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

Aaron Fait[a], Ayelet Yellin[b], Hillel Fromm[b,*]

[a] *Department of Plant Sciences, Weizmann Institute of Science, 76100 Rehovot, Israel*
[b] *Department of Plant Sciences, Tel Aviv University, 69978 Tel Aviv, Israel*

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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.

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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 $\mu mol\ m^{-2}\ s^{-1}$ (HL) and 10 $\mu mol\ m^{-2}\ s^{-1}$ (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

*Corresponding author. Fax: +972 3 6406816.
E-mail address: hillelf@post.tau.ac.il (H. Fromm).

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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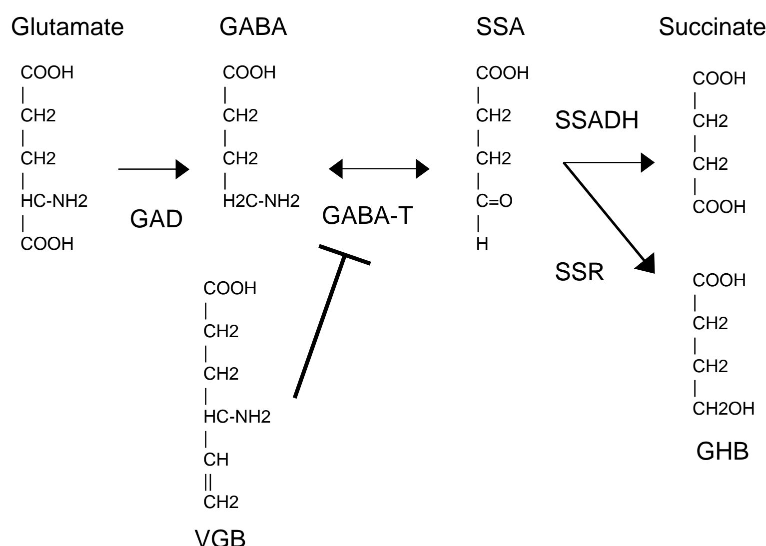

Fig. 1. Schematic presentation of the GABA shunt. Glutamate decarboxylase (GAD; EC 4.1.1.15), GABA-transaminase (GABA-T; EC 2.6.1.19), succinic-semialdehyde dehydrogenase (SSADH; EC 1.2.1.16) and succinic semialdehyde reductase (SSR; EC 1.1.1.61). Vigabatrin (VGB, γ-vinyl-GABA) is a GABA-T-specific inhibitor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

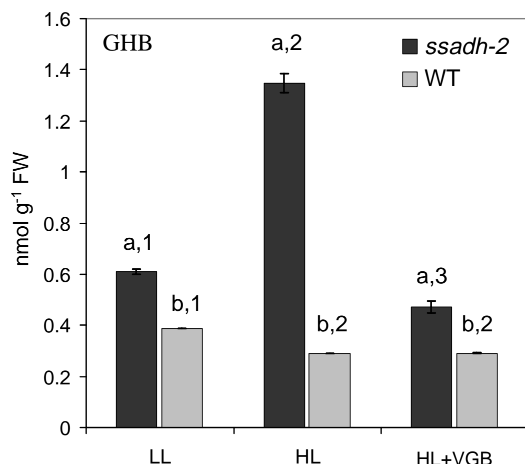

Fig. 2. GHB in the *ssadh* mutant and WT plants. Plants were grown as indicated (Section 2). Metabolites were extracted and GHB content (±S.E.) calculated based on external standard calibration curve. Statistical significance ($P < 0.05$) among lines and among treatments within one line is marked by low case letters and numbers, respectively. Data were analyzed by two-way ANOVA and SS-STP statistical (Section 2).

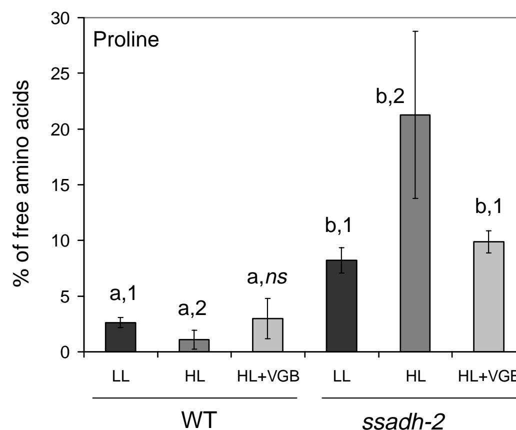

Fig. 4. Proline in the *ssadh* mutant and WT plants. Plants were grown as described (Section 2). Metabolites were extracted and proline content was determined (Section 2). Data are presented as % of proline per total free amino acids ± S.E. Statistical significance ($P < 0.05$) among lines and among treatments within one line is marked by lower case letters and numbers, respectively. Data were analyzed by two-way ANOVA and SS-STP statistical (Section 2). ns: statistically non-significant.

