Running title: 3-Hydroxyisobutyrate Dehydrogenase of Arabidopsis thaliana

To whom correspondence should be addressed: Hans-Peter Braun, Institut für Pflanzenge-netik, Leibniz Universität Hannover, Herrenhäuser Str. 2, 30419 Hannover, Germany, Tel.: +49 511 7622674, Fax: +49 511 76214351, mail: braun@genetik.uni-hannover.de

3-Hydroxyisobutyrate Dehydrogenase is involved in both, Valine and Isoleucine degradation in Arabidopsis thaliana

Peter Schertl¹, Lennart Danne¹, Hans-Peter Braun¹

¹ Institut für Pflanzengenetik, Leibniz Universität Hannover, Herrenhäuser Str. 2, 30419 Hannover, Germany

One Sentence Summary:
3-Hydroxyisobutyrate Dehydrogenase, an enzyme of the branched chain amino acid breakdown pathway, is involved in valine and isoleucine but not in leucine degradation in Arabidopsis thaliana.

Author’s contribution: PS: planned and performed experiments (enzyme purification, enzyme activity assays, substrate screening, SDS-PAGE, Western blotting, Blue native (BN) PAGE and in-gel activity staining, Mutant screening, Gene expression analyses) and wrote the paper. LD: performed Gateway cloning experiments. HPB: initiated the project, wrote the paper.

Funding: This research has been funded by the “Wege in die Forschung II” program of Leibniz University Hannover to PS and by a research grant of the „Fonds der Chemischen Industrie“ im Verband der Chemischen Industrie e.V. to PS.

Keywords
40  *Arabidopsis thaliana*, amino acid catabolism, isoleucine, mitochondria, plant respiration,
41  valine
Abstract

In plants, amino acid catabolism is especially relevant at metabolic stress situations, e.g. limited carbohydrate availability during extended darkness. Under these conditions, amino acids are used as alternative substrates for respiration. Complete oxidation of the branched chain amino acids (BCAAs) Leucine, Isoleucine and Valine in the mitochondria efficiently allows formation of ATP by oxidative phosphorylation. However, the metabolic pathways for BCAA breakdown are largely unknown so far in plants. A systematic search for Arabidopsis genes encoding proteins resembling enzymes involved in BCAA catabolism in animals, fungi and bacteria as well as proteomic analyses of mitochondrial fractions from Arabidopsis allowed identification of a putative 3-Hydroxyisobutyrate dehydrogenase, AtHDH1 (At4g20930), involved in Valine degradation. Systematic substrate screening analyses revealed that the protein uses 3-Hydroxyisobutyrate but additionally 3-Hydroxypropionate as substrate. This points to a role of the enzyme not only in Valine but possibly also in Isoleucine metabolism. At4g20930 knock-down plants were characterized to test this conclusion. Root toxicity assays revealed increased root growth inhibition of the mutants if cultivated in the presence of Valine or Isoleucine, but not in the presence of Leucine. We conclude that AtHDH1 has a dual role in BCAA metabolism in plants.
Plants can synthesize all 20 proteinogenic amino acids. Their carbon skeletons and amino groups directly or indirectly derive from photosynthesis. Besides amino acid biosynthesis, plants also can breakdown all 20 amino acids. Amino acid catabolism is especially relevant in the context of germination (conversion of storage proteins into carbohydrates), senescence (recycling of energy-rich compounds) and in the context of stress reactions (Hildebrandt et al. 2015). Synthesis of some amino acids is massively induced upon drought and salt stress, most of all proline, because they can serve as compatible osmolytes (Szabados and Savouré, 2010). Upon stress release, these amino acids are rapidly degraded. Furthermore, amino acid catabolism is essential for respiration at low light conditions or extended darkness. Upon light shortage, availability of carbohydrates for respiration is limited and amino acids can be used as alternative respiratory substrates (Araújo et al. 2011). Amino acid catabolism mainly takes place in the mitochondria of plants but most enzymatic reactions so far have not been characterized (Hildebrandt et al. 2015). They may or may not resemble reactions taking place in mammalian cells, which have been extensively investigated (Harper et al. 1984).

The branched chain amino acids (BCAAs) Leucine, Isoleucine and Valine have aliphatic and comparably short side chains. Their concentrations increase under various stress conditions (Zhao et al., 1998; Joshi et al., 2010). Complete oxidation of BCAAs in the mitochondria allows generating high amounts of ATP (Hildebrandt et al. 2015). Indeed, it has been shown that the degradation pathways for BCAAs are upregulated at extended darkness (Binder 201; Däschner et al. 2001; Ishizaki et al. 2005; Araújo et al. 2010). The BCAA catabolic pathways are especially complicated and so far only fragmentarily understood in plants.

The first two steps in the degradation of Valine, Isoleucine and Leucine are identical (see the very last figure of this publication for summarizing the known and the putative steps of BCAA catabolism in plants). After an initial transamination reaction the BCAAs are decarboxylated. Several transaminases that use BCAAs as substrates have been identified in plants. As a result branched chain 2-Oxoaicids are produced (Angelovic et al. 2013). The decarboxylation of the BCAAs is carried out by the branched-chain α-Ketoacid dehydrogenase complex (BCKDH), which is a very large complex and consists of multiple copies of the three enzymes E1, E2 and E3 (Mooney et al. 2002). The third enzyme in the degradation of Leucine is the Isovaleryl-CoA dehydrogenase (IVDH). This enzyme most likely also is involved in the parallel step of Valine and Isoleucine breakdown pathways. IVDH is a flavoenzyme that transfers electrons to the Electron Transfer Flavoprotein (ETF) (Däschner et al. 2001, Araújo et al. 2010). The forth biochemical step, a carboxylation reaction, is only known for Leucine catabolism (Alban et al. 1993, Anderson et al. 1998). Starting from this point, further steps in the degradation of the three BCAAs are not known in plants. Only putative enzymes showing some degree of sequence similarity to already identified and characterized mammalian and bacterial enzymes have been reported (Hildebrand et al. 2015). Most likely, an Enoyl-CoA hydratase is involved in converting Methylglutaconyl-CoA coming from Leucine as well as 2-Methylbutanoyl-CoA and 2-Methylpropanoyl-CoA coming from Isoleucine and Valine catabolism, respectively. From this step, the three degradation pathways are predicted to differ. The final reaction in the Leucine catabolism is the conversion of 3-Hydroxymethylglutaryl-CoA to Acetyl-CoA and Acetoacetate. In Isoleucine catabolism probably a 3-Hydroxyacyl-CoA dehydrogenase and a 3-Ketoacyl-CoA thiolase are involved in the formation of Acetyl-CoA. The terminal steps of Valine catabolism seem to be most complex. Probably, 3-Hydroxyisobutyrate is formed from 3-Hydroxyisobutyryl-CoA by a hydrolyzation reaction. Next, an
oxidation takes place which is catalyzed by 3-Hydroxyisobutyrate dehydrogenase. This step is special in the degradation of BCAAs because 3-Hydroxyisobutyrate is not coupled to Coenzyme A. Different mammalian as well as bacterial 3-Hydroxyisobutyrate dehydrogenases have been characterized in detail (Hawes et al. 1995, 1996 and 2000, Murin et al. 2008, Rougraff et al. 1988, Chowdhury et al. 2003, Park et al. 2016 Lee et al. 2014). In contrast, this enzyme has not been characterized in plants so far. The product of the reaction catalyzed by 3-Hydroxyisobutyrate dehydrogenase is Methylmalonate semialdehyde. A final decarboxylase of the Valine catabolic pathway catalyzes the conversion of Methylmalonate semialdehyde into Propionyl-CoA. The breakdown of Propionyl-CoA in plants results in Acetyl-CoA. It has been suggested that at least parts of this pathway in plants are carried out by the same set of enzymes that convert 3-Hydroxyisobutyryl-CoA to Propionyl-CoA (Hildebrandt et al. 2015).

