THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 278, No. 42, Issue of October 17, pp. 41552–41556, 2003

Printed in U.S.A.

A Novel γ-Hydroxybutyrate Dehydrogenase

IDENTIFICATION AND EXPRESSION OF AN *ARABIDOPSIS* cDNA AND POTENTIAL ROLE UNDER OXYGEN DEFICIENCY*

Received for publication, June 2, 2003
Published, JBC Papers in Press, July 25, 2003, DOI 10.1074/jbc.M305717200

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In plants, γ-aminobutyrate (GABA), a non-protein amino acid, accumulates rapidly in response to a variety of abiotic stresses such as oxygen deficiency. Under normoxia, GABA is catabolized to succinic semialdehyde and then to succinate with the latter reaction being catalyzed by succinic semialdehyde dehydrogenase (SSADH). Complementation of an SSADH-deficient yeast mutant with an *Arabidopsis* cDNA library enabled the identification of a novel cDNA (designated as *AtGHBDH* for *Arabidopsis thaliana* γ-hydroxybutyrate dehydrogenase), which encodes a 289-amino acid polypeptide containing an NADP-binding domain. Constitutive expression of *AtGHBDH* in the mutant yeast enabled growth on 20 mM GABA and significantly enhanced the cellular concentrations of γ-hydroxybutyrate, the product of the GHDBH reaction. These data confirm that the cDNA encodes a polypeptide with GHBDH activity. *Arabidopsis* plants subjected to flooding-induced oxygen deficiency for up to 4 h possessed elevated concentrations of γ-hydroxybutyrate as well as GABA and alanine. RNA expression analysis revealed that *GHBDH* transcription was not up-regulated by oxygen deficiency. These findings suggest that GHBDH activity is regulated by the supply of succinic semialdehyde or by redox balance. It is proposed that GHBDH and SSADH activities in plants are regulated in a complementary fashion and that GHBDH and γ-hydroxybutyrate function in oxidative stress tolerance.

γ-Aminobutyrate (GABA)[1] is a four-carbon non-protein amino acid that is present in virtually all of the prokaryotic and eukaryotic organisms as a significant component of the free amino acid pool (1, 2). In bacteria, it is involved in carbon and nitrogen metabolism (3), whereas in mammals, it functions as an inhibitory neurotransmitter (4). The role of GABA in plants is uncertain; however, GABA accumulates rapidly in response to a variety of abiotic stresses such as oxygen deficiency or cold temperature (1, 2, 5, 6). These stresses initiate a signal transduction pathway in which increased cytosolic Ca^{2+} stimulates Ca^{2+}/calmodulin-dependent activity of the anabolic enzyme, glutamate decarboxylase (Fig. 1). Under normoxia, GABA is catabolized via GABA transaminase (GABA-T, EC 2.6.1.19) to succinic semialdehyde (SSA), which in turn is oxidized via an NAD-dependent succinic semialdehyde dehydrogenase (SSADH, EC 1.2.2.16) to succinate. Under oxygen deficiency, SSADH activity is probably restricted by increases in reducing potential and adenylate energy charge (7, 8), thereby contributing to the accumulation of GABA.

Research on bacterial and animal systems indicates the existence of an alternative pathway for SSA catabolism to γ-hydroxybutyrate (GHB) that involves the enzyme γ-hydroxybutyrate dehydrogenase (GHBDH, might also be designated as succinic semialdehyde reductase, EC 1.1.1.61) (GenBank™ accession numbers AJ250267, L21902, and AAC41425) (9, 10). Mamelak (11) reviewed evidence for elevated GHB levels in mammalian tissues in response to anoxia or excessive metabolic demand and suggested that GHB functions as an endogenous protective agent when energy supplies are limited. Recently, Allan *et al.* (12, 13) detected GHB in plant tissues and reported that it accumulates in response to oxygen deficiency. The conversion of SSA to GHB is reductive (*i. e.* fermentative), and like other common fermentation reactions such as lactate and alcohol dehydrogenases (14), GHBDH may be involved in the stress tolerance of plants.

In this report, we identify an *Arabidopsis GHBDH* cDNA by functional complementation of an SSADH-deficient yeast mutant in conjunction with metabolite analysis. We further demonstrate that GHB accumulation in *Arabidopsis* subjected to oxygen deficiency does not result from up-regulation of gene expression and propose that GHBDH activity and GHB play a role in oxidative stress tolerance.

