Characterization of the Branched-Chain Amino Acid Aminotransferase Enzyme Family in Tomato1[W][OA]

Gregory S. Maloney2, Andrej Kochevenko2, Denise M. Tieman, Takayuki Tohge, Uri Krieger, Dani Zamir, Mark G. Taylor, Alisdair R. Fernie2, and Harry J. Klee2*

Horticultural Sciences Department and Plant Molecular and Cellular Biology Program, University of Florida, Gainesville, Florida 32611 (G.S.M., D.M.T., M.G.T., H.J.K.); Max Planck Institute of Molecular Plant Physiology, D–14476 Potsdam-Golm, Germany (A.K., T.T., A.R.F.); and Hebrew University of Jerusalem, Faculty of Agriculture, Rehovot 76100, Israel (U.K., D.Z.)

Branched-chain amino acids (BCAAs) are synthesized in plants from branched-chain keto acids, but their metabolism is not completely understood. The interface of BCAA metabolism lies with branched-chain aminotransferases (BCAT) that catalyze both the last anabolic step and the first catabolic step. In this study, six BCAT genes from the cultivated tomato (Solanum lycopersicum) were identified and characterized. SIBCAT1, -2, -3, and -4 are expressed in multiple plant tissues, while SIBCAT5 and -6 were undetectable. SIBCAT1 and -2 are located in the mitochondria, while SIBCAT3 and -4 are located in chloroplasts, while SIBCAT5 and -6 are located in the cytosol and vacuole, respectively. SIBCAT1, -2, -3, and -4 were able to restore growth of Escherichia coli BCAA auxotrophic cells, while SIBCAT1 and -2 were less effective than SIBCAT3 and -4 in growth restoration. All enzymes were active in the forward (BCAA synthesis) and reverse (branched-chain keto acid synthesis) reactions. SIBCAT3 and -4 exhibited a preference for the forward reaction, while SIBCAT1 and -2 were more active in the reverse reaction. While overexpression of SIBCAT3 or -4 in tomato fruit did not significantly alter amino acid levels, an expression quantitative trait locus on chromosome 3, associated with substantially higher expression of Solanum pennellii BCAT4, did significantly increase BCAA levels. Conversely, antisense-mediated reduction of SIBCAT1 resulted in higher levels of BCAAs. Together, these results support a model in which the mitochondrial SIBCAT1 and -2 function in BCAA catabolism while the chloroplastic SIBCAT3 and -4 function in BCAA synthesis.

The branched-chain amino acids (BCAAs) Leu, Ile, and Val are primary metabolites synthesized in plants and are essential nutrients in animals. They are synthesized from Thr or pyruvate in plastids (Schulze-Siebert et al., 1984; Hagelstein et al., 1997). Thr feeds into the Ile pathway and pyruvate into the Val pathway, after which the same four enzymatic steps are shared to form these two amino acids. Leu is synthesized by a branch of the Val pathway starting with 3-methyl-2-oxobutanoic acid in four enzymatic steps (Holmberg and Petersen, 1988; Kohlhuber, 2003; Fig. 1).

Although synthesis of BCAAs is well characterized in plants, regulation of catabolism is not completely understood. Catabolism is believed to be initiated in mitochondria, where the branched-chain keto acid (BCKA) dehydrogenase complex is located (Taylor et al., 2004). The primary fates of BCAAs in plant cells are peptide elongation, glutamate recycling, Glc- and Suc-linked branched-chain esters, branched-chain fatty acid synthesis, and respiration through the synthesis of tricarboxylic acid cycle intermediates (Kandra et al., 1990; Walters and Steffens, 1990; Kroumova et al., 1994; Daschner et al., 1999; Li et al., 2003; Beck et al., 2004; Taylor et al., 2004; Engqvist et al., 2009). BCAA catabolism likely has other functions in plant metabolism. For example, Gu et al. (2010) showed that a mutation in isovaleryl-CoA dehydrogenase, an enzyme in the BCAA catabolic pathway, influences the metabolism of many unrelated compounds in Arabidopsis (Arabidopsis thaliana) seeds, including 12 amino acids.

Branched-chain aminotransferase (BCAT) enzymes are at the interface of BCAA synthesis and catabolism, reversibly catalyzing the interconversion of BCAAs to BCKAs. Leu is converted to 4-methyl-2-oxopentanoic acid (KIC), Ile to 3-methyl-2-oxopentanoic acid (KMV), and Val to 3-methyl-2-oxobutanoic acid (KIV). BCATs have been studied in only a few plant species. In spinach (Spinacia oleracea), there are two known BCATs, one with a higher affinity toward KIV and
the other with a higher affinity toward KIC and KMV, indicating substrate preference (Binder et al., 2007). In Arabidopsis, there are six BCATs. AtBCAT1 localizes to mitochondria and is thought to be active primarily in catabolism. AtBCAT2, -3, and -5 localize to chloroplasts, suggesting roles in BCAA synthesis (Diebold et al., 2002). AtBCAT4 is cytosolic (Schuster et al., 2006), and the location of AtBCAT6 is suggested to be cytosolic because of its lack of a defined target peptide sequence (Diebold et al., 2002). Complementation analysis in BCAT-deficient yeast strains confirmed the functions of AtBCAT1, -2, -3, -5, and -6 but not AtBCAT4 (Diebold et al., 2002). AtBCAT1 is the most likely candidate for initiating BCAA breakdown (Schuster and Binder, 2005), although AtBCAT5 has also been found in mitochondrial fractions (Binder et al., 2007). AtBCAT1 catabolizes all BCAAs in almost all tissue types, and its affinity is greatest in the order Ile > Leu > Val. AtBCAT2 expression is observed only in flowers and is elevated under stress, while AtBCAT6 is expressed in flowers and siliques. Expression of the other AtBCATs is not as tissue specific (Liepman and Olsen, 2004). Two studies in Arabidopsis showed that both the chloroplastic AtBCAT3 and the cytosolic AtBCAT4 also participate in Met chain elongation and the production of aliphatic glucosinolates (Schuster et al., 2006; Knill et al., 2008). In another example of diverse BCAT function, a Nicotiana benthamiana chloroplastic BCAT was implicated in transcriptional regulation of KNOX genes that affect levels of gibberellins. This enzyme was also able to restore the growth of a BCAT-deficient yeast and was expressed highly in young leaves, suggesting the primary role in BCAA synthesis (Gao et al., 2009). Together, these studies show that BCATs have functions beyond amino acid metabolism, making it important to understand the characteristics of each enzyme form.