We here describe the identification and characterization of a 3-Hydroxyisobutyrate dehydrogenase (AtHDH1) in Arabidopsis thaliana. The enzyme was recombinantly expressed in E. coli and affinity purified. The native state and the enzyme properties of AtHDH1 were determined. Besides 3-Hydroxyisobutyrate, AtHDH1 converts 3-Hydroxypropionate and Methyl-3-hydroxy-2-methylpropionate as revealed by systematic substrate screening analyses. This points to a role of AtHDH1 not only in Valine, but also in Isoleucine metabolism. Root toxicity assays using AtHDH1 knock-down plants were employed to evaluate the physiological role of AtHDH1 in plants. Results indicate a role of AtHDH1 in both, Valine and Isoleucine catabolism, but not in the breakdown of Leucine.
Results

Identification of a putative 3-Hydroxyisobutyrate dehydrogenase from Arabidopsis thaliana

To better understand amino acid degradation in plants, the Arabidopsis genome sequence was systematically searched for genes encoding proteins similar to known enzymes involved in amino acid catabolism in animals, fungi or bacteria (Hildebrandt et al. 2015). Furthermore, genes encoding mitochondrial dehydrogenases were systematically searched based on sequence comparisons (Schertl and Braun 2014). Finally, shot gun proteome datasets of mitochondrial fractions from Arabidopsis thaliana were searched for putative enzymes involved in amino acid catabolism (Schertl 2015). One identified gene encodes a “6-phosphogluconate dehydrogenase family protein” (annotation by The Arabidopsis Information Resource [TAIR], accession At4g20930). Besides resembling 6-phosphogluconate dehydrogenase family proteins, the protein was found to exhibit sequence similarity to mammalian 3-Hydroxyisobutyrate dehydrogenases (HDH) (Supp. Fig. 1). The protein At4g20930 has been identified previously in a mitochondrial fraction of Arabidopsis thaliana in the course of a 2D-gel based proteome project (Taylor et al. 2011, Supp. Fig. 2). It has an apparent molecular mass of 34 kDa. Furthermore, At4g20930 has been identified in a mitochondrial fraction of Arabidopsis thaliana by a complexome-profiling approach (Senkler et al. 2017).

Recombinant expression and purification of At4g20930

A full length Gateway clone (G83598) encoding At4g20930 was ordered from the Arabidopsis Biological Resource Center (Ohio State University, US) (Kayoko et al. 2003). The open reading frame, which encodes a protein of 37.4 kDa, was amplified by PCR, but excluding the N-terminal 84 bp which encode a predicted mitochondrial presequence of 28 amino acids (Supp. Figure 3). For overexpression of At4g20930 in E. coli, the Gateway® pDEST™17 vector was used which allows expression of the protein in-frame with an N-terminal 6x His-tag. The overexpressed fusion protein was partly soluble if E. coli cells were cultivated at 16°C (at higher temperatures the protein formed inclusion bodies). The protein was successfully affinity purified as documented by SDS PAGE and immunoblotting using an IgG directed against the His-tag (Figure 1). Furthermore, MS analysis of the purified protein revealed that the generated transcripts are translated in the correct frame and that the protein is complete (Table 1, Supp. Figure 4, Sup. Table 1). The overexpressed protein is very pure (Figure 1). MS analyses of two minor protein bands at 75 and 30 kDa did not led to the detection of any endogenic E. coli dehydrogenases (Sup. Table 1).

Enzyme properties of At4g20930

Substrate specificity of the purified protein was systematically tested by photometric activity assays using NAD⁺ as a co-substrate (Table 2). Tested compounds were selected either because they were found to be substrates of previously characterized bacterial and/or mammalian 3-Hydroxyisobutyrate dehydrogenases, or because they exhibit structural similarities to 3-Hydroxyisobutyrate. The substrate screening revealed highest activity of the purified enzyme for 3-Hydroxyisobutyrate (4.61 µM mg⁻¹ min⁻¹ = 100 %). To a minor extend also 3-Hydroxypropionate is converted by the enzyme (13% activity compared with 3-Hydroxyisobutyrate). 3-Hydroxypropionate is an intermediate of the suggested conversion of Propionyl-CoA into Acetyl-CoA, which represents the final phase of the Valine and Isoleucine catabolic pathways. Furthermore, purified At4g20930
showed some low activity with Methyl-3-hydroxy-2-methylpropionate as substrate (1.2 %). Methyl-3-hydroxy-2-methylpropionate has an additional methyl group at the carboxyl group in comparison to 3-Hydroxyisobutyrate and was reported to be one of the major volatile components in the mamee apple (*Mammea americana*) fruit (Morales and Duque 2002). Remarkably, the R-enantiomer of 3-
Hydroxisobutyrate was only converted with 2% activity in comparison to the S-enantiomer. The R-enantiomer of Methyl-3-hydroxy-2-methylpropionate was not converted at all by the enzyme. These results reflect a very high stereo specificity of the enzyme. Other tested compounds did not reveal any activity (see Table 2). For instance, Glycerate, Serine as well as β-Hydroxypyruvate are no substrates for the enzyme, although these compounds have similar structures like 3-Hydroxisobutyrate.

We conclude that At4g20930 encodes a 3-Hydroxisobutyrate dehydrogenase. The enzyme was designated AtHDH1.