EXPERIMENTAL PROCEDURES

Isolation of a Putative GHBDH cDNA from Arabidopsis by Complementation of an SSADH-deficient Yeast Mutant—A *Saccharomyces cerevisiae* mutant of *uga2* is unable to use GABA as a nitrogen source and is defective in SSADH activity, suggesting that *UGA2* is the structural gene for this enzyme (15). To demonstrate that SSADH activity is attributable to *UGA2* and resides at the YBR006w locus, a *ura3 uga2* mutant (strain 22641c) was transformed by a centromere-based plasmid library representing the genome of strain Σ1278b (16). Several genomic clones restored normal growth to the *uga2* mutant on GABA (0.1%) as the sole nitrogen source.

* This work was supported by the Natural Sciences and Engineering Research Council of Canada in the form of an operating grant (to B. J. S.) and post-graduate scholarships (to K. E. B. and O. R. V. C.) and by the Ontario Ministry of Agriculture and Food. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AY044183.

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[1] The abbreviations used are: GABA, γ-aminobutyrate; SSA, succinic semialdehyde; SSADH, succinic semialdehyde dehydrogenase; GHB, γ-hydroxybutyrate; GHBDH, γ-hydroxybutyrate dehydrogenase; GABA-T, γ-aminobutyrate transaminase; RT, reverse transcriptase.

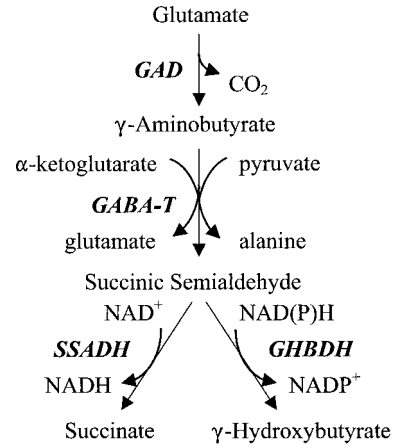

FIG. 1. **Alternative pathways for γ-aminobutyrate metabolism via succinic semialdehyde.** Enzymes are in *boldface italics*.

An *Arabidopsis thaliana* [L.] Heynh (Landsberg *erecta* ecotype) cDNA library (entire seedlings at two-leaf stage) constructed in a yeast expression vector (pFL61) containing the phosphoglycerate kinase promoter and the yeast *URA3* gene (17) was kindly provided by M. Minet. Wild type yeast Σ1278b and its mutant strain 22641c (15) were maintained on yeast minimal medium (18). Competent yeast cells were made and transformed with the *Arabidopsis* cDNA expression library (19). *URA*$^+$ transformants were selected on solid SD medium (0.67% bacto-yeast nitrogen base without amino acids, 2% glucose, 2% bacto-agar) supplemented with 0.5% ammonium sulfate and 0.35 mM uracil (18), washed from each plate with liquid SD medium, and re-selected on SD medium supplemented with 20 mM GABA as the sole nitrogen source. Plasmids were isolated from selected colonies, amplified in *Escherichia coli* strain dH5α cells on LB medium supplemented with ampicillin (50 μg/ml) according to standard protocols (20), and re-introduced into the yeast mutant for re-selection on 20 mM GABA. Plasmid DNA was isolated from a 2-ml overnight culture derived from a single colony (21).

Plasmids bearing one of four independent cDNA clones isolated by complementation were sent to Genologics (Agricultural and Food Laboratory Services Branch, Guelph, Ontario, Canada) for sequencing (ABI PRISM Dye Terminator Cycle Sequencing Ready reaction kit on the ABI PRISM Sequencer Model 377, PerkinElmer Life Sciences). Vector primers used for sequencing were PGK5′ (5′-TCA AGA TCA TCA AGG AAG TAA TTA T-3′) and PGK3′ (5′-TAT TTT AGC GTA AAG GAT GAG GAS A-3′). All four of the cDNAs encoded the gene product.

Growth and Metabolite Analyses of an SSADH-deficient Yeast Mutant Expressing the Putative Arabidopsis GHBDH cDNA—The transformed yeast strains were grown in 50 ml of liquid SD medium to an A_{600} of 0.5. Cells were washed twice and resuspended in nitrogen-free SD medium. Approximately equal volumes (full inoculating loop) were streaked onto nitrogen-free SD plates supplemented with GABA, proline, or $(NH_4)_2SO_4$ (20 mM N). Growth on the plates was checked after 4 days at 28 °C.