This study focuses on characterization of the tomato (Solanum lycopersicum) BCAT gene family, their enzymatic properties, and their role in BCAA metabolism. Our results provide insight into the specific functions of BCAT isoforms in tomato. We show that tomato BCATs are diverse in subcellular location, substrate preference, and expression. Finally, we provide evidence that different BCAT alleles influence BCAA content in fruit.

Figure 1. Synthetic pathways to BCAAs in plants. 1. Thr deaminase. 2. Acetolactase synthase. 3. Acetolactate isomeroreductase. 4. Dihydroxy acid dehydratase. 5. BCAT. 6. 2-Isopropylmalate synthase. 7. Isopropylmalate isomerase. 8. Isopropylmalate dehydrogenase.

RESULTS

Cloning of SIBCAT cDNAs

In order to better understand the dynamics of BCAA metabolism, we identified six unique tomato sequences potentially encoding BCAT enzymes in the SOL Genomics Network tomato EST database (http://solgenomics.net/index.pl; Mueller et al., 2005). Full-length cDNAs of each gene were cloned and sequenced. The unigene SGN-U569828 (SIBCAT1; 45 members) has the most ESTs of all putative SIBCATs, while the unigene SGN-U569952 (SIBCAT3; 27 members) has the second highest, both far surpassing the numbers of ESTs of the other putative SIBCATs. Phylogenetic analysis of all putative SIBCATs and comparisons with Arabidopsis BCATs (Diebold et al., 2002) revealed that SIBCAT1 is most similar to the AtBCAT2 and AtBCAT1 genes from Arabidopsis. The unigene SGN-U569830 (SIBCAT2; seven members) is most similar to AtBCAT3. SIBCAT3 and the unigene SGN-U569953 (SIBCAT4; seven members) are highly similar to each other and most similar to AtBCAT5. The unigenes SGN-U569831 (SIBCAT5; five members) and SGN-U569829 (SIBCAT6; two members) are most similar to AtBCAT2 and most similar to each other within the putative SIBCATs (Supplemental Figs. S1 and S2).

Expression of SIBCATs

To gain a better understanding of the different roles of each SIBCAT family member, expression analysis was performed by quantitative reverse transcription (qRT)-PCR on all six SIBCAT cDNAs. Tissues tested were young leaves, inflorescences at 1 d post anthesis (dpa), and mature green, breaker, turning, and red ripe fruit stages (Fig. 2). Expression of SIBCAT1 is higher in ripening and red fruit than all other SIBCATs, is very low in leaves and inflorescences, and is undetectable in green fruit. SIBCAT2 is expressed in all tissues at similar levels except for inflorescences, where it is much more highly expressed. SIBCAT3 is expressed in all tissues and is most highly expressed in leaves. Expression of SIBCAT4 is highest in inflorescences but relatively low in all other tissues compared with the other
SlBCATs. No SlBCAT5 and SlBCAT6 transcripts were detected in any of the tissues tested.

**BCAA Levels in Various Tissues of Tomato**

We next determined the levels of Val, Ile, and Leu in leaf tissue, flowers, and various stages of fruit (10 dpa, 20 dpa, 30 dpa, breaker, and 40 dpa). Stem and leaf tissue contained relatively low levels of Val, Leu, and Ile, which ranged between 24.4 and 98.2 μmol g⁻¹ fresh weight, with considerably higher levels of Val than Leu or Ile (Fig. 3). Floral tissues contained approximately twice the content of all three amino acids as leaves. The relatively high expression of SlBCAT2 and SlBCAT4 in inflorescences may be related to the higher concentration of BCAAs in this tissue than in leaves. Young fruits (10 dpa) displayed three to five times the content observed in leaves. Indeed, the levels of Val peaked at this time point, whereas those of Ile and Leu increased until 20 dpa before declining considerably, with all three amino acids being present at contents similar to, or lower than, those observed in leaves by 40 dpa. These data on fruit development are consistent with those reported previously (Carrari et al., 2006).

**Subcellular Localization of SlBCATs**

Plant organelles have specific functions that can change during cell and organ development, as is the case with ripening tomato fruit. Therefore, the subcellular locations of metabolic enzymes can be important in predicting function. All six SlBCAT cDNAs were cloned with a C-terminal E-GFP gene fusion, expressed in N. benthamiana leaf protoplasts, and analyzed with confocal microscopy (Fig. 4).

SlBCAT3 and SlBCAT4 were localized to plastids, consistent with localization algorithm software and homology with the chloroplast-localized AtBCAT pro-
Proteins. SIBCAT1 and SIBCAT2 were localized to mitochondria, consistent with localization prediction software outputs. Mitochondrial localization was confirmed with MitoTracker Orange stain. SIBCAT5 appeared to be localized to the cytosol, consistent with the lack of an N-terminal targeting signal. SIBCAT6 appeared to be localized to the vacuole, based on the E-GFP signal filling the majority of the space inside the protoplasts, typical of vacuoles in leaf cells.

The mitochondria and chloroplast locations of SIBCAT1 to -4 suggest that each may have specific functions in BCAA catabolism and anabolism, respectively. Similarly, the cytoplasmic and vacuolar locations of SIBCAT5 and SIBCAT6, respectively, suggest unique metabolic functions for these two enzymes.

Functional Verification by Complementation

In order to demonstrate BCAT function in vivo, a complementation assay was performed in *Escherichia coli*. The *E. coli* genome contains one BCAT gene, *ilvE*. A second gene, *tyrB*, encoding an aromatic amino acid aminotransferase (EC 2.6.1.57), can partially restore BCAT activity in Δ*ilvE* cells (Gelfand and Steinberg, 1977; Powell and Morrison, 1978; Vartak et al., 1991). The knockout strains for each gene were obtained from the Keio Collection (Baba et al., 2006), and a double knockout strain was constructed. The strain Δ*ilvE*/Δ*tyrB* completely lacks BCAT activity and does not grow on medium lacking all BCAAs.

