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1 The gene annotated by the locus tag At3g08860 encodes a β-alanine/L-alanine aminotransferase
2 in *Arabidopsis thaliana*

3

4 Runining Title: At3g08860 encodes a β-alanine/L-alanine aminotransferase

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25 **Abstract**

26 The aminotransferase gene family in the model plant *Arabidopsis thaliana* consists of 44 genes
27 some of which remain uncharacterized. This study elucidates the function of an uninvestigated
28 aminotransferase annotated by the locus tag At3g08860. The cDNA was shown to functionally
29 complement two *E. coli* mutants auxotrophic for the amino acids β-alanine (non-proteogenic)
30 and L-alanine (proteogenic). The elucidation of At3g08860 activity has the potential to facilitate
31 experiments for the optimization of plant lines involved in nitrogen utilization efficiency,
32 response to hypoxia, osmo-protection, vitamin B5 and coenzyme A metabolism.

33

34 **Keywords**: At3g08860, β-alanine aminotransferase, L-alanine aminotransferase,
35 Aminotransferase, Transaminase

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52 **INTRODUCTION**

53 Aminotransferases or transaminases (EC 2.6.1.X) are pyridoxal-5'-phosphate (PLP) dependent
54 enzymes that catalyze reversible reactions between amino acids and alpha keto acids by
55 transferring an amine group from a donor to an acceptor. These enzymes function via a bi-
56 molecular double displacement ping-pong mechanism where an amino acid usually serves as the
57 amino donor and a α-keto acid serves as the amino acceptor (Nelson and Cox, 2000).
58 Aminotransferases are ubiquitous in the three kingdoms and life and are involved in a variety of
59 metabolic pathways including amino acid metabolism, nitrogen assimilation, gluconeogenesis,
60 and responses to a number of biotic/abiotic stresses, among other pathways (de Sousa and Sodek,
61 2003; Liepman and Olsen, 2004; Rocha et al., 2010; McAllister et al., 2013). The genome of the
62 model plant *Arabidopsis thaliana* contains 44 annotated genes as part of the aminotransferase
63 gene family. In this family, 8 of the 44 genes are annotated as putative alanine aminotransferases.
64 The loci tags are At2g13360, At4g39660, At2g38400, At1g23310, At1g70580, At1g17290,
65 At1g72330 and At3g08860 (Liepman and Olsen, 2004; Niessen et al., 2012). In plants, the
66 biosynthesis of non-proteogenic amino acid β-alanine can be anabolized from four different
67 precursors: (1) the polyamines spermine and spermidine, (2) the nucleotide base uracil, (3)
68 propionate and (4) L-aspartate. However, only the propionate pathway involves a β-alanine
69 aminotransferase (Fig 1A). In contrast, L-alanine is synthesized by the transamination of
70 pyruvate, where L-glutamate serve as the amino donor. This reaction is catalyzed by the enzyme
71 alanine aminotransferase (Fig 1B).

72 In plants, alanine aminotransferases are important because they are involved in a number of
73 important pathways. For example, it was identified in *A. thaliana* and *Oryza sativa* that two
74 mitochondrial L-alanine/glyoxylate aminotransferases link glycoxylate oxidation to glycine
75 formation (Niessen et al., 2012). The phenotypes of various alanine aminotransferase
76 overexpressed in the *A. thaliana* Col-0 background and in the alanine aminotransferase
77 (At1g17290 and At1g72330) knockouts suggests that nitrogen use efficiency (NUE) could be
78 improved in plants by the overexpression of alanine aminotransferases (McAllister and Good,
79 2015). Gene regulation studies of alanine aminotransferase in response to low-oxygen stress,
80 light and nitrogen has been studied in many plants and it was shown that hypoxia induced the
81 expression of two distinct alanine aminotransferase genes (At1g17290 and At1g72330) in *A.*
82 *thaliana* (Miyashita et al., 2007). The function of the gene product from the locus tag At3g08860

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83 has not been experimentally elucidated. Here we present data to show that the gene product of
84 one of the putative alanine aminotransferase genes annotated by the locus tag At3g08860
85 encodes a β-alanine/L-alanine aminotransferase using an *in vivo* functional complementation
86 experiment.

87 **Materials and Methods**

88 *Plant Growth and Conditions*

89 *A. thaliana* Col 7 from the Arabidopsis Biological Resource Center (ABRC) was grown on
90 Murashige and Skoog (MS) medium with a 16-hour light (light intensity was approximately
91 120 Em^{-2} s^{-1}) and an 8-hour dark period, with temperatures of 24ºC during the light period and
92 20ºmC during the dark period.