Single colonies from wild type yeast (Σ1278b) and 22641c yeast transformed with the empty yeast vector (pFL61) or *Arabidopsis* complement (*GHBDH*) were used to inoculate 1-ml aliquots of liquid SD medium and grown overnight in a rotary shaker (150 rpm, 28 °C). The overnight culture was divided into three aliquots and used to inoculate 50 ml of nitrogen-free liquid SD medium supplemented with GABA, proline, or $(NH_4)_2SO_4$ (20 mM N). The cells were grown to mid-log phase (A_{600} of 1.0 ± 0.1) at 28 °C for 12–96 h depending on the strain. Approximately 1.2×10^9 cells were harvested by centrifugation (5000 × *g*, 4 °C, 5 min), and the pellet was washed twice with 50 ml of cold 0.3 M sorbitol and suspended in 1 ml of 95% methanol and 1 g of silica sand. This mixture was vigorously vortexed (3 × 20 s) and centrifuged (20,500 × *g*, 5 min). The supernatant then was transferred to a second Microfuge tube, and the pellet was extracted with an additional 1 ml of 70% methanol by vortexing. After centrifugation (20,500 × *g*, 5 min), the second supernatant was combined with the first. These methanol-derived extracts were analyzed for GHB and GABA levels by gas chromatography-mass spectrometry (22, 23).

Metabolite and Expression Analyses of Arabidopsis—*Arabidopsis* seeds were stratified at 4 °C for 48 h and then grown in Fox sandy loam (pH 6.5) at 22/18 °C day/night temperature and a 11/13-h day/night photoperiod. Plants were grown individually and with sufficient spacing in seedling trays to preclude shading between adjacent plants. They were fertilized twice weekly with a half-strength modified nutrient solution (24) and subirrigated as needed with water. To investigate gene expression as a function of development and organ, rosette leaves 1–3, rosette leaves 4–6, rosette leaves 7–8, roots, flowers, and siliques were collected from three 6-week-old plants. To investigate the response to oxygen deficiency, all of the rosette leaves were harvested from six 4-week-old plants at time zero with the 24 plants remaining divided equally and randomly between two washbasins covered with tin foil. One contained sufficient water to cover the entire plants (*i.e.* flooded or treated plants), whereas the other did not contain water (*i.e.* control plants). At 2 or 4 h, leaves from six plants in each basin were quickly harvested and frozen in liquid nitrogen. For each harvest, the leaves were divided into three replicates of two plants each (mean fresh weight ± S.E. of 0.27 ± 0.002 g) and stored at −80 °C until analysis.

For metabolite analysis, the frozen leaf tissue was extracted with 80% ethanol containing 5 nmol of GHB-d6 as an internal control and the water-soluble fraction after washing with chloroform was dried in a Speedvac concentrator. The dried residue was suspended in 500 μl of de-ionized water, and then the extract was filter-sterilized through a 45-μm membrane. Analysis and quantification of GHB content were performed using a Hewlett-Packard 1100 series liquid chromatography/mass spectrometer (Agilent Technologies Inc.) as described previously (12). For GABA and alanine analysis, a 100-μl aliquot of each extract was derivatized on-line with *o*-phthalaldehyde and separated by reverse-phase high pressure liquid chromatography using a 4.6 × 150 mm, 3.5-μm Zorbax Eclipse AAA column (Agilent Technologies Inc.) and fluorescence was detected at 340 nm essentially as described by Henderson *et al.* (25).

For expression analysis by relative quantification reverse transcriptase (RT)-PCR, total RNA was extracted from leaf tissue using reagents in the RNeasy plant mini kit (Qiagen, Inc.). The RNA was treated with 1 unit of DNase I (MBI Fermentas) in the presence of reaction buffer (100 mM Tris-HCL (pH 8.0), 10 mM $MgSO_4$, 1 mM $CaCl_2$) and 40 units of RNase inhibitor (MBI Fermentas) for 30 min at 37 °C to remove any contaminating genomic DNA. First strand cDNA synthesis was prepared using a two-step RT-PCR protocol as described in the Enhanced Avian RT-PCR kit (Sigma) using 1 μg of the purified RNA and 2.5 μmol of random nonamers during the RT step. Two microliters of cDNA was used for each subsequent PCR reaction. The PCR protocol was as described in the QuantumRNA 18s Internal Standards kit (Ambion, Inc). To obtain relative quantification of gene expression, 18 S ribosomal cDNA was co-amplified, producing a 315-bp fragment along with the gene of interest using an optimized primers:competimers mixture of 8:2 ratio as provided in the kit. Optimized amplification of these two genes was ensured by adding the gene-of-interest primers and primers:competimers mixture to the PCR reaction at a ratio of 5:1. The primers used to amplify *GHBDH* and *GABA-T* (pyruvate-dependent isoform is described elsewhere (26)) cDNAs, respectively, were as follows: 5′-ATG GAA GTA GGG TTT CTG G-3′ (forward), 5′-CAA GTA AAA CGA TCT CTT CC-3′ (reverse) and 5′-ATG GTC GTT ATC AAC AGT C-3′ (forward), 5′-ATC TCT AAA AGA ACC TTA GC-3′ (reverse). These primers amplify gene fragments of 480 and 428 bp for GHBDH and GABA-T, respectively. The PCR-cycling parameters were 94 °C for 5 min, 35 cycles at 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, and a 5-min extension period at 72 °C for 5 min. Electrophoresis was performed with 1.5% agarose gels as described previously (20). Negative control reactions did not contain reverse transcriptase, whereas positive control reactions contained the cDNA of interest in a custom vector. Quantification of amplified signals were performed using Spot densitometry (Fluorchem™ 8800 Imaging System, Alpha Innotech Inc.).