$K_m$ and $K_{cat}$ values of AtHDH1 from Arabidopsis were determined using freshly purified protein, 3-Hydroxisobutyrate as substrate and NAD$^+$ as co-substrate. The calculated $K_m$ values were 686 µM and 350 µM for 3-Hydroxisobutyrate and NAD$^+$, respectively (Table 3). The turnover rate $K_{cat}$ was 3.430 s$^{-1}$ for 3-Hydroxisobutyrate and 2.652 s$^{-1}$ for NAD$. This implies a catalytic efficiency ($K_{cat}/K_m$) of 4.993 and 7.571 for 3-Hydroxisobutyrate and NAD$, respectively. The reaction mechanism catalyzed by AtHDH1 is a sequential bi-bi mechanism common for most dehydrogenases (Supp. Figure 5). Both substrates (3-Hydroxisobutyrate and NAD$^+$) have to bind before either product is released. The pH optimum of AtHDH1 is 8.5 (Figure 2A), which is close to the estimated pH value of 8.1 for the mitochondrial matrix in Arabidopsis as determined by a mitochondria-specific fluorescence pH sensor (Shen et al. 2013). Enzyme activity measurements at varying temperatures revealed a wide temperature range. The optimum activity is at about 40 °C (Figure 2b). Above 50 °C the enzyme activity declines.

Arabidopsis AtHDH1 is highly specific for NAD$^+$ as a cofactor. NADP$^+$ is not a suitable cofactor for AtHDH1. Also, FAD, oxidized cytochrome c as well as the artificial electron acceptors phenazinemethosulfate (PMS) and dichlorophenolindophenol (DCPIP) cannot be reduced by AtHDH1 (data not shown).

In parallel, another Arabidopsis protein, At4g29120, which slightly resembles AtHDH1, was overexpressed in E. coli and affinity purified (Supp. Figure 6). This protein did not exhibit any 3-hydroxisobutyrate dehydrogenase activity. We conclude that HDH is encoded by single-copy gene. Furthermore, lack of activity in overexpressed At4g29120 fractions further excludes that the E. coli background of our protein expression system interfered with our biochemical characterization of overexpressed AtHDH1

The native state of AtHDH1

The native state of AtHDH1 from Arabidopsis was tested by analyzing the purified recombinant protein using Blue-native PAGE in combination with a 3-Hydroxisobutyrate dehydrogenase in-gel activity assay (Figure 3). Due to a high Coomassie-blue background in the low molecular mass region of the gel, activity of the monomer could not be seen. Activity signals were visible at about 150, 187, 200 and 224 kDa. Since the overexpressed protein is of high purity, we conclude that AtHDH1 can form homooligomeric protein complexes. Dimeric and tetrameric forms of HDH1 were described previously for several organisms (Rougraff et al. 1988, Lokanath et al. 2005). The 150 kDa band nicely corresponds to the expected mass of a tetramer. The 187 and 224 kDa bands could represent penta- or hexamers. The identity of the 200 kDa band is not clear.

Characterization of Arabidopsis AtHDH1 knock-down lines
Arabidopsis lines carrying T-DNA insertions in the gene encoding At4g20930 were ordered to investigate the physiological role of AtHDH1. Overall, six mutant lines are available, termed ΔHDH1-1 to ΔHDH1-6 (Figure 4). Exact positions of the insertions were determined by DNA sequencing for lines ΔHDH1-2, ΔHDH1-4 and ΔHDH1-6. Line ΔHDH1-4 carries an insertion 471 bp upstream of the
transcription initiation site, which is usually considered to be outside the promoter region in Arabidopsis (Kleinboelting et al. 2012, Shahmuradov et al. 2017). Insertions in ΔHDH1-1, ΔHDH1-5 are even further upstream (Figure 4). All three lines were discarded for further analyses because the insertions probably do not affect expression of the AtHDH1 gene. The T-DNA insertion of another line (ΔHDH1-3) could not be confirmed by the GABI-Kat consortium. For these reasons, all further experiments were carried out using lines ΔHDH1-2 (GK-911G06) and ΔHDH1-6 (GK-710H08). Line ΔHDH1-6 carries the insertion within the 5' UTR (9 bp upstream of the transcript initiation site) and Line ΔHDH1-2 within intron 6 (Figure 4). Both lines are homozygous with respect to the T-DNA insertion as confirmed by PCR (Supp. Fig. 7). HDH1 expression is reduced by around 50 % in the two mutant lines (Figure 5). We conclude that both lines represent knock-down lines. HDH1 knock-out lines possibly are not viable because disruption of the hdh1 gene causes an embryo-lethal defect.

ΔHDH1 knock-down lines are deficient in Valine and Isoleucine but not in Leucine breakdown.

Plants of the Arabidopsis lines ΔHDH1-2 and ΔHDH1-6 had no visible phenotypes under the conditions tested. Plants were cultivated at long-day conditions and short-day conditions. Even upon extended darkness for 7 days, no phenotypic differences were visible between Wild-type plants and the knock down mutants (Supp. Fig 8). Progress of chlorosis was similar in all lines. We conclude that reduction in AtHDH1 expression is not strong enough to cause a visible phenotype. Therefore, root toxicity assays were carried out to use a more sensitive experimental system for monitoring phenotypic effects of the T-DNA insertion line.

Wild-type plants and plants of the AtHDH1 knock-down lines ΔHDH1-2 and ΔHDH1-6 were cultivated under sterile conditions on agar plates for 4 days. Subsequently, plant cultivation was continued on
agar plates, which were supplemented with either 250 µM and 500 µM Valine or with 50 µM and 100 µM Leucine/Isoleucine. After 10 days of further cultivation, roots were scanned and root length was calculated using the AxioVision Microscope Software (Carl Zeiss, Oberkochen, Germany). Cultivation of plants in the presence of excess Valine and Isoleucine clearly increased root growth inhibition in the mutant with respect to wild-type plants (Figure 6). In contrast, root growth in the presence of Leucine did not cause a decrease in root growth in the mutant line compared to the wt-line. The observed root inhibition effects in the AtHDH1 knock-down line were of moderate intensity, which probably is caused by remaining AtHDH1 knock-down line were of moderate intensity, which probably is caused by remaining AtHDH1 expression (Figure 5). We conclude that AtHDH1 is involved in the breakdown of Valine and Isoleucine but not of Leucine.