SIBCAT1, SIBCAT2, SIBCAT3, and SIBCAT4 were cloned into the *E. coli* expression vector pBAD24 under control of the P_bad promoter (Guzman et al., 1995) and transformed into the Δ*ilvE*/Δ*tyrB* strain. Protein gel blots of cell extracts confirmed that BCAT concentrations did not vary greatly (data not shown). The growth rates of all four lines in minimal medium lacking all amino acids were compared with the wild type (Table I). SIBCAT3 and SIBCAT4 were significantly better at restoring growth (52% and 39% of the wild-type rate, respectively) than SIBCAT1 and SIBCAT2 (18% and 11% of the wild-type rate, respectively). The more effective restoration of growth by the chloroplastic SIBCAT3 and SIBCAT4 supports the hypothesis that they are the major BCAA-synthesizing enzymes in tomato. The relatively reduced growth of...
cells expressing *SlBCAT1* and *SlBCAT2* suggests that these mitochondrial enzymes are less efficient in the forward direction.

**Enzyme Activity of SlBCATs**

In order to determine the kinetic properties of each SlBCAT, proteins were expressed in *E. coli* cells and purified. Enzyme assays were performed with each recombinant SlBCAT in both the forward (amino acid-forming) and reverse (amino acid-degrading) directions. Table II shows the *K* <sub>m</sub>, *V* <sub>max</sub>, *K* <sub>cat</sub>, and *K* <sub>cat</sub>/*K* <sub>m</sub> values with all six branched-chain substrates. All SlBCATs functioned in both forward and reverse directions with all six substrates. SlBCAT3 has the highest efficiency in the forward direction than the reverse, having higher affinity for the BCKAs than BCAAs. SlBCAT4 exhibits a slight preference in the forward direction for KMV and Ile in the reverse direction. Like SlBCAT3, its most closely related form, SlBCAT4 is most efficient in the forward direction, consistent with the role of chloroplastic BCATs in BCAA synthesis in tomato. SlBCAT1 showed relatively low efficiency in the forward direction and much higher efficiency on Leu and Ile in the reverse direction. This preference, together with its mitochondrial location, supports a primarily catabolic function. SlBCAT2, also located in mitochondria, has a much higher affinity for the BCAAs than the corresponding BCKAs, similar to SlBCAT1, suggesting that it also principally functions in BCAA catabolism. SlBCAT5 was most efficient in the forward direction, with highest affinities towards KMV and KIC. SlBCAT6 was relatively highly efficient in both the forward and reverse directions, with highest efficiency when providing KIV as substrate.

Taken together, the data are consistent with the chloroplastic enzymes SlBCAT3 and -4 functioning principally in BCAA anabolism and the mitochondrial enzymes SlBCAT1 and -2 functioning principally in BCAA catabolism. The functions of SlBCAT5 and -6 are not apparent.

**SlBCAT Transgenic Analysis**

In order to further evaluate the functions of BCAT enzymes in vivo, transgenic plants overexpressing or underexpressing *BCAT* cDNAs were generated. A 1,138-bp *SlBCAT1* fragment was cloned in the antisense orientation into the transformation vector pK2WG7 under the control of the cauliflower mosaic virus promoter. Nine selected lines were amplified in tissue culture, and six plants per line were grown in the greenhouse. No apparent phenotype was observed in any of these lines. Evaluation of the relative levels of *SlBCAT1* gene expression in leaves of 6-week-old plants is presented in Figure 5A. Having identified lines with reduced expression of *SlBCAT1*, we determined the levels of amino acids in red ripe pericarp fruit. The levels of Leu, Ile, and to a lesser extent Val increased in the transgenic lines (Fig. 5B). This observation is consistent with a catabolic function for SlBCAT1 and loss of function resulting in higher amino acid levels.

In order to determine if an increase in either a single synthetic or catabolic *SlBCAT* could alter fruit metabolism, constitutive overexpression (OE) constructs of two cDNAs, *SlBCAT1* and *SlBCAT3*, were transformed into tomato plants. These two cDNAs were chosen due to their high expression in ripening fruits and because they represent a primarily catabolic and primarily anabolic enzyme, respectively. Ripe fruits from field-grown T1 plants were analyzed for amino acid content in comparison with M82 controls. Three independent lines from each construct were chosen for this analysis, and RNA from each line was analyzed by qRT-PCR. Although there were significant increases in expression of the transgenes (Supplemental Fig. S3), there were no consistent differences from the control in amino acid content in any of the *SlBCAT1-OE* lines (Supplemental Table S1), nor was there any visible phenotype. The only significant change was an increase in Ile in the *SlBCAT3-OE* lines, which is consistent with SlBCAT3 having the highest enzyme activity on KMV, the precursor to Ile. Increased expression of an individual *SlBCAT* does not necessarily change amino acid metabolism in tomato fruit, likely due to a tight enzymatic regulation of the BCAA pathways and/or redundancy of individual *SlBCATs* in fruit.

**Genetic Analysis of BCATs in Tomato**

We previously identified quantitative trait loci (QTLs) for the BCAAs in tomato fruit pericarp of introgression lines (ILs) resulting from the interspecific cross of *S. lycopersicum* and its wild relative *Solanum pennellii* (Schauer et al., 2006, 2008). There are 13 Ile, 17 Leu, and 18 Val QTLs, with seven of these coordinately altering all three BCAAs (Fig. 6). Given that BCAT enzymes participate both in the biosynthesis and degradation of all three BCAAs, we determined whether any of the *SlBCAT* genes colocalized with the seven QTLs simultaneously affecting all three
amino acids. A draft tomato genome is available (http://solgenomics.net/). We were able to precisely map five of the *SlBCAT* genes by their proximities to previously mapped markers within scaffolds (Fig. 6). The scaffold containing *SlBCAT2* did not contain any mapped marker. *SlBCAT2* was mapped to an IL bin on chromosome 7 using a polymorphic marker. Two of the major coordinate QTLs for BCAA content (i.e. those in which the change in amino acid content was consistent for Ile, Leu, and Val) were found to colocalize with *SlBCAT* genes (IL3-2:SlBCAT4 and IL12-3: SlBCAT1).

