GABA shunt deficiencies and accumulation of reactive oxygen intermediates: insight from *Arabidopsis* mutants

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Abstract In plants, succinic semialdehyde dehydrogenase (SSADH)-deficiency results in the accumulation of reactive oxygen intermediates (ROI), necrotic lesions, dwarfism, and hypersensitivity to environmental stresses [Bouché, N., Fait, A., Moller, S.G. and Fromm, H. (2003) Proc. Natl. Acad. Sci. USA. 100, 6843–6848]. We report that *Arabidopsis ssadh* knockout mutants contain five times the normal level of γ-hydroxybutyrate (GHB), which in SSADH-deficient mammals accounts for phenotypic abnormalities. Moreover, the level of GHB in *Arabidopsis* is light dependent. Treatment with γ-vinyl-γ-aminobutyrate, a specific γ-aminobutyrate (GABA)-transaminase inhibitor, prevents the accumulation of ROI and GHB in *ssadh* mutants, inhibits cell death, and improves growth. These results provide novel evidence for the relationship between the GABA shunt and ROI, which may, in part, explain the phenotype of SSADH-deficient plants and animals.

Keywords: γ-Aminobutyrate; Reactive oxygen intermediate; Succinic semialdehyde dehydrogenase; γ-Hydroxybutyrate; *Arabidopsis*

1. Introduction

Succinic semialdehyde dehydrogenase (SSADH) is a key enzyme in the metabolic pathway known as the GABA shunt. This pathway begins with the decarboxylation of glutamate in the cytosol by glutamic acid decarboxylase (GAD) and ends in the mitochondria by the catabolism of GABA to succinic semialdehyde and its conversion to succinate, which enters the TCA cycle, and the production of NADH (Fig. 1, reviewed by [2]). In mammals, a deficiency in the capacity to catabolize GABA through GABA-transaminase (GABA-T) and SSADH leads to severe clinical implications including mental retardation, developmental delay, and occasional seizures [3,4]. Mutant mice deficient in SSADH are neurologically impaired, fail to gain weight, and manifest a critical period at 16–22 days of life associated with 100% mortality [5]. The SSADH deficiency is coupled to the accumulation of gamma-hydroxybutyric acid (GHB) in physiological fluids. GHB is a by-product of the GABA-shunt and is suspected to cause the pathologic phenotype known as GHB aciduria. GHB has been shown to bind $GABA_B$ receptors [4,6,7], and recent studies revealed the presence of GHB-specific receptors which can mediate some of the physiological and pharmacological effects of GHB [8]. Based on these and other evidences, GHB has been proposed as the primary cause of the aberrant phenotype of SSADH-deficiency. Consequently, numerous studies attempted to find ways to reduce its content for clinical purposes. One of the compounds found to be effective is γ-vinyl-GABA (Vigabatrin; VGB), a specific inhibitor of GABA-T (Fig. 1). Indeed, use of VGB partially relieved some of the symptoms of GHB-aciduria in mammals [4,5,9]. However, in spite of suggestions for a relationship between GHB-aciduria and the oxidative state of the cell [10], a direct relationship between excess GHB and levels of reactive oxygen intermediate (ROI) has not been reported to our knowledge.

In plants, disruption of the gene encoding SSADH leads to high levels of ROI associated with dwarfism, chlorotic leaves, and extensive necrotic lesions [1]. This phenotype is exacerbated upon exposure to environmental stresses such as high-fluence white light, UV-B and heat [1]. A recent study revealed a plant gene encoding succinic semialdehyde reducate (SSR; 11) that catalyzes the conversion of SSA to GHB (Fig. 1), which might be responsible for the accumulation of GHB in plants undergoing hypoxia (e.g., flooding) [11,12]. We sought to test the levels of GHB and the effect of VGB in an *Arabidopsis* mutant deficient in SSADH to assess the relationships between the GABA shunt, GHB, ROI, and plant growth.

2. Materials and methods

2.1. Plant culture and growth conditions

Surface-sterilized seeds were plated on Gamborg B5 medium, pH 6.4 (Sigma), containing 1% sucrose and 0.8% agar (plant cell culture tested, Sigma), incubated at 4 °C for 48 h and grown in vitro in controlled-environment Binder/Brinkmann growth chambers (model KBWF720, Germany) as described [1]. At day/night cycle of 16/8 h, light intensity was set to 100 μmol m^{-2} s^{-1} (HL) and 10 μmol m^{-2} s^{-1} (LL), temperature day/night was 20/15 °C. Seedlings were grown vertically. Provided a similar trend (see also [1]) of different alleles, *ssadh*-4 and *ssadh*-2, additional experiments were conducted on *ssadh*-2 allele and WT (Columbia). Treatment with VGB (10, 100 μM and 1 mM) was performed on 10 day plants 24 h following transfer to HL. A concentration of 100 μM was used for the following experiments. Prior to incubation at LL and HL ± VGB, young plants were

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Abbreviations: SSA, succinic semialdehyde; SSADH, SSA dehydrogenase; GABA, γ-aminobutyrate; GABA-T, GABA transaminase; GHB, γ-hydroxybutyrate; VGB, γ-vinyl-GABA; ROI, reactive oxygen intermediates