**Figure 4.** Model of the gene encoding 3-Hydroxyisobutyrate Dehydrogenase from Arabidopsis thaliana. The promoter region is shown in light grey. The 5' and 3' UTR are shown in dark grey. Exons are indicated by black arrows and introns by a grey line. Available T-DNA insertion lines are indicated by triangles (ΔHDH1-1 – ΔHDH1-6 are Salk, SAIL or GABI-Kat lines). The exact positions of the T-DNA insertions were determined by sequence analysis for lines ΔHDH1-2, ΔHDH1-4 and ΔHDH1-6. All experiments of our study were carried out using lines ΔHDH1-2 and ΔHDH1-6 (insertions indicated by blue triangles. TIS: Transcription Initiation Site).
Figure 5. Relative AtDH1 expression in ΔHDH1-2 and ΔHDH1-6 knock-down lines. Relative expression was determined by RT-qPCR (n = 3). Actin was used for normalization.
Plants can generate ATP from ADP and P\textsubscript{i} by various ways. In the light, ATP mainly comes from photophosphorylation taking place in the chloroplasts. At the same time, like in heterotrophic eukaryotes, ATP is generated in the mitochondria by oxidative phosphorylation. The latter process is the main ATP generating process in plants in the absence of light (e.g. at night) or in non-green tissue (e.g. roots). For oxidative phosphorylation, plants have to fuel the electron transport chain with electrons, which mainly come from the oxidation of carbohydrates via glycolysis and the citric acid cycle. However, under special abiotic as well as biotic stress conditions, carbohydrates can become limiting and oxidation of other compounds becomes important for providing electrons to the mitochondrial electron transport chain. The oxidation of BCAAs was found to be very important for respiration of plant cells under carbon starvation conditions (Ishizaki et al. 2005, Ishizaki et al. 2006, Araujo et al. 2010). In BCAA catabolism, BCKDH generates NADH. We here report identification of a second NADH-generating enzyme of the BCAA degradation pathway, AtHDH1. The suggested role of the enzyme in BCAA catabolism is summarized in Figure 7. Besides BCKDH and AtHDH1 the existence of a third enzyme, Methylmalonate-semialdehyde dehydrogenase, is predicted to produce NADH. The formed NADH can transfer electrons to the NADH dehydrogenase complex (complex I) or to one of the alternative NAD(P)H dehydrogenases forming part of the respiratory chain in plants (Rasmusson et al. 2004). Furthermore, IVDH, another enzyme of the BCAA catabolic pathway, contributes electrons to the respiratory chain. However, this enzyme directly transfers electrons via the ETF/ETFQO system to Ubiquinone. Finally, BCAA catabolism leads to the formation of Acetyl-CoA, which is a substrate of the citric acid cycle. Complete oxidation of the acetyl group generates another 3 NADH and one FADH\textsubscript{2}. 
In summary, BCAA degradation can greatly contribute to oxidative phosphorylation at carbohydrate limitation. Nevertheless, Leucine, Isoleucine and Valine catabolism has not been much studied in plants so far. We here report the characterization of one of the so-far unknown enzymes of the BCAA degradation pathway in plants, AtHDH1 (At4g20930). A second putative HDH gene has been recently predicted, At4g29120 (Hildebrandt et al. 2015). The amino acid sequence identity between...

Figure 7: Branched-chain amino acid catabolism in plants (from Hildebrandt et al. 2015, modified). Enzymes are underlayed by gray circles (BCAT: Branched-chain amino acid transaminase, BCKDH: Branched-chain α-keto acid dehydrogenase complex, IVDH: Isovaleryl-CoA-dehydrogenase, MCCase: Methylcrotonyl-CoA carboxylase, ECH: Enoyl-CoA hydratase, HL: Hydroxymethylglutaryl-CoA lyase, HCDH: 3-Hydroxyacyl-CoA dehydrogenase, KCT: 3-Ketoacyl-CoA thiolase, HADH: Hydroxacyl-CoA hydrolase, HDH: 3-Hydroxisobutyrate dehydrogenase, MMSDH: Methylmalonate semialdehyde dehydrogenase, ACOX: Acyl-CoA oxidase, ACAT: Acetyl-CoA acetyltransferase). Some key substrates, co-substrates and products are underlayed in blue (Leu: Leucine, Ile: Isoleucine, Val: Valine). The two enzymatic steps catalyzed by HDH are underlayed in green.
At4g29120 and At4g20930 is 32%. We also recombinantly expressed At4g29120 in E. coli (Supp. Fig. 6). However, the purified protein did not exhibit 3-hydroxyisobutyrate dehydrogenase activity (Supp. Fig. 6). We cannot exclude that the overexpressed protein was inactive due to misfolding or aggregation. However, our current results rather indicate that HDH is encoded by a single-copy locus in Arabidopsis. In contrast HDH isoforms have been described for mammalian mitochondria (Loupatty et al. 2006).

AtHDH1 was shown to be a highly active 3-Hydroxyisobutyrate dehydrogenase. In addition, 3-Hydroxypropionate and Methyl-3-hydroxy-2-methylpropionate are also substrates for AtHDH1. No activity could be measured using Glycerate or Serine as substrates. This is in contrast to HDH from mammalian mitochondria. The enzyme from rat is active with a series of 3-hydroxyacid substrates including S- and R-Hydroxyisobutyrate, L-Glycerate, and L-Serine (Haws et al. 1996). In our study, AtHDH1 shows high specificity for S-enantiomers like it was reported for the majority of HDHs from microorganisms and mammals (Hasegawa 1981). AgNO₃ is a strong inhibitor of AtHDH1 activity (data not shown). This points to the presence of sulfhydryl groups close to the 3-hydroxyisobutyrate or NAD⁺ binding sites. The optimal pH for AtHDH1 is around 8.5. Also HDH from different mammals show a basic pH optimum (Robinson and Coon 1957, Tasi et al. 2013). The temperature optimum of AtHDH1 is around 40 °C and the enzyme shows a wide activity range between 20 °C and 50 °C, which reflects the conditions of natural Arabidopsis habitats. The AtHDH1 knock-down plants used in our study had no visible phenotype under the conditions tested, even under extended darkness, which most likely is due to remaining AtHDH1 expression. However, more sensitive root toxicity assays revealed a role of AtHDH1 in branched chain amino acid catabolism.

The 3-hydroxyisobutyrate dehydrogenase from Thermus thermophilus has been crystallized and shown to be a homotetrameric enzyme (Lokanath et al. 2005). Similarly, AtHDH1 might form homooligomeric complexes. Activity signals of the recombinantly expressed protein could be detected in the molecular mass range from 150 to 225 kDa upon blue native PAGE (Figure 3). This might indicate that AtHDH1 forms tetra- to hexahomomeric complexes. However, results have to be taken with caution because artificial aggregations of AtHDH1 overexpressed in E. coli cannot be excluded.