### Table II. Kinetic parameters of SlBCATs

Activities of purified recombinant SlBCAT proteins on all BCAA and BCKA substrates. *K*<sub>m</sub> is presented as average ± SE. *K*<sub>m</sub> data were obtained using GraphPad Prism5 software. Other parameters were obtained by calculations listed in “Materials and Methods.” Substrate indicates substrate used in the assay, and Enzyme indicates SlBCAT isoform used in the assay.

| Substrate | Enzyme | *K*<sub>m</sub> | *V*<sub>max</sub> | *K*<sub>catalytic</sub> | *K*<sub>cat</sub>/*K*<sub>m</sub> |
|-----------|--------|----------------|-----------------|---------------------|------------------|
| KIC       |        |                |                |                     |                  |
| 1         | 7.09 ± 0.92 | 0.5             | 11.7            | 0.002               |
| 2         | 7.90 ± 0.80 | 3.5             | 84.8            | 0.011               |
| 3         | 0.35 ± 0.06 | 1.1             | 28.1            | 0.080               |
| 4         | 0.41 ± 0.02 | 1.4             | 35.0            | 0.085               |
| 5         | 0.34 ± 0.06 | 2.2             | 54.6            | 0.160               |
| 6         | 0.22 ± 0.02 | 1.2             | 28.7            | 0.130               |
| KMV       |        |                |                |                     |                  |
| 1         | 11.65 ± 1.89 | 0.7             | 16.3            | 0.001               |
| 2         | 12.40 ± 0.90 | 2.8             | 76.7            | 0.006               |
| 3         | 0.19 ± 0.02 | 1.0             | 23.6            | 0.120               |
| 4         | 0.14 ± 0.01 | 0.8             | 18.4            | 0.131               |
| 5         | 0.19 ± 0.02 | 1.0             | 23.6            | 0.120               |
| 6         | 0.16 ± 0.02 | 1.0             | 22.7            | 0.140               |
| KIV       |        |                |                |                     |                  |
| 1         | 5.57 ± 0.75 | 1.0             | 23.0            | 0.004               |
| 2         | 5.50 ± 0.60 | 3.5             | 84.3            | 0.015               |
| 3         | 0.65 ± 0.07 | 1.9             | 46.5            | 0.070               |
| 4         | 0.37 ± 0.02 | 2.5             | 60.6            | 0.164               |
| 5         | 1.20 ± 0.10 | 0.9             | 22.1            | 0.020               |
| 6         | 0.15 ± 0.01 | 4.6             | 109.8           | 0.730               |
| Leu       |        |                |                |                     |                  |
| 1         | 0.56 ± 0.04 | 1.6             | 39.1            | 0.070               |
| 2         | 0.20 ± 0.02 | 0.3             | 8.1             | 0.040               |
| 3         | 2.70 ± 0.30 | 4.8             | 121.0           | 0.045               |
| 4         | 0.57 ± 0.03 | 0.7             | 17.9            | 0.031               |
| 5         | 1.80 ± 0.10 | 4.7             | 118.0           | 0.066               |
| 6         | 0.21 ± 0.02 | 0.6             | 15.7            | 0.075               |
| Ile       |        |                |                |                     |                  |
| 1         | 0.67 ± 0.09 | 1.6             | 40.8            | 0.061               |
| 2         | 0.31 ± 0.02 | 0.3             | 7.6             | 0.025               |
| 3         | 4.90 ± 0.90 | 6.9             | 174.0           | 0.036               |
| 4         | 0.43 ± 0.03 | 0.8             | 20.0            | 0.047               |
| 5         | 3.20 ± 0.20 | 6.5             | 163.0           | 0.051               |
| 6         | 0.34 ± 0.03 | 0.8             | 20.0            | 0.059               |
| Val       |        |                |                |                     |                  |
| 1         | 1.00 ± 0.10 | 2.0             | 50.5            | 0.050               |
| 2         | 1.40 ± 0.50 | 0.2             | 3.8             | 0.003               |
| 3         | 2.00 ± 0.20 | 4.4             | 111.0           | 0.056               |
| 4         | 1.40 ± 0.10 | 0.8             | 20.6            | 0.015               |
| 5         | 2.60 ± 0.20 | 4.9             | 123.0           | 0.047               |
| 6         | 1.20 ± 0.10 | 1.0             | 24.0            | 0.020               |

Evaluation of Amino Acid and Transcript Levels of Sublines of IL3-2 Containing BCAT4

To gain better genetic resolution of the BCAA QTLs, we searched for recombinant lines containing smaller *S. pennellii* introgressions in the region of interest. Only for the QTL identified in IL3-2 was such material available, with three as yet undescribed sublines spanning this region (Fig. 7A). Only two of these lines were marker delimited, subIL5197 and subIL4376; therefore, we chose these for further characterization. While subIL5197 expressed the *S. pennellii* BCAT4
allele, subIL4376 did not. IL3-2, subIL4376-1, and subIL5197-5 carried the coordinate QTL for Ile, Leu, and Val, displaying more than 160% of the levels of these amino acids than the *S. lycopersicum* control (Fig. 7B). Semi-quantitative comparisons of the expression levels of *BCAT4* in IL3-2 and the two sublines to M82 revealed that all three displayed enhanced expression of *BCAT4* (Fig. 7D). Further quantitative analysis by qRT-PCR revealed that the expression level of *BCAT4* is 120.1-fold (±7.4 se) higher in IL3-2 than M82, as determined by the cycle thresholds of the samples normalized to the ubiquitin endogenous control gene (ΔCt; Fig. 7E). Fold difference was derived from the ΔCt values of *BCAT4* in IL3-2 and M82 as described in “Materials and Methods.” Sequencing of the *S. lycopersicum* and *S. pennellii* *BCAT4* revealed no polymorphism in translated regions of the gene (data not shown). This finding is highly suggestive of an expression QTL mediated by a polymorphism in the promoter or another regulatory element that directly controls *BCAT4* expression. We were able to delimit this QTL to a 1-centimorgan region (distance between markers C2A15g23880 and TG542) immediately adjacent to the *BCAT4* gene.