RESULTS AND DISCUSSION

Identification of an Arabidopsis GHBDH cDNA by Yeast Complementation—Functional complementation of an NADP-dependent SSADH-deficient yeast mutant 22641c was used to isolate an *Arabidopsis GHBDH* cDNA. This mutant had only approximately 10% of the SSADH activity of the wild-type strain when grown on GABA as the sole nitrogen source (2.3 and 24.8 μmol/h/mg protein, respectively) and was found to overlap the YBR006W locus (data not shown), confirming that UGA2/YBR006w is the yeast SSADH (27). The mutant was transformed with a cDNA expression library synthesized from entire seedlings of *Arabidopsis*, and several plasmids containing a 1032-bp cDNA were recovered. These plasmids, when reintroduced into mutant 22641c, allowed growth on GABA.

A

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M E V G F L G L G I M G K A M S M N L L K N G F K V T V W N   30
R T L S K C D E L V E H G A S V C E S P A E V I K K C K Y T   60
I A M L S D P C A A L S V V F D G G V L E Q I C E G K G Y I   90
D M S T V D A E T S L K I N E A L T G K G G R F V E G P V S  120
G S K K P A E D G Q L I I L A A G D K A L F E E S I P A F D  150
V L G K R F Y L G Q V G N G A K M K L I V N M I M G S M M N  180
A F S E G L V L A D K S G L S S D T L L D I L D L G A M T N  210
P M F K G K G P S M N K S S Y P P A F P L K H Q K D M R L A  240
L A L G D E N A V S M P V A A A A N E A F K K A R S L G L G  270
D L D F S A V I E A V K F S R E *
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B

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3   VGFLGLGIMGKAMSMNLLKNGFKVTVWNRT  GHBDH (At)
3   VGFIGLGHLGRAIAKRLIEQGVELIVWNRT  6-PGDH (Aa)
5   VGVVGLGVMGANLALNIAEKGFKVAVFNRT  6-PHDH (Tb)
29  IGWIGTGVMGRSMCGHLIKAGYTVTVFNRT  CAB43926 (At)
6   IAVLGTGMMGLPMARRLAQAGHEVIHAWNRT  THRDH (Pc)
39  VGFIGLGNMGFRMVNNLIRAGYKVTVHDIN  CAB45888 (At)
52  VGFIGLGNMGNPMAKNLIKHGYPLILYDVF  3-HIBDH (Rn)
5   VGFIGLGNMGNPMAKNLMKHGYPLIIYDVF  3-HIBDH (Oc)
3   IAFLGLGNMGPMAANLLKAGHRVNVFDLQ  3-HIBDH (Pa)
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FIG. 2. ***AtGHBDH* cDNA.** *A*, deduced amino acid sequence. A putative cyclic nucleotide-binding domain is *underlined*, and the stop codon is marked with an *asterisk*. *B*, sequence alignment of the putative cyclic nucleotide-binding domain of GHBDH with the corresponding domains from 6-phosphogluconate dehydrogenase, threonine dehydrogenase, and 3-hydroxyisobutyrate dehydrogenase. Amino acids are *numbered* from the Met codon. Potential residues that are conserved in 50% or more of the sequences are shown in *shaded text*. The alignment was created using LASERGENE software (DNAstar) with a gap penalty of 12 and a gap length penalty of 4. All of the sequences were obtained from the NCBI data base. *6-PGDH* (*Aa*) and *6-PGDH* (*Tb*) denote 6-phosphogluconate dehydrogenase from *Aquifex aeolicus* (GenBank™ accession number AAC06408) and *Trypanosoma brucei* (GenBank™ accession number A48565), respectively. *THRDH* (*Pc*) denotes threonine dehydrogenase from *Pseudomonas cruciviae* (GenBank™ accession number BAA34184). *3-HIBDH* (*Rn*) and *3-HIBDH* (*Oc*) denote 3-hydroxyisobutyrate dehydrogenase from *Rattus norvegicus* (GenBank™ accession number P29266) and *Oryctolagus cuniculus* (GenBank™ accession number P32185), respectively. A putative protein (GenBank™ accession number CAB43926) and a 3-hydroxyisobutyrate dehydrogenase-like protein (GenBank™ accession number CAB4588) from *A. thaliana* are denoted as *CAB43926* (*At*) and *CAB4588* (*At*), respectively.