93

94 *RNA Isolation from Arabidopsis thaliana*

95 Total RNA was isolated from 7 day old Col 7 *A. thaliana* seedlings grown on MS medium using
96 TriZol reagent (Life Technologies). One hundred milligrams of seedlings was ground in liquid
97 nitrogen and homogenized in 1 mL of TriZol, followed by incubation at room temperature for
98 two minutes. Total RNA was precipitated using 1 mL 100% (v/v) isopropanol. The RNA pellet
99 was washed three times with 1 mL 75% (v/v) ethanol. The air-dried RNA pellet was re-
100 suspended in 30 µL of Diethyl Pyro carbonate (DEPC)-treated water and quantified using a
101 NanoDrop spectrophotometer.

102 *cDNA Synthesis*

103 The Reverse Transcription System Kit (Promega) was used to synthesize a cDNA library
104 following the manufacturer's protocol. One microgram of total RNA from 7 day old seedlings
105 was used to synthesize cDNA. The reaction contained 1µL oligo-dT primer, 1 µg total RNA,
106 1µ L of 10 mM dNTP mix, and DEPC-treated water up to 13 µL. The mixture was incubated
107 at 65ºC for 5 minutes followed by an incubation on ice for 5 minutes.

108

109 *Amplification and Cloning of At3g08860 cDNA*

110 The protein full length protein encoded by At3g08860 is predicted to be 481 amino acids in
111 length. The protein was predicted to be localized to the mitochondria using the TargetP and
112 SUBA subcellular localization prediction tools (Emanuelsson et al., 2007; Heazlewood et al.,

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113 2007). The first 93 nucleotides of the full length cDNA was predicted to encode the signal
114 sequence that denote localization to the mitochondria. As such, the first 93 nucleotides was
115 excluded when cloning the cDNA. The At3g08860 cDNA was amplified via PCR. The PCR (50
116 μL) reaction contained 1 μL (12 pm/ μL) each of the forward primer 5'-
117 CACCATGTCCTCCGTCCGCGAGACCGAGACCGAA-3' and the reverse primer 5'-
118 *CTGCAG*TCACATCTTGGACATGGCGTGATCCATCAC-3', 1 mM $MgSO_4$, 0.4 mM of each
119 of the four deoxynucleotide triphosphates, 2 μL of cDNA library and 1 unit of platinum *Pfx*
120 DNA polymerase (Invitrogen Corporation, Carlsbad, CA, USA). The following PCR conditions
121 were used: 1 cycle at 94ºC for 3 minutes, followed by 35 cycles of 94ºC for 30 seconds, 60ºC for
122 30 seconds, and 72ºC for 2 minutes and an indefinite soak of 4ºC. The cDNA amplicon was
123 ligated into the pET100/D-TOPO vector (Invitrogen Corporation, Carlsbad, CA, USA). The
124 fidelity of the of the pET100D::At3g08860 construct was confirmed via Sanger nucleotide
125 sequencing using T7 promoter (5'-TAATACGACTCACTATAGGG-3') and the T7 terminator
126 (5'-TATGCTAGTTATTGCTCAG-3') primers located on the pET100/D-TOPO plasmid
127 backbone.

128 *Plasmid for Functional Complementation*

129 The plasmid pBAD33::At3g08860 used for functional complementation experiments of the *E.*
130 *coli* mutants auxotrophic for β-alanine (*panD*) and L-alanine (*HYE032*) was constructed by sub-
131 cloning Xba1, Pst1 sites from the pET100D::At3g08860 construct into pBAD33 (Guzman et al.,
132 1995).

133 *Functional Complementation*

134 Auxotrophic *E. coli* mutants for L-alanine synthesis (*HYE032*) (avtA::GM, yfbQ::KM,
135 yfdZ::FRT, Ala^-) was obtained from Dr. Dr. Hiroshi Yoneyama from Tohoku University
136 (Yoneyama et al., 2011). β-alanine synthesis (*panD*) (F-, *Δ(araD-araB)567*, *ΔpanD748::kan*,
137 *ΔlacZ4787c* (::rrnB-3), λ^-, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*) was obtained from the Coli
138 Genetic Stock Center (CGSC #8404) (http://cgsc2.biology.yale.edu/) (Baba et al., 2006). The
139 auxotrophic strains were transformed with pBAD33 or pBAD33::At3g08860. Transformants
140 were selected on LB media supplemented with chloramphenicol (34 μg/mL). Colonies were then
141 replica-plated on M9 agar plates containing M9 salts (1X), 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and

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142 0.1% glycerol (w/v), +/- 0.2% glucose or arabinose, +/- β-alanine/L-alanine (10 μg/μL). In
143 testing of the *panD* (β-alanine auxotroph), uracil was also required (10 μg/μL).