**Figure 5.** Characterization of *SIBCAT1* antisense lines. A, RT-PCR analyses of *SIBCAT1* expression in transgenic plants. B, Content of BCAAs in red tomato fruits (40 dpa). Data represent means ± se from three independent biological replicates with two technical replicates for each. Asterisks show statistically significant changes according to Student’s *t* test (*P* < 0.05). FW, Fresh weight; WT, wild type.

Therefore, we evaluated the nucleotide sequences of the translated regions of *BCAT1* amplified from M82 and IL12-3-1 (Supplemental Fig. S3). This analysis revealed the presence of six nucleotide polymorphisms between the *S. lycopersicum* and *S. pennellii* alleles resulting in three amino acid changes; the *S. lycopersicum* allele harbors Ser-220, Asp-236, and Arg-237, whereas the *S. pennellii* allele harbors Ala-220, Glu-236, and Lys-237. Interestingly, all three residues lie on the fourth and fifth α-helices of the protein, very close to the conserved active site Lys residue (Tremblay and Blanchard, 2009).

In order to determine if differences in these three residues confer altered enzymatic function, both forms were expressed and purified from *E. coli* cells and assayed. Since *SIBCAT1* is thought to function primarily in amino acid catabolism in fruit, assays were performed using BCAA substrates (Table III). The *S. pennellii* *Km* values were significantly (*P* < 0.05) higher than the *S. lycopersicum* enzyme. Consistent with the *Km* values, the *S. pennellii* catalytic efficiencies were lower with all three substrates. These small but significantly different values suggest that the *S. lycopersicum* enzyme is more catabolically efficient and could explain the higher BCAA levels in IL12-3-1.

**DISCUSSION**

Here, we describe six tomato *BCAT* cDNAs, distinguished by their unique patterns of expression and subcellular locations. *SIBCAT1* and *SIBCAT2* localize to mitochondria. Given that mitochondria are the primary location of BCAA catabolism in plant cells and that the activities of these two enzymes are primarily catabolic, only partially restoring growth in *E. coli* auxotrophs, these are likely to be the primary BCAA catabolic enzymes in tomato. Since *SIBCAT1* has the highest expression in ripening fruit, it likely is the primary enzyme for recycling of BCAAs generated by protein degradation. This conclusion is further supported by the elevation of BCAAs in *SIBCAT1* antisense transgenic tomato. Since *SIBCAT2* is expressed in all green tissues examined, it is likely a more general contributor to BCAA catabolism. We expect a catabolic mitochondrial housekeeping enzyme to exist, since BCAAs are used as precursors to the tricarboxylic acid cycle intermediates succinyl-CoA and acetyl-CoA as well as direct electron donors of the mitochondrial electron transport chain (Ishizaki et al., 2005). This enzyme may also function in the crucial process of regulating steady-state levels of BCAAs in cells.

*SIBCAT3* and *SIBCAT4* are localized to chloroplasts and were able to effectively restore the growth of *E. coli* BCAA auxotrophs, consistent with a primary role in BCAA synthesis. The kinetic properties of these two enzymes, especially of *SIBCAT3*, further support this conclusion. It is possible, however, that under certain conditions these two enzymes may be active in BCAA
catabolism, evident from their ability to use BCAAs as substrates. **SlBCAT3** likely functions as the major enzyme for BCAA synthesis, since it is expressed nearly equally in all tissues, including all stages of fruit development. **SlBCAT4** appears to be more specialized in function, given that its expression is by far highest in flowers and is relatively low in other tissues compared with the other **SlBCATs**. This expression pattern may reflect high demand for amino acid synthesis in reproductive tissue. Alternatively, it may have a specific role beyond primary metabolism, as has been suggested for Arabidopsis AtBCAT3 and AtBCAT4 (Schuster et al., 2006; Knill et al., 2008). The NbBCAT, recently found to have a role in hormonal regulation, is also expressed highly in flowers and localized to chloroplasts (Gao et al., 2009).

**SlBCAT5** and **SlBCAT6** expression was not detected in any of the tissues analyzed. These two unigenes are represented by only five and two ESTs, respectively. Both ESTs for **SlBCAT6** were isolated from callus tissue. These genes may be expressed under specific growth, hormonal, or environmental conditions or may be linked to specific secondary metabolic pathways. Their distinct localization patterns and demonstrated functionality on branched-chain substrates makes these two genes interesting candidates for future research. The vacuolar localization of **SlBCAT6** suggests that it may function in the recycling of proteolytically derived BCAAs. This function is further supported by its relatively high affinity for all branched-chain substrates and by the fact that BCAAs accumulate in this organelle (Farre et al., 2001). Of particular interest is this enzyme’s efficiency using KIV, which is extremely high compared with all other **SlBCATs** and all other substrates. It is unclear why this enzyme is more active in Val synthesis.

Overexpression of the most highly expressed anabolic (**SlBCAT3**) or catabolic (**SlBCAT1**) cDNAs in fruits did not significantly alter the BCAA pool. As in bacteria (Massey et al., 1976), the enzymes in the pathway of BCAA synthesis are tightly regulated by substrate feedback. Increased BCAA catabolism may
stimulate coincident increased BCAA synthesis. The opposite effect may occur with overexpression of \( \text{SlBCAT3} \), a primarily anabolic enzyme, where increased BCAA synthesis might have an effect on feedback to earlier steps in the BCAA synthesis pathway, such as Thr deaminase or acetolactate synthase, inhibiting BCAA synthesis.