The cDNA insert in the plasmids was sequenced and found to encode a hydrophobic polypeptide, which contains 289 amino acids (Fig. 2*A*) and has a predicted molecular mass of 30.3 kDa, an isoelectric point of 5.59, and a net charge of −2.88 at neutral pH (Edit Sequence, DNAstar software, London, UK). The gene encoding the polypeptide has been designated *AtGHBDH* for *A. thaliana GHBDH*. A search of the GenBank™ data base does not identify significant homology with NADP-dependent SSA reductase from rat (9) or human brain (10) and NAD-dependent GHBDHs from *Clostridium aminobutyricum* (GenBank™ accession number AJ250267), *Clostridium kluyveri* (GenBank™ accession number L21902), or *Ralstonia utropha* (GenBank™ accession number AAC41425). However, the predicted amino sequence of AtGHBDH does exhibit 20–33% similarity to several hypothetical and known dehydrogenases, including 3-hydroxyisobutyrate dehydrogenase, threonine dehydrogenase, and 6-phoshogluconate dehydrogenase from several sources (data not shown). The highest degree of similarity between AtGHBDH and these dehydrogenases is found at the N terminus (Fig. 2*B*), a region containing a strict consensus sequence (A/G)*XX*GL(A/L)*X*MGX_5NX_4G) that is typical of the dinucleotide cofactor-binding fold of many dehydrogenases (28). Preceding this motif by seven amino acids is an arginine residue that is conserved in AtGHBDH, threonine dehydrogenase, and all of the NADP-dependent 6-phosphogluconate dehydrogenases. This residue is implicated in the specific binding of NADP. In contrast, all of the NAD-dependent

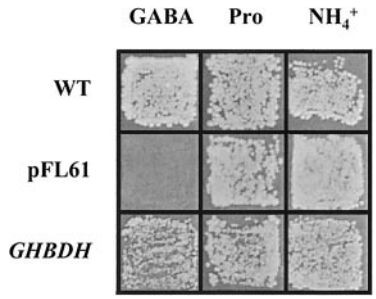

FIG. 3. **Growth of wild-type yeast (Σ1278b) and the SSADH-deficient mutant (22641c) transformed with the empty vector (pFL61) or *AtGHBDH* on medium supplemented with various nitrogen sources (20 mM).**

3-hydroxyisobutyrate dehydrogenases examined thus far have an aspartate residue at this position. Several other residues that have been implicated in substrate binding in 6-phosphogluconate and 3-hydroxyisobutyrate dehydrogenases appear to be strictly conserved within AtGHBDH. These include Val-119, Ser-120, Gly-121, Lys-168, and Asn-172 numbered according to AtGHBDH. Thus, the AtGHBDH polypeptide appears to possess an NADP-binding domain and to belong to a family of dehydrogenases that has not been previously identified.

The wild-type yeast and the mutant 22641c expressing *AtGHBDH* grew well on 20 mM GABA as well as 20 mM proline or NH_4^+, whereas mutant cells expressing the empty vector, pFL61, did not grow on GABA (Fig. 3). Mutant cells expressing either *AtGHBDH* or pFL61 had significantly higher concentrations of GABA than wild-type cells (Table I). This was accompanied by higher concentrations of GHB; however, the concentrations in cells expressing *AtGHBDH* were 8-fold higher than in those expressing pFL61. Negligible GHB was synthesized by all of the strains when grown on proline or NH_4^+ as the sole nitrogen source. These experimental data confirm that the isolated *Arabidopsis* cDNA encodes a polypeptide with GHBDH activity.

Role of GHBDH/GHB in Arabidopsis—To investigate the role of GHBDH *in planta*, we first demonstrated using relative RT-PCR that the expression of *GHBDH* or *GABA-T* was similar among various plant parts (leaves 1–3, leaves 4–6, leaves 7–8, roots, flowers, and siliques) from untreated *Arabidopsis* plants (data not shown) and therefore independent of organ-specific and developmental regulation. We then subjected *Arabidopsis* plants to flooding for up to 4 h to induce oxygen deficiency and determined the expression of both genes in leaves as well as the concentrations of GHB, the product of the GHBDH reaction, and related metabolites (Fig. 1). The relative levels of both gene transcripts at 2 and 4 h were lower in flooded plants than in control plants (Fig. 4), whereas the concentrations of GABA and alanine as expected (5) were significantly higher in leaves of flooded plants (Fig. 5). This was accompanied by a 60% greater accumulation of GHB in flooded plants. Green tea and soybean sprouts exhibit a similar increase in the pool size of GHB during exposure to oxygen deficiency (13).