144 **RESULTS AND DISCUSSION**

145 Literature mostly supports the idea of alanine accumulation during hypoxia (unknown reasons)
146 and an increase in alanine aminotransferase activity as plants return to normoxia (de Sousa and
147 Sodek, 2003). This is perhaps a mechanism for maintenance via an increase of the nitrogen
148 pool/skeletons, since the assimilation of inorganic nitrogen affects anaerobic tolerance
149 (Miyashita et al., 2007). During hypoxia/anoxia in plant tissues, fermentative products such as
150 acetaldehyde, ethanol, and lactate can accumulate where the regeneration of NAD^{+} by lactate
151 dehydrogenase and alcohol dehydrogenase enhances seedling survival (Ismond et al., 2003). In
152 fact, in *A. thaliana,* it was reported that pyruvate decarboxylase was specifically induced during
153 oxygen limitation, but not other stresses (Kürsteiner et al., 2003). An alternative way to counter
154 hypoxia would be through alanine aminotransferase, which could reduce the flux of carbon
155 through lactate (which is acidic and has the potential to regulate the cytoplasmic pH) and prevent
156 the buildup of toxic acetaldehyde (Ricoult et al., 2006). It appears that alanine fermentation
157 primarily functions to regulate the level of pyruvate. Pyruvate is not only a known activator of
158 the alternative oxidase (Vanlerberghe et al., 1999), but has also recently been shown to interfere
159 with the hypoxia-induced inhibition of respiration (Gupta et al., 2009; Zabalza et al., 2009).
160 Therefore, in order to control the rate of respiratory oxygen consumption when the oxygen
161 availability is low, it is important to prevent pyruvate accumulation. Alanine fermentation has
162 the potential to accomplish pyruvate accumulation with the additional advantage that alanine can
163 accumulate to high concentrations without the detrimental side effects that go along with the
164 lactate or ethanol fermentation pathways (Rocha et al., 2010). Alanine enters the propionate
165 pathway of β-alanine synthesis via the enzyme β-alanine aminotransferase [EC 2.6.1.18],
166 exchanging an amino group with malonate semialdehyde, and generating pyruvate and β-alanine
167 (Fig 1A).
168

169 Functional complementation assays showed that the plasmid harboring the *At3g08860* cDNA
170 was able to rescue both the L-alanine and β-alanine auxotrophs. The assay show that both *E. coli*
171 mutants were able to grow on β-alanine and L-alanine free media compared to the vector only

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172 control, which needed both amino acids to grow (Fig 2A and 2B). Interestingly, in the *panD*
173 background, the cDNA rescued growth under repressible conditions (plus glucose) and not under
174 inducible conditions (plus arabinose) (Fig 2A) whereas the opposite is true in the *HYE032*
175 background (Fig 2B). This result suggests that the enzyme is probably involved in β-alanine
176 metabolism and not L-alanine metabolism. The results of the assays demonstrated a definitive
177 preference of enzymatic activity towards the synthesis of β-alanine suggesting that At3g08860 is
178 maybe involved in osmo-protection, formation of vitamin B5 and coenzyme A, given the fact
179 that β-alanine is involved in these pathways.
180
181 Studies investigating genes (across a wide array of metabolic/cellular processes) have identified
182 the *At3g08860* locus as responsive to changes in light, which indirectly could affect
183 carbon/oxygen availability/concentration (Thum et al., 2008). Previous work suggested that the
184 protein is localized in either the mitochondria or the peroxisome (Niessen et al, 2012). The
185 visualization of cellular localization using GFP-tagged transcripts was unsuccessful, however
186 based on its sequence; this aminotransferase is believed to be localized in the mitochondria. This
187 suggests an involvement in photorespiration, particularly as it relates to glycine synthesis
188 (following glycolate oxidation to form glyoxylate) as detailed by Niessen et al., 2007, who also
189 demonstrated that alanine:glyoxylate aminotransferase activity was the only aminotransferase
190 activity detected within the mitochondria (Niessen et al., 2012). They also demonstrated that
191 alanine degradation resulted in an increase in CO_2 release following addition of alanine to
192 mitochondrial extract, implying that alanine degradation increased photorespiratory activity
193 (Niessen et al., 2007). Further suggesting the involvement of At3g08860 in photorespiration
194 based on the gene expression in hydathode tissue (Wenzel et al., 2008).
195

