhibitor of β-Alanine Methylation in Limonium Latifolium, Plumbaginaceae." *Journal of Plant Physiology* 159, no. 6: 671–674. https://doi.org/10.1078/0176-1617-0742.

Renault, H., V. Roussel, A. El Amrani, et al. 2010. "The Arabidopsis Pop2-1 Mutant Reveals the Involvement of GABA Transaminase in Salt Stress Tolerance." *BMC Plant Biology* 10, no. 1: 20. https://doi.org/10.1186/1471-2229-10-20.

Rizhsky, L., H. Liang, J. Shuman, V. Shulaev, S. Davletova, and R. Mittler. 2004. "When Defense Pathways Collide. The Response of Arabidopsis to a Combination of Drought and Heat Stress." *Plant Physiology* 134, no. 4: 1683–1696. https://doi.org/10.1104/pp.103.033431.

Senkler, J., M. Senkler, H. Eubel, et al. 2017. "The Mitochondrial Complexome of Arabidopsis Thaliana." *Plant Journal* 89, no. 6: 1079–1092. https://doi.org/10.1111/tpj.13448.

Stinson, R. A., and M. Spencer. 1969. "β-Alanine as an Ethylene Precursor. Investigations Towards Preparation, and Properties, of a Soluble Enzyme System From a Subcellular Particulate Fraction of Bean Cotyledons." *Plant Physiology* 44, no. 9: 1217–1226. https://doi.org/10.1104/pp.44.9.1217.

Sun, X., J. Zhang, H. Zhang, et al. 2010. "The Responses of Arabidopsis Thaliana to Cadmium Exposure Explored via Metabolite Profiling." *Chemosphere* 78, no. 7: 840–845. https://doi.org/10.1016/j.chemosphere.2009.11.045.

Terano, S., and Y. Suzuki. 1978. "Formation of β-Alanine From Spermine and Spermidine in Maize Shoots." *Phytochemistry* 17, no. 1: 148–149. https://doi.org/10.1016/S0031-9422(00)89700-9.

Tsai, C. S., and B. Axelrod. 1965. "Catabolism of Pyrimidines in Rape Seedlings." *Plant Physiology* 40, no. 1: 39–44. https://doi.org/10.1104/pp.40.1.39.

Walsh, T. A., S. B. Green, I. M. Larrinua, and P. R. Schmitzer. 2001. "Characterization of Plant β-Ureidopropionase and Functional Overexpression in Escherichia Coli." *Plant Physiology* 125, no. 2: 1001–1011. https://doi.org/10.1104/pp.125.2.1001.

Wang, L., Y. Mao, Z. Wang, H. Ma, and T. Chen. 2021. "Advances in Biotechnological Production of β-Alanine." *World Journal of Microbiology and Biotechnology* 37, no. 5: 79. https://doi.org/10.1007/s11274-021-03042-1.

White, W. H., P. L. Gunyuzlu, and J. H. Toyn. 2001. "Saccharomyces Cerevisiae Is Capable of de Novo Pantothenic Acid Biosynthesis Involving a Novel Pathway of β-Alanine Production From Spermine." *Journal of Biological Chemistry* 276, no. 14: 10794–10800. https://doi.org/10.1074/jbc.M009804200.

Winter, D., B. Vinegar, H. Nahal, R. Ammar, G. V. Wilson, and N. J. Provart. 2007. "An "Electronic Fluorescent Pictograph" Browser for Exploring and Analyzing Large-Scale Biological Data Sets." *PLoS ONE* 2, no. 8: e718. https://doi.org/10.1371/journal.pone.0000718.

Witte, C.-P., and M. Herde. 2020. "Nucleotide Metabolism in Plants." *Plant Physiology* 182, no. 1: 63–78. https://doi.org/10.1104/pp.19.00955.

Yao, X., W. He, and C.-D. Lu. 2011. "Functional Characterization of Seven γ-Glutamylpolyamine Synthetase Genes and the bauRABCD Locus for Polyamine and β-Alanine Utilization in Pseudomonas Aeruginosa PAO1." *Journal of Bacteriology* 193, no. 15: 3923–3930. https://doi.org/10.1128/jb.05105-11.

Zrenner, R., H. Riegler, C. R. Marquard, et al. 2009. "A Functional Analysis of the Pyrimidine Catabolic Pathway in Arabidopsis." *New Phytologist* 183, no. 1: 117–132. https://doi.org/10.1111/j.1469-8137.2009.02843.x.

Supporting Information

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