These metabolic responses can be largely attributed to: 1) a stimulation of glutamate decarboxylase activity by two mechanisms, increasing cytosolic acidification or calcium in conjunction with calmodulin and corresponding increases in GABA and alanine formation (Fig. 1) (1, 2, 6); 2) restricted SSADH activity due to altered NAD/NADH ratios (7, 8), thereby causing the accumulation of SSA and the feedback inhibition of GABA-T activity (29); and 3) the induction of alanine transaminase, an enzyme that catalyzes the formation of alanine from pyruvate and glutamate (30). Despite uncertainty regarding the relative contribution to pool size of these mechanisms, it

TABLE I

Comparison of GABA and GHB pool sizes in wild type yeast (Σ1278b) and the SSADH-deficient mutant (22641c) transformed with the empty vector (pFL61) or AtGHBDH

Cells were grown on various nitrogen sources (20 mM N). These treatments were harvested at different times when growth was in mid-log phase. N.D. indicates not detected. Data represent mean ± S.E. of four replicates.

Yeast strain	Nitrogen source	GABA pool	GHB pool
		μmol g^{-1} DW	*μmol g^{-1} DW*
Wild type	GABA	59.8 ± 2.0	0.199 ± 0.042
	Proline	0.666 ± 0.079	0.082 ± 0.051
	NH_4SO_4	0.081 ± 0.007	0.012 ± 0.012
pFL61	GABA	604 ± 26	10.9 ± 0.3
	Proline	0.445 ± 0.026	0.117 ± 0.042
	NH_4SO_4	0.096 ± 0.013	N.D.
GHBDH	GABA	561 ± 29	90.0 ± 5.9
	Proline	0.467 ± 0.020	0.059 ± 0.031
	NH_4SO_4	0.091 ± 0.004	0.023 ± 0.012

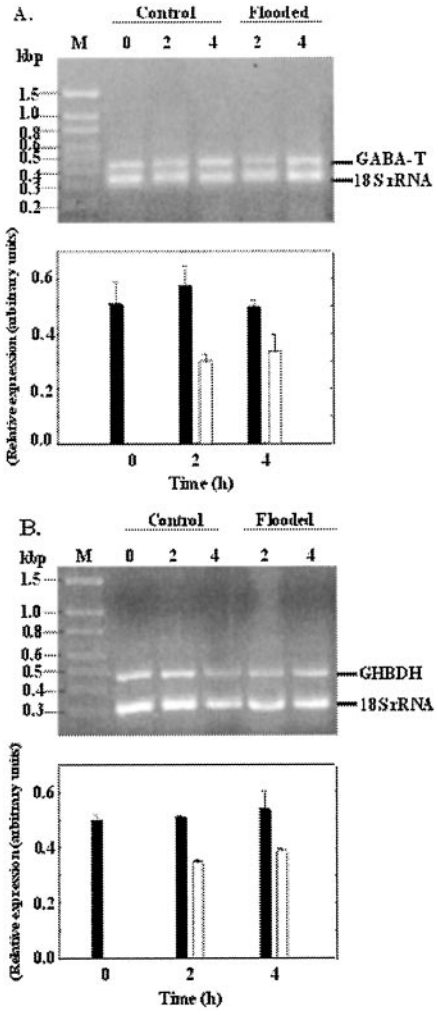

FIG. 4. **Relative expression of *GABA-T* and *GHBDH* in *Arabidopsis* plants subjected to flooding up to 4 h.** The *upper panel* in both *A* and *B* represents a typical relative RT-PCR gel of GABA-T (428 bp) or GHBDH (480 bp) and 18 S rRNA (315 bp). *Lane M* is a 100-bp ladder. The *bottom panel* of both *A* and *B* presents the mean ± S.E. of three replicates. The *closed* and *open bars* represent control and flooded plants, respectively.

seems probable that a significant portion of the SSA derived form GABA under flooding-induced oxygen deficiency was converted to GHB and that this result could not be attributed to up-regulation of gene transcription.