According to currently available information, the first four enzymatic steps of Valine, Isoleucine and Leucine degradation are carried out by the same set of enzymes in plants (Hildebrandt et al. 2015), with an Enoyl-CoA hydratase representing the last enzyme acting in all three degradation pathways (Figure 7). In Valine degradation, Hydroxyisobutyrate is produced from Hydroxyisobutyryl-CoA. This step distinguishes the Valine catabolic pathway from Leucine and Isoleucine degradation. In Leucine as well as Isoleucine degradation, no Coenzyme A liberation takes place at this step. AtHDH1 converts 3-Hydroxyisobutyrate to Methylmalonate semialdehyde. Root toxicity experiments with mutant lines show the involvement of AtHDH1 in Valine degradation. Cultivation of AtHDH1 knock down mutants in the presence of excess of Valine inhibits root growth stronger than in wild type plants (Figure 6). In the next step, Coenzyme A is again attached. This step is carried out by the Methylmalonate semialdehyde dehydrogenase. The mammalian enzyme is unique among known Aldehyde dehydrogenases because it is Coenzyme A-dependent (Kedishvili et al. 1992). It is assumed that Propionyl-CoA is produced in Valine as well as in Isoleucine degradation (Figure 7). Finally, it has been suggested that at least five enzymes are required for converting Propionyl-CoA into Acetyl-CoA (Hildebrandt et al. 2015). We present evidence that AtHDH1 is among these five enzymes. AtHDH1 is capable converting 3-Hydroxypropionate into Malonate semialdehyde (Table 2). Indeed, root toxicity
experiments using the AtHD1 knock-down mutant revealed that growth inhibition not only increased upon cultivation of plants in the presence of Valine, but also Isoleucine (Figure 6). This double role of HDH has also been reported for 3-Hydroxyisobutyrate dehydrogenase from microorganisms (Chowdhury et al. 1996, Yao et al. 2010). In contrast, according to available experimental data, HDH seems to be only involved in Valine degradation in animals. Further investigation of BCCA catabolism in plants will be required to better understand the plant-specific features and the physiological function of BCAA catabolism in the context of photoautotrophic life.
Material and Methods

Expression and Purification of recombinant AtHDH1

The nucleotide sequence encoding Arabidopsis AtHDH1 was amplified from a Gateway clone (G83598; Arabidopsis Biological Resource Center Ohio State University). The first 84 bp of the ORF were excluded because they encode the N-terminal presequence as predicted by Mitofates (http://mitf.cbrc.jp/MitoFates/cgi-bin/top.cgi, see Supp. Fig. 3). The forward PCR-primer contained the sequence CACC at the 5´-end to enable directional TOPO® cloning. The following primers were used: AtHDH1_FWD_w/o_Mito: 5´-CACCTCTTCGTCTCAAAATTCAA-3´ and AtHDH1_RV_w/o_Mito: 5´-TCAGACCTCATCTTCCCATTG-3´. The PCR reaction was carried out using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, USA) to create blunt end PCR products. The following conditions were applied: initial denaturation at 95 °C for 5 min, followed by 37 cycles of denaturation at 95 °C for 30 s, annealing at 67.5 °C for 30 s, elongation at 72 °C for 1:30 min, and a final elongation step at 72 °C for 5 min. The obtained DNA fragment was cloned into the pENTR/D-TOPO vector using the pENTR™ Directional TOPO® Cloning Kit (Thermo Fisher Scientific, Waltham, USA). The resulting plasmid was called pENTR-D-TOPO-AtHDH1-w/o-28-AA. After transformation of the plasmid in One Shot® chemically competent E. coli cells (contained in the pENTR™ Directional TOPO® Cloning Kit) and overnight culturing on LB-agar plates supplemented with 50 µg/ml kanamycin, single colonies were picked and propagated over night at 37 °C in LB medium at 170 rpm. Afterwards, the plasmid DNA was isolated using the GeneJet Plasmid Miniprep Kit (Thermofisher Scientific, Waltham, USA). The plasmid was sequenced by SEQLAB (SEQLAB Sequence Laboratories, Göttingen, Germany). Afterwards, the LR recombination reaction using the Gateway™ LR Clonase™ II Enzyme Mix (Thermo Fisher Scientific, Waltham, USA) was performed to clone AtHDH1 into the Gateway® pDEST™17 vector so that AtHDH1 can be expressed in frame with a N-terminal 6x-His-tag. The resulting expression plasmid was designated pDest17-AtHDH1-w/o-28-AA. Next, the plasmid was transformed into NEB S-alpha (New England Biolabs, Ipswich, USA) competent cells and grown overnight on LB-agar plates supplemented with 50 µg/ml carbenicillin. Single colonies were picked and grown in LB medium at 37 °C and shaking at 170 rpm in the presence of 50 µg/ml carbenicillin. The correct sequence of plasmids was checked by sequencing (SEQLAB Sequence Laboratories, Göttingen, Germany).

For AtHDH1 expression, pDest17-AtHDH1-w/o-28-AA was transformed in BL21-AI™ One Shot® Chemically Competent E. coli cells (Thermo Fisher Scientific, Waltham, USA). In these cells, the T7-RNA polymerase gene is under control of the araBAD promoter. Transformed cells were grown in LB medium supplemented with 50 µg/ml carbenicillin at 37 °C until the OD₆₀₀ reaches 0.6–1.0. AtHDH1 expression was induced using 0.05 % L-arabinose. Starting from this time point, bacteria were transferred to 16 °C for 24 h in order to decrease the risk of inclusion body formation. Bacteria were lysed according to the manufacturer’s instructions using BugBuster® Master Mix (Merck Millipore, Darmstadt, Germany). The soluble fraction was used for affinity purification using immobilized metal ion chromatography on Ni-NTA Agarose (Qiagen, Hilden, Germany). 1 M NaCl and 20 mM Imidazol as final concentration were added to the supernatant to prevent unspecific binding of non His-tagged proteins. The beads were equilibrated and washed with 33 mM NaH₂PO₄, 2 M NaCl and 53 mM Imidazol. The protein was eluted using 50 mM NaH₂PO₄, 300 mM NaCl and 400 mM Imidazol. De-salting was achieved by using Microcon-30kDa centrifugal filters (Merck Millipore, Darmstadt, Germany).

After 10 min centrifugation at 14.000 g, 200 µl of 20 mM K₂HPO₄, pH 8 was added and again centri-
fuged for 10 min at 14.000 g. The freshly desalted protein was immediately used to determine $K_M$ and $K_{cat}$ or 20% glycerol was added for protein storage at -20 °C for short term.