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200 **COMPETING INTERESTS**

201 The authors declare no competing or financial interests.

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288 **Fig 1**. (A) β-alanine synthesis from the propionate pathway. β-Alanine aminotransferase
289 catalyzes the synthesis of β-alanine and pyruvate using L-alanine as the amino donor and
290 malonate semialdehyde as the amino acceptor. (B) Pyruvate is transaminated by alanine
291 aminotransferase to synthesize L-alanine using L-glutamate as the amino donor. The EC
292 numbers of the enzymes shown in brackets correspond to the following enzymes: 6.2.1.17 =
293 propionyl-CoA synthetase, 1.3.99.3 = acyl-CoA dehydrogenase, 4.2.1.116 = 3-
294 hydroxypropionyl-CoA dehydratase, 3.2.1.4 = 3-hydroxyisobutyryl-CoA hydrolase, 1.1.1.59 =
295 3-hydroxypropionate dehydrogenase, 2.6.1.18 = β-alanine-pyruvate aminotransferase and 2.6.1.2
296 = alanine aminotransferase. The yellow arrows indicate the reactions catalyzed by β-alanine and
297 L-alanine aminotransferase.
298

299 **Fig 2.** Functional complementation assay. (A) Functional complementation of the *panD E. coli*
300 mutant, which is auxotrophic for β-alanine. (B) Functional complementation of the *E. coli*
301 *HYE032* mutant, which is auxotrophic for L-alanine. The plasmids used were pBAD33 and
302 pBAD33::At3g08860. Transformants harboring pBAD33 or pBAD33::At3g08860 were grown
303 to an OD of 1.0 measured at 600nm. The strains were serially diluted to 10^{-1}, 10^{-2}, 10^{-3}, and 10^{-4}
304 using 0.85% (w/v) saline. Five□µL was replica plated on M9 medium with or without β-alanine
305 or L-alanine supplemented with 0.2% (w/v) arabinose or glucose.

A

Propionate

Propionyl-CoA

Acrylyl-CoA

3-hydroxypropionyl-CoA

[6.2.1.17]

ATP + CoA

AMP + diphosphate

CoA

[1.3.99.3]

FAD

$FADH_2$

CoA

[4.2.1.116]

H_2O

CoA

OH

H_2O

CoA + H^+

[3.1.2.4]

3-hydroxy-propionate

HO

O^-

[1.1.1.59]

NAD$^+$

NADH+ H^+

Malonate semialdehyde

O^-

[2.6.1.18]

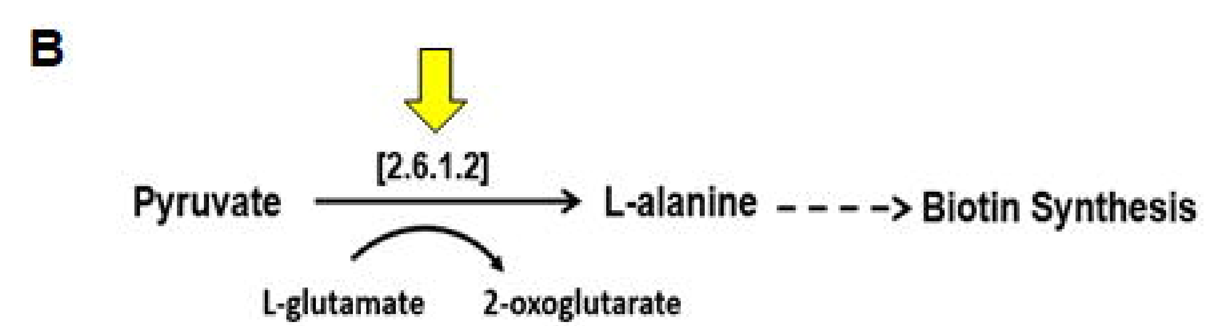
L-alanine

Pyruvate

β-alanine

NH_3^+

O^-



A

10-1 10-2 10-3 10-4 10-1 10-2 10-3 10-4 10-1 10-2 10-3 10-4

pBAD33

pBAD33::At3g08860

+ β-alanine

-β-alanine + glucose

-β-alanine + arabinose

panD

B

10-1 10-2 10-3 10-4 10-1 10-2 10-3 10-4 10-1 10-2 10-3 10-4

pBAD33

pBAD33::At3g08860

+ L-alanine

-L-alanine + glucose

-L-alanine + arabinose

HYE032