\( \text{BCAT4} \) transcript is substantially increased in IL3-2 and the sublines relative to \( \text{S. lycopersicum} \). These lines also have elevated levels of Leu, Ile, and Val (Fig. 7B). However, sequencing of the coding region of the gene amplified from IL3-2 and from \( \text{S. lycopersicum} \) revealed no polymorphisms. Furthermore, subline 4376 carries the \( \text{S. lycopersicum BCAT4} \) allele. One possible explanation for the \( \text{BCAT4} \) expression QTL is that this very large increase in expression is caused by a difference in the noncoding region of the gene. Although subline 4376 contains the \( \text{S. lycopersicum} \) allele, the point of recombination is in the vicinity of the structural gene. Analysis of the genome scaffolds in the region indicates that this is the only structural gene associated with BCAA metabolism mapping to this locus. We cannot, however, exclude the possibility that the region between markers C2At5g23880 and TG542 encodes a trans-acting factor that is responsible for elevated \( \text{BCAT4} \) expression. These data, alongside the localization of the protein in the chloroplast, are consistent with \( \text{BCAT4} \) operating predominantly in the synthetic direction in vivo. By contrast, \( \text{BCAT1} \), which encodes a mitochondria-localized protein, is

### Table III. Kinetic parameters of SlBCAT1 enzymes

| Allele     | Substrate | \( K_m \) (mM) | \( V_{max} \) (nkat mg\(^{-1}\) s\(^{-1}\)) | \( K_{cat} \) (s\(^{-1}\)) | \( K_{cat}/K_m \) (\( \mu \text{kat}^{-1} \text{s}^{-1} \)) |
|------------|-----------|----------------|---------------------------------|----------------------------|----------------------------------|
| M82        | Leu       | 0.56 ± 0.05    | 2.7                            | 63.1                       | 0.113                             |
|            | Ile       | 0.60 ± 0.09    | 2.8                            | 66.4                       | 0.111                             |
|            | Val       | 0.92 ± 0.13    | 1.9                            | 45.8                       | 0.050                             |
| **S. pennellii** | Leu | 0.84 ± 0.07*   | 3.4                            | 80.2                       | 0.095                             |
|            | Ile       | 0.84 ± 0.09*   | 2.9                            | 69.9                       | 0.083                             |
|            | Val       | 1.32 ± 0.25*   | 2.3                            | 55.0                       | 0.042                             |
equivalently expressed in IL12-3 and *S. lycopersicum*. However, sequencing of the IL12-3 and *S. lycopersicum* BCA1 coding regions revealed three variant amino acid residues. Intriguingly, when aligning these two protein sequences with that from *Mycobacterium tuberculosis*, for which a high-resolution crystal structure exists (Tremlay and Blanchard, 2009), the variant residues are very close to the active site of the protein. The *S. pennellii* allele exhibits higher *Kₘ* values for Leu, Ile, and Val that may explain the increased levels of BCAAs in fruits. However, results from the SIBCAT1 antisense transgenic lines do indicate that SIBCAT1 activity is a major determinant of BCAA levels in mature tomato fruits.

CONCLUSION

The results of these experiments give important new information about BCAA metabolism and the BCAT gene family. They support anabolic functions for SIBCAT3/SIBCAT4, catabolic functions for SIBCAT1/SIBCAT2, and potentially novel functions for SIBCAT5/SIBCAT6. They also suggest possible roles of SIBCAT1 and SIBCAT4 in the regulation of BCAAs in tomato.

MATERIALS AND METHODS

All chemicals and reagents used were purchased from Sigma-Aldrich unless otherwise noted. Oligonucleotides were purchased from Integrated DNA Technologies and are listed in Supplemental Table S2.

Cloning of SIBCAT cDNAs

ESTs for each SIBCAT were found by searching the SOL Genomics Network tomato (*Solanum lycopersicum*) EST database (http://solgenomics.net/index.pl) for sequences that share homology with known plant BCA1s. The full-length clones of each SIBCAT were obtained using RACE PCR with the SMART RACE cDNA synthesis kit (Clontech Laboratories). PCR with Advantage HF polymerase (Clontech Laboratories) was used to amplify the full-length open reading frames from cDNA. These were cloned into pGEMT (Promega) and sequenced. Alignments of protein sequences were produced using ClustalW (Larkin et al., 2007).

Constructs

Open reading frames for each construct were amplified from cDNA by PCR and cloned into pGEMT-Easy vector (Promega). SIBCAT expression constructs were made by subcloning into the *Nel* and *Sall* restriction sites of pET-28b (Invitrogen), which contains an N-terminal 6xHis tag. Protein expression constructs for assaying allelics of SIBCAT1 and *Solanum pennellii* BCA1 were constructed by subcloning into pENTR/SD/D-TOPO (Invitrogen) and then into pDEST15 (Invitrogen), which contains an N-terminal glutathione S-transferase tag. Primers were designed to omit signal peptides, as predicted by SignalP software (Emanuelsson et al., 2007), and are listed in Supplemental Table S2.

Bacterial complementation constructs were made by excising the inserts from pET28b and inserting them into pBAD24 (Guzman et al., 1995) using *Sall* and *Nel* restriction sites, resulting in a pBAD24 construct containing a 6xHis tag.

For plant overexpression constructs, SIBCAT1 and SIBCAT3 cDNAs were cloned in the sense orientation into pENTR/D-TOPO and cloned using Gateway LR Recombinase (Invitrogen) into a vector containing the figwort mosaic virus promoter (Richins et al., 1987), a kanamycin resistance gene, and an *Agrobacterium tumefaciens* nopaline synthase 3' terminator. The overexpres-
described previously (Lisec et al., 2006; Schauer et al., 2006), with the exception that, for low-abundance metabolites, a substantially higher extract concentration was injected onto the GC-MS system. The absolute concentration of metabolites was determined by comparison with standard concentration curves as defined by Schauer et al. (2005b). Metabolites were identified in comparison with database entries of authentic standards (Kopka et al., 2005; Schauer et al., 2005a). In addition, the metabolites KIC, KMW, and KIV, for which no mass spectral tag information was available, were identified by analysis of identically derivatized authentic standards.