**Enzyme activity assays and substrate screening**

The standard 3-Hydroxyisobutyrate Dehydrogenase assay took place in a buffer composed of 250 mM Tris-HCl pH 8.5, 5 mM NAD$^+$, ~200 ng AtHDH1 (at least from a minimum of three different enzyme batches from elution fraction 2) and 5 mM 3-HIB. Elution fraction 2 was used in order to have a very pure fraction for experiments. Assays were carried out in a total Volume of 300 µl in the wells of a 96-well plate. The production of NADH ($\varepsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) was measured at 340 nm using an Epoch Microplate Spectrophotometer (Biotek, Winooski, USA). Enzyme assays were carried out at 25 °C. Kinetic values were corrected by values obtained in parallel control experiments (assays without substrate or added protein). Protein quantification was carried out using Pierce™ Coomassie (Bradford) Protein Assay (Thermo Fisher Scientific, Waltham, USA).

The $K_m$ and $V_{max}$ values were determined at pH 8.5 varying the concentration of the substrate while keeping the concentrations of the other components constant. At least three different enzyme batches were used and kinetic constants were calculated using nonlinear regression (Graph Pad Prism 7, GraphPad Software, Inc. La Jolla, CA, USA).

Substrate screening was carried out in a buffer containing 250 mM Tris-HCl pH 8.5, 5 mM NAD$^+$, different amounts of recombinantly expressed AtHDH1 and 10 mM substrate. The substrates tested were 3-Hydroxyisobutyrate, 3-Hydroxypropionate, Methyl-3-Hydroxy-2-Methylpropionate, Glycerate, Serine, β-Hydroxypyruvate, Threonine, 2-Hydroxybutyrate, Malate, Glycolate, β-Hydroxybutyrate, Lactate and 6-Phosphogluconate.

For determining the pH optimum of AtHDH1 a buffer mixture was used containing 250 mM Tris-HCl pH 7 – 9.5, 5 mM NAD$^+$ and different amounts of recombinantly expressed AtHDH1. The reaction was started with 5 mM 3-Hydroxyisobutyrate and took place a final volume of 300 µl.

The temperature optimum of AtHDH1 was tested using a buffer including 250 mM K$_2$HPO$_4 \times$KH$_2$PO$_4$, pH 7.5 (since the pH of a Tris-HCl buffer changes with different temperatures we decided to use a potassium phosphate buffer).

Cofactors were analyzed using the standard assay conditions. However, NAD$^+$ was replaced by either NADP$^+$ ($\varepsilon_{340} = 6.22 \text{ mM}^{-1} \text{ cm}^{-1}$), cytochrome c ($\varepsilon_{550} = 19 \text{ mM}^{-1} \text{ cm}^{-1}$) or FAD, phenazine methosulfate (PMS) and dichlorophenolindophenol (DCPIP) ($\varepsilon_{600} = 19.1 \text{ mM}^{-1} \text{ cm}^{-1}$).

**SDS-PAGE and Western blotting**

Protein concentration was determined using Pierce™ Coomassie (Bradford) Protein Assay (Thermo Fisher Scientific, Waltham, USA). SDS-PAGE was performed using pre-casted gels from BIO-RAD (BIO-RAD, Hercules, USA). Gel runs were performed according to the manufacturer’s instructions. After completion of the gel run the gel was either stained using by the Coomassie colloidal procedure (Neuhoff et al. 1988) or electroblotted onto a nitrocellulose membrane for immunoblotting. Western blots were incubated with an HRP-conjugated 6*His, His-Tag Mouse Monoclonal antibody (Proteintech Europe, Manchester, UK) and developed using nickel chloride combined with DAB according to Coventry et al. 1995. The Amersham ECL Rainbow Marker - High Range was used (GE Healthcare UK Limited Amersham Place Little Chalfont, Buckinghamshire, UK).

**Protein identification by mass spectrometry**
For identification, protein bands were cut out from polyacrylamide gels and included proteins were fragmented into peptides by trypsin treatment as described previously (Klodmann et al. 2011). Peptide mixtures were purified, peptides separated by liquid chromatography and analyzed by tandem MS using a micrOTOF Q-II mass spectrometer (Bruker, Bremen/Germany) as outlined before (Klodmann et al. 2011).

**Blue native (BN) PAGE and in-gel activity staining**

Blue native PAGE was performed employing a pre-casted 4–16 % Bis-Tris Native Gel (Thermo Fisher Scientific, Waltham, USA). The BN cathode buffer (50 mM tricine, 15 mM BisTris, 0.02 % (w/v) Coomassie G250, pH 7.0) and the BN anode buffer (50 mM BisTris, pH 7.0) were used. The Coomassie containing cathode buffer was replaced after half of the run by a cathode buffer without Coomassie in order to strongly reduce excess Coomassie-blue. The gel run was carried out according to the manufacturer’s instructions at 4°C. After the gel run was completed, *in-gel* activity assays were carried out by incubating gels in the dark at room temperature with 20 ml staining solution containing 250 mM Tris-HCl pH 8.5, 0.4 mg/ml nitro blue tetrazolium (NBT), 0.2 mM PMS, 2 mM NAD⁺ and 5 mM 3-HIB. As native marker the Amersham High Molecular Weight Calibration Kit for native electrophoresis was used (GE Healthcare UK Limited Amersham Place Little Chalfont, Buckinghamshire, UK).

**Mutant screening**

Seeds of T-DNA insertion lines ΔHDH1-1 (SALK_009001C), ΔHDH1-2 (GK-911G06), ΔHDH1-4 (SAIL_31_B02), and ΔHDH1-5 (SAIL_657_E01) were obtained from the Nottingham Arabidopsis Stock Center (http://arabidopsis.info/). Lines ΔHDH1-6 (GK-710H08) and ΔHDH1-3 (GK-350A08) were directly ordered from GABI-Kat (https://www.gabi-kat.de/). Genomic DNA was isolated according to Edwards et al. 1991. Plants homozygous with respect to the T-DNA insertion were isolated by multiplex PCRs using following primers. ΔHDH1-2_Fw: 5´-TGGAGAAAATGATAAGACCTGC-3´, ΔHDH1-2_Rv: 5´-TGTCTGAAAACACAAGAGAAGTC-3´, T-DNA Primer: GABI_Kat_o8474 5´-ATAATAACGCTGCGGACAT-3´. For ΔHDH1-6 following primers were used. ΔHDH1-6_Fw: 5´-AAATTATTTTACATGGTTTACACGG-3´, ΔHDH1-6_Rv: 5´-CATTTTTACATAACATCAGGTTCT-3´, T-DNA Primer: GABI_Kat_o8474 5´-ATAATAACGCTGCGGACAT-3´. Annealing temperatures were calculated using the Tm Calculator www.thermoscientific.com/pcrwebtools (Thermo Fisher Scientific, Waltham, USA). In order to determine the exact T-DNA insertion site PCR products were sequenced by Seqlab (Göttingen, Germany).