in response to an incompatible pathogen–plant interaction [22]. We found that transfer of *ssadh* seedlings from LL to HL caused a substantial increase in the level of proline, which was almost completely prevented by treatment with VGB (Fig. 4). WT plants did not show any significant change in their proline content following transfer to HL and HL + VGB treatment (Fig. 4). We then tested the effect of VGB on ROI.

3.2. VGB inhibits ROI accumulation and improves plant growth

WT and *ssadh* plants were transferred from LL to HL with or without VGB, and hydrogen peroxide was monitored by in situ staining with DAB as previously described [1] (Section 2). Under LL, WT and mutant plants showed a similar low background level of DAB staining (Fig. 5A, LL) with no leaf showing staining that reached 50% of the leaf surface area (26 and

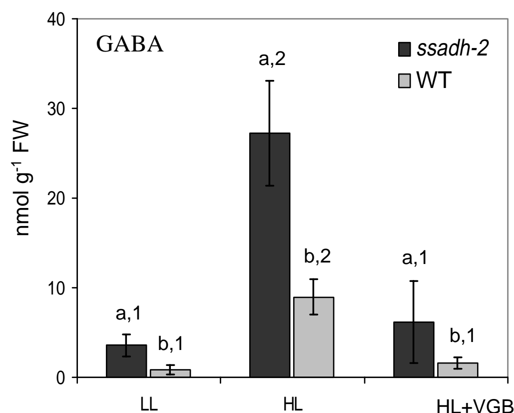

Fig. 3. GABA in the *ssadh* mutant and WT plants. Plants were grown as described (Section 2). Metabolites were extracted and GABA content (±S.E.) was calculated based on external standard calibration curve. Statistical significance ($P < 0.05$) among lines and among treatments within one line is marked by lower case letters and numbers, respectively. Data were analyzed by two-way ANOVA and SS-STP statistical (Section 2).

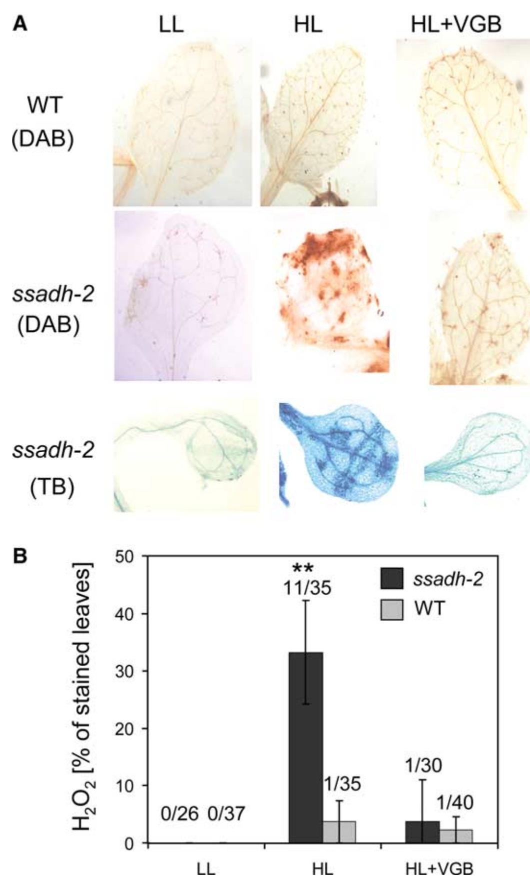

Fig. 5. Hydrogen peroxide and cell death in the *ssadh* mutant and WT plants. Plants were grown as described (Section 2) and stained with DAB for in situ H_2O_2 measurements and TB to visualize dead cells (Section 2). (A) Plants grown under LL, HL ± VGB. (B) Quantitative analysis of DAB stain presented as the occurrence [average % ± S.E.] of stained leaves per plant (Section 2). The numbers of stained leaves and the total number of leaves tested are presented above each treatment histogram. '**', Statistically significant data ($P < 0.05$). Data were analyzed by two-way ANOVA and SS-STP (Section 2).