Recent research using SSADH knock-out mutants in yeast

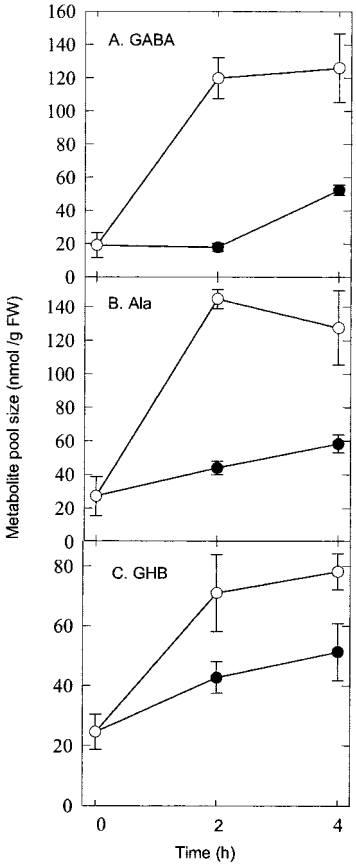

FIG. 5. **Metabolite pool sizes in *Arabidopsis* plants subjected to flooding for up to 4 h.** All of the plants were placed in the dark during the time course. The *closed* and *open symbols* represent control and flooded plants, respectively. Data are the mean ± S.E. of three replicates.

(27) and plants (31) established that the GABA shunt plays a role in preventing the accumulation of reactive oxygen species, probably by providing a source of reducing equivalents for the maintenance of antioxidant pools or by scavenging SSA. Earlier research on mammals suggested that GHB also functions in detoxification of reactive oxygen species, probably by providing NADPH (11). Interestingly, oxygen deficiency increases the production of NADPH (32, 33) and reactive oxygen species such as superoxide and hydrogen peroxide in plants (34); however, the oxidation of NADPH as well as NADH via the mitochondrial respiratory chain is limited (34). Taken together, these findings could suggest that GHBDH and SSADH activities in plants are regulated in a complementary fashion by redox balance and that GHB functions in oxidative stress tolerance.

Other studies using anaerobic bacteria such as *Clostridium* sp. and *R. utropha* have shown that GHB is part of a fermentation pathway from SSA to 4-hydroxybutyryl-CoA, crotonyl-CoA, acetate, and butyrate (35–38). 4-Hydroxybutyryl-CoA is also a substrate for polyhydroxyalkanoic acid synthase (38, 39). In animals, GHB serves as both product and precursor of the neurotransmitter GABA (40–42). It may also function as a neurotransmitter (43, 44). Indeed, its sedative and mood-elevating properties have contributed to its use as a recreational drug (45, 46). A better understanding of pathways associated with GHB in plants may uncover a specific function or receptor for GHB.

Acknowledgments—We thank Drs. M. D. McLean and A. Makhmoudova for technical advice and Dr. M. Minet for providing the *Arabidopsis* cDNA library constructed in a yeast expression vector.