**Gene expression analyses**

To determine gene expression in the lines ΔHDH1-2 and ΔHDH1-6 lines, a qPCR analysis was carried out according to Heimann et al. 2013. First, total RNA was isolated from 100 mg of leaves using the GeneJET Plant RNA Purification Kit (Thermo Fisher Scientific, Waltham, USA). Subsequently, RNA was converted into first strand cDNA. cDNA synthesis was performed with approximately 1 μg of total RNA and 50 pmol of random nonamer primer. Reactions were incubated for 5 min at 70°C and cooled down on ice before adding 200 units of Moloney murine leukemia virus reverse transcriptase.
Quantitative PCR was performed on an ABI PRISM 7300 sequence detection system (Thermo Fisher Scientific, Waltham, USA) using SYBR Green fluorescence (Platinum SYBR Green QPCR Mix Thermo Fisher Scientific, Waltham, USA). Following primers were used: \( \Delta \text{HDH1}_q\text{PCR_FW }_{2} \ 5'\text{-CTAGTGCCGTTGGAGCAGTGA-3'} \) and \( \Delta \text{HDH1}_q\text{PCR_RV }_{2} \ 5'\text{-TCAGCTGAGGCTGCTGAGGT-3'} \). Amplification conditions were 2 min of initial denaturation at 95°C, followed by 40 cycles of 15 s at 95°C, 1 min at 60°C. Afterwards, a melting curve was recorded. General reaction conditions were 3 mM MgCl\(_2\) and 200 nM of each oligonucleotide. Sizes of the amplified molecules were confirmed by gel electrophoresis. At least three biological replicates were carried out for each measurement.

**Cultivation of plants on agar plates and Root toxicity assays**

Seeds of \( \Delta \text{HDH1-2}, \Delta \text{HDH1-6} \) and Col-0 were surface sterilized and sown on half MS agar plates. After two days at 4°C plates were transferred to a climate chamber (16/8 h light/dark) for continuation of plant cultivation at 22 °C and 85 μmol s\(^{-1}\) m\(^{-2}\) light. After four days in the climate chamber, 10 seedlings with approximately the same size and the same root length were transferred to half MS agar plates which were supplemented with 250 μM and 500 μM Valine or 50 μM and 100 μM Leucine/Isoleucine. Control plates did not contain any amino acids. After 10 days, plates were scanned and the root lengths were determined with the AxioVision Microscope Software (Carl Zeiss, Oberkochen, Germany). Results are derived from three different biological experiments with each 20 plants (two plates) per treatment (in total, 60 plantlets which were measured per treatment and concentration).

**Supplemental Material:**

**Supp. Figure 1:** Alignment of At4g20930 and human 3-Hydroxyisobutyrate Dehydrogenase (EAL24214) using ClustalΩ.

**Supp. Figure 2:** HDH1 has been identified in a mitochondrial fraction of Arabidopsis thaliana.

**Supp. Figure 3:** HDH1 has a predicted presequence comprising 28 amino acids.

**Supp. Figure 4:** Sequence coverage of HDH1 from Arabidopsis by peptides identified by MS.

**Supp. Figure 5:** The reaction mechanism catalyzed by AtHDH1 is a sequential bi-bi mechanism.

**Supp. Figure 6:** Affinity purification of recombinant At4g29120 and activity with 3-Hydroxyisobutyrate as substrate.

**Supp. Figure 7:** Identification of Arabidopsis lines homozygous for T-DNA insertions in At4g20930.

**Supp. Figure 8:** HDH1 knock-down mutants under extended darkness conditions.
Supp. Table 1: Proteins identified in the 75 kDa, 37 kDa and 30 kDa bands of the SDS gel shown in Fig. 1 by mass spectrometry using SwissProt Database.

Acknowledgments: We thank Dr. Stefanie Fromm for support with respect to the Gateway cloning system, Marianne Langer and Christa Ruppelt for expert technical assistance, Dr. Jennifer Senkler for protein identifications by mass spectrometry and Dr. Tatjana Hildebrandt for critically reading the manuscript. Gateway clones were obtained from the Arabidopsis Biological Resource Center (ABRC), Joe Ecker/SALK.
Table 1. Identification of overexpressed AtHDH1 (Fig. 1, protein band visible in lane E1) by mass spectrometry.

<table>
<thead>
<tr>
<th>Accession</th>
<th>Name</th>
<th>MW [kDa]</th>
<th>Mascot Score</th>
<th>Peptides</th>
<th>SC [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>At4g20930</td>
<td>AtHDH1 (currently annotated as “6-phosphogluconate dehydrogenase family protein”)</td>
<td>37.4</td>
<td>1296</td>
<td>31</td>
<td>54.9</td>
</tr>
</tbody>
</table>

*a* accession number, *b* name of identified protein, *c* calculated molecular mass, *d* probability score for the protein identification based on the MS data and MASCOT search, *e* number of unique identified peptides, *f* sequence coverage of the protein by identified peptides (see Supp. Figure 4)
Table 2. Enzymatic activities of recombinant AtHDH1 using different substrates and NAD$^+$ as electron acceptor. Activity was measured using the standard conditions described in the material and methods section. The activity of the substrate S-3-Hydroxyisobutyrate was defined to be 100%.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>S-3-Hydroxyisobutyrate</td>
<td>100% S-Enantiomer</td>
</tr>
<tr>
<td></td>
<td>2% R-Enantiomer</td>
</tr>
<tr>
<td>3-Hydroxypropionate</td>
<td>13%</td>
</tr>
<tr>
<td>Methyl-3-Hydroxy-2-Methylpropionate</td>
<td>1.2% S-Enantiomer</td>
</tr>
<tr>
<td></td>
<td>0% R-Enantiomer</td>
</tr>
<tr>
<td>Glycerate</td>
<td>0% S-Enantiomer</td>
</tr>
<tr>
<td></td>
<td>0% R-Enantiomer</td>
</tr>
<tr>
<td>Serine</td>
<td>0% S-Enantiomer</td>
</tr>
<tr>
<td></td>
<td>0% R-Enantiomer</td>
</tr>
<tr>
<td>Beta-Hydroxypyruvate</td>
<td>0%</td>
</tr>
<tr>
<td>Threonine</td>
<td>0% S-Enantiomer</td>
</tr>
<tr>
<td></td>
<td>0% R-Enantiomer</td>
</tr>
<tr>
<td>2-Hydroxybutyrate</td>
<td>0% S-Enantiomer</td>
</tr>
<tr>
<td></td>
<td>0% R-Enantiomer</td>
</tr>
<tr>
<td>Malate</td>
<td>0% S-Enantiomer</td>
</tr>
<tr>
<td></td>
<td>0% R-Enantiomer</td>
</tr>
<tr>
<td>Glycolate</td>
<td>0%</td>
</tr>
<tr>
<td>Beta-Hydroxybutyrate</td>
<td>0% S-Enantiomer</td>
</tr>
<tr>
<td></td>
<td>0% R-Enantiomer</td>
</tr>
<tr>
<td>Lactate</td>
<td>0% S-Enantiomer</td>
</tr>
<tr>
<td></td>
<td>0% R-Enantiomer</td>
</tr>
</tbody>
</table>
Table 3. Kinetic parameters of recombinant 3-Hydroxyisobutyrate Dehydrogenase from *Arabidopsis thaliana* using 3-Hydroxyisobutyrate (3-HIB) and Nicotinamide adenine dinucleotide (NAD$^+$) as substrates. Kinetic data were fitted by using nonlinear regression analysis. The values represent the mean ± SE of three independent enzyme preparations.