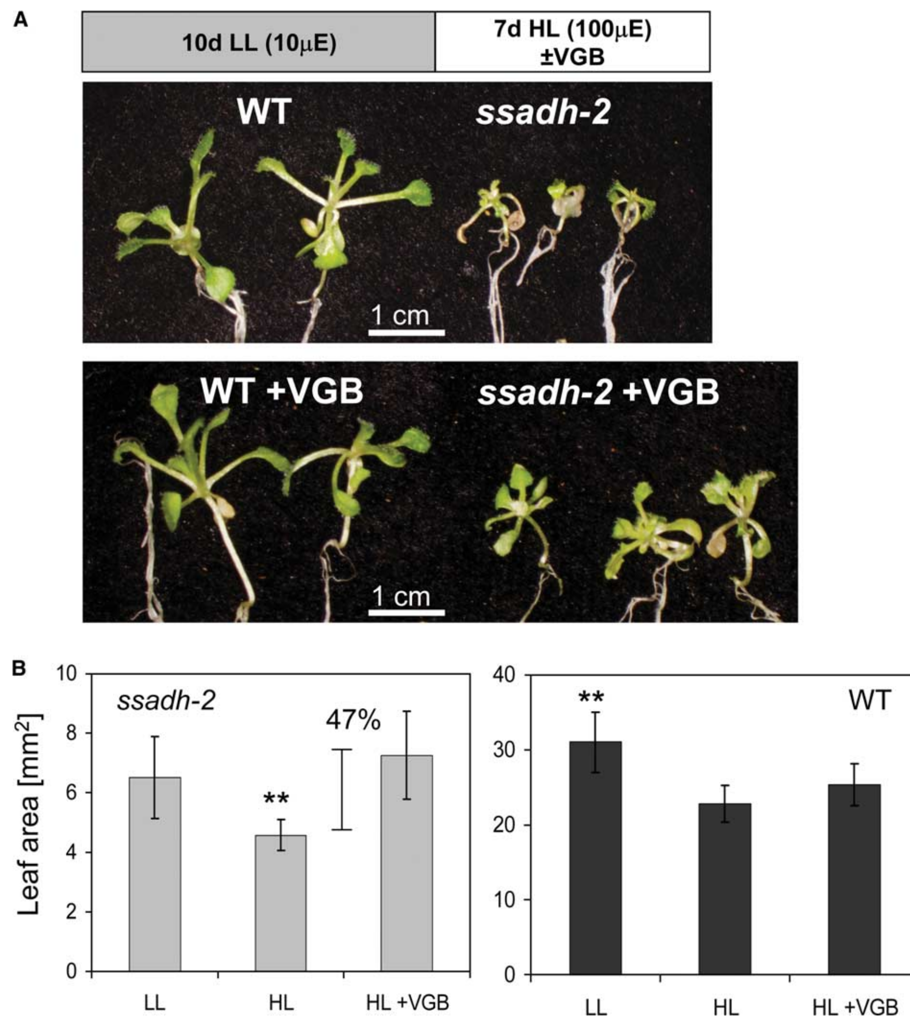

Fig. 6. Effect of VGB on plant growth. (A) Representative samples grown for 10 days under LL and then transferred for 7 days to HL ± VGB. (B) Quantitative analysis of plant growth (leaf area ± S.E.). '**', Statistically significant data ($P < 0.01$). Data were analyzed by one-way ANOVA and SS-STP (Section 2).

30 leaves tested in the mutant and WT, respectively; Fig. 5B). Upon exposure to HL, there was no statistically significant change in the extent of DAB staining in the WT (only one leaf out of 35 tested was stained with DAB over 50% of its surface area; Fig. 5B). In contrast, a dramatic increase in DAB staining of leaves was observed in the mutant upon transfer to HL (Fig. 5A and B, HL). Application of VGB (Section 2) significantly reduced DAB staining of mutant leaves to the extent found in WT leaves (Fig. 5A and B HL + VGB). Furthermore, staining of cells with TB revealed that VGB reduced the extent of necrotic lesions in the mutant (Fig. 5A, TB row). VGB had no effect on DAB staining of WT plants grown at HL (Fig. 5B). The WT was free of lesions under all conditions (not shown). The quantitative analysis of DAB staining (see Section 2) in WT and mutant plants under all experimental conditions is presented in Fig. 5B.