Expression Analysis

RNA was isolated from tomato fruit tissue using the RNeasy Plant RNA Extraction Kit (Qiagen) followed by DNase treatment to rid samples of contaminating DNA. RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Omniscript reverse transcriptase (Qiagen) was used with 1 μg of each RNA sample to synthesize oligo(dT)-primed cDNA. 1× SYBR Green Master Mix (Applied Biosystems) was used with 1 μL of each cDNA sample and 500 nM gene-specific primers for qRT-PCR on the Applied Biosystems StepOnePlus real-time PCR machine. For RT-PCR of SIBCATs in different plant tissues and for overexpression transgensics, live-point standard curves were made for each SIBCAT to calculate the ratio of transcript. For comparison of SIBCATs in IL3 and antisense transgensics, ΔCt values were determined by comparison with the ubiquitin control gene. For BCAT4 analysis in IL3-2 and M82, fold differences were derived by comparison with the ubiquitin calibrator and calculated using the ΔΔCt quantification algorithm (Livak and Schmittgen, 2001). Amplification conditions were as follows: 2 min at 50°C; 10 min at 95°C; 40 cycles each of 15 s at 95°C followed by 1 min at 60°C; 15 s at 95°C; 20 s at 60°C; 15 s at 95°C. Primer specificity was confirmed with melting curve analysis on the StepOnePlus real-time PCR machine.

Escherichia coli Complementation

E. coli strain BW25131 with knockouts in ilvE (JW5060-1) or tyrB (W4014-2) were purchased from the Keio Collection (Baba et al., 2006). Double knockouts were constructed as described previously (Cherepanov and Wackernagel, 1995; Baba et al., 2006) and validated by PCR with primers flanking the sites of the two genes (Supplemental Table S2). Constructs of SIBCATs in pBAD24 were transformed into ΔilvE/ΔilvB cells. Cells were first grown in liquid M9 minimal medium supplemented with 0.2% casamino acids and 1 mM thiamine hydrochloride and then were washed and transferred to M9 minimal medium lacking amino acids (Sambrook et al., 1989) and supplemented with 0.2% (w/v) Ara for induction, 0.4% (w/v) glycerol for carbon source, and 50 μg mL⁻¹ carbenicillin. Cell culture density in minimal medium lacking amino acids (Sambrook et al., 1989) and supplemented with 0.2% (w/v) Ara for induction, 0.4% (w/v) glycerol for carbon source, and 50 μg mL⁻¹ carbenicillin. Cell culture density in minimal medium lacking amino acids was measured by OD₆₀₀ probing with mouse anti-His antibody (Invitrogen).

Amino Acid Analysis of Tomato Fruit by GC-MS

Amino acid levels in M82 control and SIBCAT1-OE and SIBCAT3-OE transgenic ripe tomato fruit were determined by derivatization with methyl chloroformate and quantification by GC-MS according to the method of Chen et al. (W.P. Chen, X.Y. Yang, W. Gray, and J.D. Cohen, unpublished data), using an Agilent 6890N gas chromatograph and 5975 mass spectrometer. Three technical replicates of each of three biological replicates were analyzed for each transgenic line. Fruits were grown in fields at the North Florida Research and Education Center.

Extraction and Analysis of BCAAs by HPLC

Amino acids were measured using HPLC after labeling with o-phthalaldehyde according to the method of Kreft et al. (2003). Detection and quantification were based on the conversion of the primary amino group with o-phthalic acid dialedehyde to a fluorescing derivative. Peak areas were integrated using Chromelone software 6.8 (Dionex) and subjected to quantification by comparison with calibration curves generated following serial runs of a dilution series of mixed standards.
Tea and KmR cassettes with the option of Flip-catalyzed excision of the antibiotic-resistance determinant. Gene 158: 9–14

Daschner K, Thalmann C, Guha C, Brenchnic A, Binder S (1999) In plants a putative isovaleryl-CoA dehydrogenase is located in mitochondria. Plant Mol Biol 39: 1275–1282

Diebold R, Schuster J, Daschner K, Binder S (2002) The branched-chain amino acid transaminase gene family in Arabidopsis encodes plastid and mitochondrial proteins. Plant Physiol 129: 540–550

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure from small amounts of fresh leaf tissue. Phytochemistry 26: 19–21

Emanuelsson O, Brunak S, von Heijne G, Nielsen H (2007) Locating proteins in the cell using TargetP, SignalP and related tools. Nat Protoc 2: 953–971

Engqvist M, Drincovic ME, Flugge UI, Maurino VG (2009) Two D-2-hydroxy-acid dehydrogenases in Arabidopsis thaliana with catalytic capacities participate in the last reactions of the methyglyoxal and beta-oxidation pathways. J Biol Chem 284: 25026–25037

Farre EM, Tiessen A, Roessner U, Geigenberger P, Trethewey RN, Kroumova AB, Xie Z, Wagner GJ (2009) Two D-2-hydroxy-acid dehydrogenases in Arabidopsis thaliana with catalytic capacities participate in the last reactions of the methyglyoxal and beta-oxidation pathways. J Biol Chem 284: 25026–25037

Gao F, Wang C, Wei C, Li Y (2009) A branched-chain aminotransferase may regulate hormone levels by affecting KNOX genes in plants. Planta 230: 611–623

Gelfand DH, Steinberg RA (1977) Escherichia coli mutants deficient in the aspartate and aromatic amino acid aminotransferases. J Bacteriol 130: 429–440

Gu L, Jones AD, Last RL (2010) Broad connections in the Arabidopsis seed metabolic network revealed by metabolite profiling of an amino acid catabolism mutant. Plant Physiol 153: 2010

Hofberg S, Petersen JG (1988) Regulation of isoleucine-valine biosynthesis in Saccharomyces cerevisiae. Curr Genet 13: 207–217

Ishizaki K, Larson TR, Schauer N, Fernie AR, Graham IA, Leaver CJ (2005) The critical role of Arabidopsis electron-transfer flavoprotein: ubiquinone oxidoreductase during dark-induced starvation. Plant Cell 17: 2587–2600

Kandra G, Severson R, Wagner GJ (1990) Modified branched-chain amino acid pathways give rise to acyl acids of scuor esters exuded from tobacco leaf trichomes. Eur J Biochem 188: 385–391

Karimi M, Inze D, Depicker A (2002) Gateway vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci 7: 193–195

Knill T, Schuster J, Reichelt M, Gershzenon J, Binder S (2008) Arabidopsis branched-chain aminotransferase 3 functions in both amino acid and glucosinolate biosynthesis. Plant Physiol 146: 1028–1039