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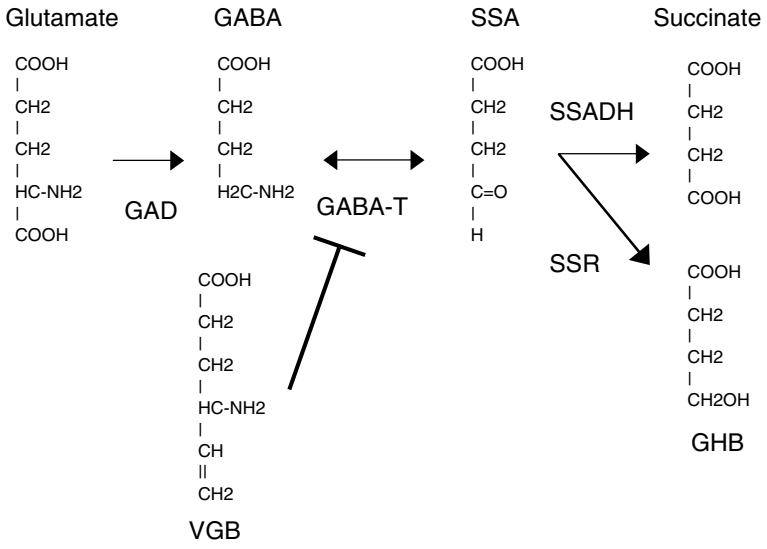

Fig. 1. Schematic presentation of the GABA shunt. Glutamate decarboxylase (GAD; EC 4.1.1.15), GABA-transaminase (GABA-T; EC 2.6.1.19), succinic-semialdehyde dehydrogenase (SSADH; EC 1.2.1.16) and succinic semialdehyde reductase (SSR; EC 1.1.1.61). Vigabatrin (VGB, γ-vinyl-GABA) is a GABA-T-specific inhibitor in animals.

placed on strips (1 × 10 cm) of filter paper on Gamborg B5 agar medium. VGB or control solution (DW) was dropped (every three days) on the filter paper and let to absorb. Plates were returned in the growth chamber. Growth was followed for additional 7–14 d. Data are representatives of at least three independent experiments and at least four repeats per experiment.

2.2. Detection of ROI and cell death

Trypan blue (TB) stain was used to visualize dying cells as described [13]. H_2O_2 was detected in situ using 3,3-diaminobenzidine (DAB) as described [14]. Quantitative analysis of DAB stain was performed by visualizing leaves (20–40 per experiment) of a minimum of five plants under a binocular. Staining pattern was measured in three independent experiments. Results are presented as the occurrence of leaves [%] per plant with at least 50% of the leaf surface stained.

2.3. Metabolite analysis

Plant tissue was extracted and extracts were derivatized as described [15]. The relative content of metabolites was determined using a GC-MS protocol [15,16]. GHB and the two possible GABA derivates were detected by targeting *m/z* 233 [17] and *m/z* 102 and 304, respectively. A retention time and mass spectral library for automatic peak quantification of metabolite derivatives were implemented within the Xcalibur processing method format. Substances were identified by comparison with authentic standards [16] and the NIST 98 library (http://www.nist.gov/srd/nist1a.htm). Automated targeting of unique fragment ions for each individual metabolite were taken as default as quantifiers, and manually corrected where necessary. The gas chromatography–mass spectrometry (GC-MS) system was composed of a Pal autosampler (CTC Analytic, Zwingen, Switzerland), a TRACE GC 2000 gas chromatograph, and a TRACE DSQ quadrupole mass spectrometer (ThermoFinnigan, Hemel, UK). The mass spectrometer was tuned according to the manufacturer's recommendations. GC was performed on 30-m Rtx_5Sil MS column with 0.25-μm film thickness (Restek). Chromatography parameters were as described elsewhere [16]. Chromatograms and mass spectra were evaluated using XCALIBUR v1.3 software (ThermoFinnigan).

2.4. Statistical analysis

Data were compared and analyzed (following *arcsin* transformation of ratios) by one or two-way ANOVA (analysis of variance) and more specifically by the sum-of-squares-simultaneous-test-procedure (SS-STP), a conservative method which tests all possible sets of comparisons among means [18].

3. Results

3.1. Accumulation of GHB in SSADH-deficient plants: the preventive effect of VGB

First, we sought to assess the occurrence of GHB in an *Arabidopsis ssadh* mutant under growth conditions that were shown to enhance ROI accumulation in the mutant and that lead to necrotic lesions appearance on the leaves [1]. Plants were grown for 10 days at low fluence white light (LL; 10 $\mu mol\ m^{-2}\ s^{-1}$), transferred to high-fluence white light (HL; 100 $\mu mol\ m^{-2}\ s^{-1}$) for an additional period of 7 days and thereafter tested for their metabolite content (see Section 2). Mutant plants grown under LL had about 50% higher levels of GHB compared to those in the WT (0.6 vs. 0.4 $nmol\ g^{-1}$ FW; Fig. 2). Transfer of plants to HL resulted in a further accumulation of GHB in the mutant as early as 5 h of exposure to HL (*data not shown*). Eventually, it reached 2–3-fold its levels at LL, following 7d of exposure to HL (Fig. 2), and five times the content found in the WT under the same conditions. In WT plants, GHB levels were barely affected by light intensity. Treatment of plants exposed to HL with VGB (see Section 2) prevented the accumulation of GHB in the mutant beyond the levels detected under LL. VGB had no effect on GHB levels in the WT plants exposed to HL (Fig. 2).

We further tested the levels of GABA. Under LL conditions, the *ssadh* mutant had much higher levels of GABA than in the WT (Fig. 3). After 7 days at HL, GABA content increased 3-fold in the mutant and similarly in the WT, thus HL did not change the 3-fold-difference in GABA levels between the WT and the mutant. Unexpectedly, treatment of the plants with VGB (Fig. 3) decreased the GABA levels both in the WT and in the mutant, suggesting that GABA levels are determined by several factors other than its transamination to SSA.

The amino acid proline is known to accumulate under various stress conditions. Under osmotic stress it may function as an osmoprotectant [19,20], but it may also function as a scavenger of ROI [19,21,22]. Moreover, proline synthesis may be induced by ROI irrespective of osmotic stress, for example,