We then tested the effect of VGB on growth of the *ssadh* mutant. Clearly, VGB partially relieved the symptoms of the *ssadh* mutant (Fig. 6A). To quantify the effect of VGB on plant growth, we measured leaf area under LL, HL and HL treated with VGB. Leaf area under HL was about 65% and 75% in the mutant and WT, respectively, compared to the leaf area under LL (Fig. 6B). Application of VGB had a profound and statistically significant effect on the leaf area in the mutant (increase of 47%), whereas it had no statistically significant effect on the area of WT leaves.

4. Discussion

In the present study, we show hyper-accumulation of GHB in SSADH-deficient *Arabidopsis* mutants. This was evident under LL conditions and enhanced in response to HL. VGB, a specific inhibitor of mammalian GABA-T [3,23], reduced GHB levels, prevented H_2O_2 accumulation and cell death, and improved plant growth. Our results link for the first time GHB and SSADH-deficiency in plants. Application of VGB partially relieved the aberrant symptoms caused by the deficiency, as was shown in mice [10]. Taken together, these findings suggest intriguing similarities in GABA metabolism between plants and animals. In animals, the effects of GHB are thought to be mediated by specific GHB or GABA receptors. In plants there is yet no conclusive evidence for their occurrence although some indications do exist [24].

In a recent study, we showed that SSADH-deficient transgenic plants are adversely affected by environmental stresses including high-fluence white light, UV-B and heat. Under these conditions, plants accumulate particularly high levels of hydrogen peroxide [1]. In the present work, we show for the first time that treatment of GABA-shunt deficient plants with VGB prevents the accumulation of hydrogen peroxide and improves growth. VGB has also been used successfully as treatment against hyperbaric oxygen toxicity [25], a condition that induces ROI accumulation. Our findings suggest that high ROI is a major cause of the *ssadh* aberrant phenotype. However, the role of proline accumulation in conjunction with the enhanced oxidative stress in the *ssadh* mutant is not clear. While an increase in proline content may reflect the increase in ROI levels, it may also reflect unfavorable osmotic conditions developing in the mutant when transferred from LL to HL. To test this, we investigated a few parameters that are typically associated with osmotic stress. GC–MS chromatograms of plant extracts were analyzed for sugar content. However, there was no apparent increase in the total sugar content in leaves of either mutant or WT plants exposed to the HL conditions for a 7 day period. In fact, a slight decrease was observed specifically in sucrose levels in the mutant (data not shown). Furthermore, while total sugar content was similar in WT and mutant plants, sucrose was specifically slightly higher in the mutant (by about 50%) under all experimental conditions. Taken together, these lines of evidence suggest the occurrence of an oxidative stress response in the *ssadh* mutant, which drastically increases upon exposure to HL. However, this increase is probably not the result of osmotic stress. Nevertheless, we cannot rule out a contribution of osmotic stress to the oxidative state of the mutant.

Earlier studies have postulated a protective effect for GHB against ROI in mammals [26]. However, recently the opposite effect has been proposed [10]. In bacteroids, GHB has been associated with growth under hypoxic conditions. The anabolism of GHB utilizes NADH. It has been hypothesized that under oxygen deficiency this reaction might reduce the NADH/NAD ratio in the cell and, consequently, promote the reactivation of key enzymes of the TCA cycle [27]. Considering the increase in the levels of ROI, GHB and proline in the *ssadh* mutant upon exposure to HL, it would be interesting to examine in greater detail the anti-oxidative defense machinery that operates in the mutant under these conditions. We found that ascorbic acid content increased similarly in both the WT and the mutant when transferred from LL to HL, with or without VGB (data not shown). Therefore, it seems that ascorbic acid is not involved in the defense responses associated with the *ssadh* phenotype. It should be noted that DAB specifically binds to peroxidase (associated with hydrogen peroxide), which is another important component of the antioxidant machinery of the plant. The substantial increase in peroxidase-H_2O_2 complexes, as shown by in situ DAB staining in the mutant (Fig. 5), may suggest that the antioxidant defense machinery associated with *ssadh* mainly involves peroxidases, rather than ascorbic acid. These observations may reflect intracellular or even intra-organellar compartmentalization of different anti-oxidant defenses vis-á-avis the site of the GABA-shunt deficiency.

In summary, we recently provided genetic evidence for the involvement of the GABA shunt in maintaining Redox equilibrium [1]. Here, based on metabolite analysis and histological and pharmacological studies, we provide novel evidence for an intriguing link between the GABA shunt, its byproduct GHB, and ROI.

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