Kohlhaw GB (2003) Leucine biosynthesis in fungi: entering metabolism through the back door. Microbiol Mol Biol Rev 67: 1–15

Konz C, Schell J (1986) The promoter of Ti-DNA gene 5 controls the tissue-specific expression of chimeric genes carried by novel types of Agrobacterium binary vector. Mol Genet 204: 383–396

Kopka J, Schauer N, Krueger S, Biekemeyer C, Usadel B, Bergmüller E, Dürmann P, Weckwerth W, Gibon Y, Stitt M, et al (2005) Gmd@csb.Db: a putative isovaleryl-CoA-dehydrogenase is located in mitochondria. Mol Biol Rev 67: 1–15

Kreft O, Hoefgen R, Hesse H (2003) Functional analysis of cystathionine γ-synthase in genetically engineered potato plants. Plant Physiol 131: 1843–1853

Kroumova AB, Xie Z, Wagner GJ (1994) A pathway for the biosynthesis of straight and branched, odd- and even-length, medium-chain fatty acids in plants. Proc Natl Acad Sci USA 91: 11437–11441

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, et al (2007) Clustal W and Clustal X version 2.0. Bioinformatics 23: 2947–2948

Li L, Thiypaopong P, Breeden DC, Steffens JC (2003) Overexpression of a bacterial branched-chain alpha-keto acid dehydrogenase complex in Arabidopsis results in accumulation of branched-chain acyl-CoAs and alteration of free amino acid composition in seeds. Plant Sci 165: 1213–1219

Lipman AH, Olsen LI (2004) Genomic analysis of aminotransferases in Arabidopsis thaliana. Crit Rev Plant Sci 23: 73–89

Lisec J, Schauer N, Kopka J, Willmitzer L, Fernie AR (2006) Gas chromatography mass spectrometry-based metabolite profiling in plants. Nat Protoc 1: 387–396

Liu KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(ΔΔCT) method. Methods 25: 402–408

Massey LH, Sokatch JR, Conrad RS (1976) Branched-chain amino-acid catabolism in bacteria. Bacteriol Rev 40: 42–54

Mc Cormick S, Niederemeyer J, Fry J, Barnason A, Horsch R, Fraley R (1986) Leaf disc transformation of cultivated tomato (L. esculentum) using Agrobacterium tumefaciens. Plant Cell Rep 5: 81–84

Mueller LA, Solow TH, Taylor N, Skwarecki B, Buelas R, Binns J, Lin CW, Wright MH, Ahrens R, Wang Y, et al (2005) The SOL Genomics Network: a comparative resource for Solanaceae biology and beyond. Plant Physiol 138: 1310–1317

Powell JT, Morrison JF (1978) Role of the Escherichia coli aromatic amino acid aminotransferase in leucine biosynthesis. J Bacteriol 136: 1–4

Prohl C, Kispal G, Lill R (2000) Branched-chain amino-acid aminotransaminase of yeasts Saccharomyces cerevisiae. Methods Enzymol 324: 365–375

Richins RD, Scholthof HB, Shepherd RJ (1987) Sequence of wigwort mosaic virus DNA (caulimovirus group). Nucleic Acids Res 15: 8451–8466

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular Cloning: A Laboratory Manual, Ed 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Schauer N, Semel Y, Balbo I, Steinfath M, Repsilber D, Selbig J, Pieban T, Zamir D, Fernie AR (2008) Mode of inheritance of primary metabolic traits in tomato. Plant Cell 20: 509–523

Schauer N, Semel Y, Roessner U, Gur A, Balbo I, Carrari F, Pieban T, Perez-Melis A, Bruedigam C, Kopka J, et al (2006) Comprehensive metabolic profiling and phenotyping of interspecific introgression lines for tomato improvement. Nat Biotechnol 24: 447–454

Schauer N, Steinhauser D, Strelkov S, Schomburg D, Alliuss G, Moritz T, Lundregen K, Roessner-Tunali U, Forbes MG, Willmitzer L, et al (2005a) GC-MS libraries for the rapid identification of metabolites in complex biological samples. FEMS Lett 579: 1332–1337

Schauer N, Zamir D, Fernie AR (2005b) Metabolic profiling of leaves and fruit of wild species tomato: a survey of the Solanum lycopersicum complex. J Exp Bot 56: 297–307

Schulze-Siebert D, Heineke D, Scharf H, Schulz G (1984) Pyruvate-derived amino acids in spinach chloroplasts: synthesis and regulation during photosynthetic carbon metabolism. Plant Physiol 76: 465–471

Schuster J, Binder S (2005) The mitochondrial branched chain amino transferase (AIBCAT-1) is capable to initiate degradation of leucine, isoleucine and valine in almost all tissues of Arabidopsis. Plant Mol Biol 57: 241–254

Schuster J, Knill T, Reichelt M, Gershzenon J, Binder S (2006) Branched-chain aminotransferase is part of the chain elongation pathway in the biosynthesis of methionine-derived glucosinolates in Arabidopsis. Plant Physiol 138: 2664–2679

Taylor NL, Heazlewood JL, Day DA, MILLAR AH (2004) Lipoic acid-dependent oxidative catabolism of alpha-keto acids in mitochondria provides evidence for branched-chain amino acid catabolism in Arabidopsis. Plant Physiol 134: 838–848

Tremblay LW, Blanchard JS (2009) The 1.9 angstrom structure of the branched chain amino acid transaminase (IlvE) from Mycobacterium tuberculosis. Acta Crystallogr Sect F Struct Biol Cryst Commun 65: 1071–1077

Vartak NB, Liu L, Wang BM, Berg CM (1991) A functional Jrk/ABCD operon is required for leucine synthesis by the tyrosine-repressible transaminase in Escherichia coli K-12. J Bacteriol 173: 3864–3871

Walters DS, Steffens JC (1990) Branched chain amino acid metabolism in the biosynthesis of Lycopersicon pennelli glucose esters. Plant Physiol 93: 1544–1551

Yoo SD, Cho YH, Sheen J (2007) Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. Nat Protoc 2: 1565–1572

936 Plant Physiol. Vol. 153, 2010