<table>
<thead>
<tr>
<th></th>
<th>3-HIB</th>
<th>NAD$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$K_m$ [mM]</td>
<td>0.687 ± 0.198</td>
<td>0.350 ± 0.152</td>
</tr>
<tr>
<td>$K_{cat}$ [s$^{-1}$]</td>
<td>3.430 ± 0.279</td>
<td>2.652 ± 0.264</td>
</tr>
<tr>
<td>$K_{cat}/K_m$ [s$^{-1}$ mM$^{-1}$]</td>
<td>4.993</td>
<td>7.571</td>
</tr>
</tbody>
</table>
Figure 1. Expression and affinity purification of recombinant AtHDH1. Part A: Progression of AtHDH1 overexpression in E. coli and subsequent steps for AtHDH1 purification. Protein fractions were resolved by SDS PAGE. The gel was Coomassie-stained. M: marker (5 µl). A: protein fraction after induction of the AtHDH1 gene by arabinose for 24 h at 16 °C (5 µg). NB: soluble protein fraction not bound to Ni-NTA agarose beads (5 µg). W1: protein fraction of the first washing step (15 µl). E1: first protein fraction eluted from the Ni-NTA agarose beads (recombinant AtHDH1). E2: second protein fraction eluted from the Ni-NTA agarose beads (recombinant AtHDH1). Part B: Corresponding Western Blot. Recombinant AtHDH1 was detected using a HRP-coupled His-antibody.

Figure 2. pH and temperature optimum of overexpressed 3-Hydroxyisobutyrate Dehydrogenase (AtHDH1) from Arabidopsis thaliana. Part A: pH optimum. Activity of AtHDH1 was measured in a buffer containing 250 mM Tris-HCl pH 7.0 – 9.5, 5 mM NAD+ and 5 mM 3-HIB. Part B: Temperature optimum of AtHDH1. Activity of AtHDH1 was measured at 20 to 65°C in a buffer containing 250 mM K2HPO4•KH2PO4 pH 7.5, 5 mM NAD+, 10 mM 3-HIB.

Figure 3. Native molecular mass of 3-Hydroxyisobutyrate Dehydrogenase. A protein fraction containing recombinant AtHDH1 was separated by blue native (BN) PAGE. The gel was stained for AtHDH1 activity. Proteins of a molecular mass standard (M) were separated in parallel (the masses of the standard proteins are given to the left in kDa). Apparent native molecular masses of HDH1 are indicated to the right of the activity stain.

Figure 4. Model of the gene encoding 3-Hydroxyisobutyrate Dehydrogenase from Arabidopsis thaliana. The promoter region is shown in light grey. The 5’ and 3’UTR are shown in dark grey. Exons are indicated by black arrows and Introns by a grey line. Available T-DNA insertion lines are indicated by triangles (ΔHDH1-1 – ΔHDH1-6 are Salk, Sail or GABI-Kat lines). The exact positions of the T-DNA insertions were determined by sequence analyses for lines ΔHDH1-2, ΔHDH1-4 and ΔHDH1-6. All experiments of our study were carried out using lines ΔHDH1-2 and ΔHDH1-6 (insertions indicated by blue triangles. TIS: Transcription Initiation Site).

Figure 5. Relative AtHDH1 expression in ΔHDH1-2 and ΔHDH1-6 knock-down lines. Relative expression was determined by RT-qPCR (n = 3). Actin was used for normalization.

Figure 6. AtHDH1-2 shows impaired root growth in the presence of Valine and Isoleucine. Arabidopsis lines (wt, AtHDH1-2, AtHDH1-6) were grown on half MS plates for four days before being transferred to half MS plates supplemented with Valine (250 µM and 500 µM), Isoleucine (50 µM and 100 µM), or Leucine (50 µM and 100 µM). Inhibition in % is shown in comparison to the plants of each line grown on control plates. Results are derived from three different experiments with 20 plants (two plates) per treatment. Shown are the mean values ± SEM. Asterisks show the significant differences compared to the wild type according to t-test (p<0.05). Images to the left of the graphs exemplarily show the effects of the treatments on root growths.

Figure 7: Branched-chain amino acid catabolism in plants (from Hildebrandt et al. 2015, modified). Enzymes are underlayed by gray circles (BCAT: Branched-chain amino acid transaminase, BCKDH: Branched-chain a-keto acid dehydrogenase complex, IVDH: Isovaleryl-CoA-dehydrogenase, MCCase: Methylcrotonyl-CoA carboxylase, ECH: Enoyl-CoA hydratase, HL: Hydroxymethylglutaryl-CoA lyase, HCDH: 3-Hydroxyacetyl-CoA dehydrogenase, KCT: 3-Ketoacyl-CoA thiolase, HADH: Hydroxacyl-CoA hydrolase, HDH: 3-Hydroxyisobutyrate dehydrogenase, MMSDH: Methylmalonate-semialdehyde dehydrogenase, ACOX: Acyl-CoA oxidase, ACAT: Acetyl-CoA acetyltransferase). Some key substrates, co-substrates and products are underlayed in blue (Leu: Leucine, Ile: Isoleucine, Val: Valine). The two enzymatic steps catalyzed by HDH are underlayed in green.


Hasegawa J (1981) Distribution in Organisms and Stereospecificity of ß -Hydroxyisobutyrate Dehydrogenase. Agricultural and
Biological Chemistry 45: 2899-2901.