REFERENCES

1. Bown, A. W., and Shelp, B. J. (1997) *Plant Physiol.* (*Bethesda*) **115,** 1–5
2. Shelp, B. J., Bown, A. W., and McLean, M. D. (1999) *Trends Plant Sci.* **4,** 446–452
3. Metzer, E., and Helpern, Y. S. (1990) *J. Bacteriol.* **172,** 3250–3256
4. Bormann, J. (1988) *Trends Neurosci.* **11,** 112–116
5. Bown, A. W., and Shelp, B. J. (1989) *Biochem.* (*Life Sci. Adv.*) **8,** 21–25
6. Kinnersley, A. M., and Turano, F. J. (2000) *Crit. Rev. Plant Sci.* **19,** 479–509
7. Busch, K. B., and Fromm, H. (1999) *Plant Physiol.* (*Bethesda*) **121,** 589–597
8. Busch, K. B., Piehler, J., and Fromm, H. (2000) *Biochemistry* **39,** 10110–10117
9. Andriamampandry, C., Siffert, J.-C., Schmitt, M., Garnier, J.-M., Staub, A., Muller, C., Gobaille, S., Mark, J., and Maitre, M. (1998) *Biochem. J.* **334,** 43–50
10. Schaller, M., Schaffhauser, M., Sans, N., and Wermuth, B. (1999) *Eur. J. Biochem.* **265,** 1056–1060
11. Mamelak, M. (1989) *Neurosci. Biobehav. Rev.* **13,** 187–198
12. Allan, W. L., Smith, R., and Shelp, B. J. (2003) *Application Bulletin AB-0015*, p. 4, Agilent Technologies Inc., Mississauga, Ontario, Canada
13. Allan, W. L., Peiris, C., Brown, A. W., and Shelp, B. J. (2003) *Can. J. Plant Sci.*, in press
14. Drew, M. C. (1997) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **48,** 223–250
15. Ramos, F., El Guezzar, M., Grenson, M., and Wiame, J.-M. (1985) *Eur. J. Biochem.* **149,** 401–404
16. Marini, A. M., Vissers, S., Urrestarazu, A., and Andre, B. (1994) *EMBO J.* **l13**, 3456–3463
17. Minet, M., Dufour, M. E., and Lacoute, F. (1992) *Plant J.* **2,** 417–422
18. Rose, M. D., Winston, F., and Hieter, P. (1990) *Methods in Yeast Genetics*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
19. Dohlmen, R. J., Strasser, A. W., Höner, C. B., and Hollenberg, C. P. (1991) *Yeast* **7,** 691–692
20. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
21. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1992) *Short Protocols in Molecular Biology*, 2nd Ed., John Wiley & Sons, Inc., New York
22. Gibson, K. M., Aramaki, S., Sweetman, L., Nyhan, W. L., DeVivo, D. C., Hodson, A. K., and Jakobs, C. (1990) *Biomed. Environ. Mass Spectrom.* **9,** 89–93
23. Kok, R. M., Howells, D. W., Van DenHeuvel, C. C. M., Guérand, W. S., Thompson, G. N., and Jakobs, C. (1993) *J. Inherit. Metab. Dis.* **16,** 508–512
24. Shelp, B. J., Penner, R., and Zhu, Z. (1992) *Can. J. Plant Sci.* **72,** 883–888
25. Henderson, J. W., Ricker, R. D., Bidlingmeyer, B. A., and Woodward, C. (2000) *Technical Note 5980-1193E*, p. 8, Agilent Technologies Inc., Mississauga, Ontario, Canada
26. Van Cauwenberghe, O. R., Makhmoudova, A., McLean, M. D., Clark, S. M., and Shelp, B. J. (2002) *Can. J. Bot.* **80,** 933–941
27. Coleman, S. T., Fang, T. K., Rovinsky, S. A., Turano, F. J., and Moye-Rowley, W. S. (2001) *J. Biol. Chem.* **276,** 244–250
28. Hawes, J. W., Harper, E. T., Crabb, D. W., and Harris, R. H. (1996) *FEBS Lett.* **389,** 263–267
29. Van Cauwenberghe, O. R., and Shelp, B. J. (1999) *Phytochemistry* **52,** 575–581
30. Good, A. G., and Muench, D. G. (1992) *Plant Physiol.* (*Bethesda*) **99,** 1520–1525
31. Bouché, N., Fait, A., Bouchez, D., Moller, S. G., and Fromm, H. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 6843–6848
32. Harding, S. A., Oh, S.-H., and Roberts, D. M. (1997) *EMBO J.* **16,** 1137–1144
33. Lee, S. H., Seo, H. Y., Kim, J. C., Heo, W. D., Chung, W. S., Lee, K. J., Kim, M. C., Cheong, Y. H., Choi, J. Y., Lim, C. O., and Cho, M. J. (1997) *J. Biol. Chem.* **272,** 9252–9259
34. Moller, I. M. (2001) *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **52,** 561–591
35. Hardman, J. K., and Stadtman, T. C. (1963) *J. Biol. Chem.* **238,** 2088–2093
36. Wolf, R. A., Urben, G. W., O'Herrin, S. M., and Kenealy, W. R. (1993) *Appl. Environ. Microbiol.* **59,** 1876–1882
37. Valentin, H. E., Zwingmann, G., Schönebaum, A., and Steinbüchel, A. (1995) *Eur. J. Biochem.* **227,** 43–60
38. Buckel, W. C. (2001) *Appl. Microbiol. Biotechnol.* **57,** 263–273
39. Valentin, H. E., Reiser, S., and Gruys, K. J. (2000) *Biotechnol. Bioeng. Symp.* **67,** 291–299
40. Rumigny, J. F., Maitre, M., Cash, C., and Mandel, P. (1980) *FEBS Lett.* **117,** 111–116
41. Hechler, V., Ratomponirina, C., and Maitre, M. (1997) *J. Pharmacol. Exp. Ther.* **281,** 753–760
42. Maitre, M. (1997) *Prog. Neurobiol.* **51,** 337–361
43. Cash, C. D. (1994) *Neurosci. Biobehav. Rev.* **18,** 291–304
44. Madden, T. E., and Johnson, S. W. (1998) *J. Parmacol. Exp. Ther.* **287,** 261–265
45. Galloway, G. P., Frederick, S. L., Staggers, F. E., Jr., Gonazales, M., Stalcup, S. A., and Smith, D. E. (1997) *Addiction* **92,** 89–96
46. Marwick, C. (1997) *J. Am. Med. Assoc.* **277,** 1505–1506