Targeted quantitative profiling of metabolites and gene transcripts associated with 4-aminobutyrate (GABA) in apple fruit stored under multiple abiotic stresses

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Abstract
4-Aminobutyrate accumulates in plants under abiotic stress. Here, targeted quantitative profiling of metabolites and transcripts was conducted to monitor glutamate- and polyamine-derived 4-aminobutyrate production and its subsequent catabolism to succinate or 4-hydroxybutyrate in apple (Malus x domestica Borkh.) fruit stored at 0 °C with 2.5 kPa O2 and 0.03 or 5 kPa CO2 for 16 weeks. Low-temperature-induced protein hydrolysis appeared to be responsible for the enhanced availability of amino acids during early storage, and the resulting higher glutamate level stimulated 4-aminobutyrate levels more than polyamines. Elevated CO2 increased the levels of polyamines, as well as succinate and 4-hydroxybutyrate, during early storage, and 4-aminobutyrate and 4-hydroxybutyrate over the longer term. Expression of all of the genes likely involved in 4-aminobutyrate metabolism from glutamate/polyamines to succinate/4-hydroxybutyrate was induced in a co-ordinated manner. CO2-regulated expression of apple GLUTAMATE DECARBOXYLASE 2, AMINE OXIDASE 1, ALDEHYDE DEHYDROGENASE 10A8 and POLYAMINE OXIDASE 2 was evident with longer term storage. Evidence suggested that respiratory activities were restricted by the elevated CO2/O2 environment, and that decreasing NAD+ availability and increasing NADPH and NADPH/NADP+, respectively, played key roles in the regulation of succinate and 4-hydroxybutyrate accumulation. Together, these findings suggest that both transcriptional and biochemical mechanisms are associated with 4-aminobutyrate and 4-hydroxybutyrate metabolism in apple fruit stored under multiple abiotic stresses.

Introduction
The non-protein amino acid 4-aminobutyrate (GABA) is derived from glutamate in plants exposed to various abiotic stresses via activity of the enzyme glutamate decarboxylase (GAD), which can be activated by Ca2+/calmodulin or stimulated by cytosolic acidification. In turn, GABA is converted to succinic semialdehyde (SSA) via GABA transaminase (GABA-T) and then to succinate via NAD+‐dependent succinic semialdehyde dehydrogenase (SSADH) or to 4-hydroxybutyrate (GHB) via NADPH-dependent glyoxylate/succinic semialdehyde reductase (GLYR). Much less attention has been paid to the derivation of GABA from polyamines. This can occur by the terminal catabolism of putrescine (Put) to 4-
aminobutanal/Δ1-pyrroline via O2-dependent copper-containing amine oxidases (AO) and spermidine to 4-
aminobutanal via FAD-dependent polyamine oxidases (PAO)7,8, or the non-enzymatic decarboxylation of 
proline to pyrrolidin-1-yl, which is easily converted to 4-
aminobutanal9. In turn, 4-aminobutanal can be converted to 
GABA via members of the aldehyde dehydrogenase 
(ALDH)10 family (i.e., NAD+)-dependent ALDH10A8 and 
ALDH10A97,8.

In developed countries apple (Malus × domestica 
Borkh.) fruit are stored under controlled-atmosphere 
(CA) conditions (i.e., low O2 and elevated CO2) at low 
temperature to delay ripening9,10. Low temperature, low 
O2 or elevated CO2 have been associated with GABA 
accumulation in many plant systems1,11–15 and limited O2 
availability has been associated with changes in redox 
balance16,17, which may in turn influence the activities of 
AO, ALDH10A, SSADH and GLYR1,18,19. If so, low-O2 
and elevated-CO2 storage of apple fruit at low temperature 
could inhibit the production of GABA from poly-
amines and divert glutamate-derived GABA catabolism to 
GHB20.

Many of the experimental approaches that have been 
used to investigate the pathways associated with GABA 
metabolism in plants subjected to abiotic stress1,20 are not 
suited or readily adapted to study their relative import-
ance in apples. For example, mutants are not available, 
and amine oxidase inhibitors and radiolabelled precursors 
cannot be supplied to intact fruit without perturbing the 
internal gaseous environment. Untargeted metabonomics 
and enzymatic approaches could provide a global view of 
the internal gaseous environment. Untargeted metabolomics 
cannot be supplied to intact fruit without perturbing the 
metabolites and gene transcripts were speci-
cally evaluated using formaldehyde RNA gel electrophoresis. RNA (1 μg) was treated with DNase I using the Turbo 
DNA-free kit (Applied Biosystems) according to the 
manufacturer’s protocol. For first strand cDNA synthesis, 
10–100 ng total RNA was incubated with oligo(dT)20 and 
Superscript III RT (Invitrogen) at 50 °C, followed by 55 °C 
for 30 min.

Identification of apple genes

The apple genes for three GADs, two GABA-Ts, two 
GLYRs, five AOs and two ALDH10As have been reported 
elsewhere5,7,15,24. Methods for identifying the putative 
materials and methods

Controlled-atmosphere storage

The harvest, low-temperature CA storage, and quality 
assessment in 2009 of the apple (Malus × domestica 
Borkh. cultivar ‘Empire’) fruit have been described else-
where9. Here we chose the fruit collected from orchard 2.

Immediately prior to storage, eight apples were randomly 
sampled from the bulk apples for assessment of physio-
logical disorders. Four apples were also sampled and 
rapidly frozen in liquid N2 for assessment of metabolite 
and gene transcript levels. The remaining apples were 
stored at 0 °C in two CA rooms (i.e., treatment blocks). 
Briefly, within each CA room, two random duplicate 
chambers were supplied with either 0.03 (control) or 5 
(kPa CO2-treated) kPa CO2 in combination with 2.5 kPa O2 
(i.e., a split-plot design) for 16 wk. At several times during 
storage, eight apples were randomly sampled from each 
treatment replicate for assessment of physiological dis-
orders, and four apples (i.e., subsamples) were randomly 
sampled from each treatment replicate and rapidly frozen 
in liquid N2. All frozen apples were stored for several 
months before being individually pulverized to powder 
using an arbor press, taking care to ensure that thawing 
did not occur, before being stored at −80 °C. None of the 
freshly collected or stored fruit showed signs of flesh 
browning or senescent breakdown21, but external CO2 
injury (i.e., bronze- to brown-colored, rough uneven 
lesion with sunken areas on the peel) was evident on fruit 
receiving 5 kPa CO2; the incidence was 37, 51, 70 and 
85%, respectively, after 2, 4, 8 and 16 weeks of storage9.

Extraction and analysis of metabolites

The frozen apple fruit powders were extracted within 
1–3 years of harvest using various protocols, depending 
on the metabolites under consideration. The levels of 
various amino acids, including GABA, and the free forms 
of putrescine, spermidine and spermine were determined 
by reverse-phase high performance liquid chromatogra-
phy as described elsewhere14,23. Detailed protocols for 
the extraction and GC-MS determination of GHB, and 
the enzymatic determination of succinate and pyridine 
dinucleotides are given in the Supplementary Information 
Materials and Methods S1.

RNA extraction and cDNA synthesis

RNA was isolated from the frozen apple fruit powders 
for three treatment blocks within a year of harvest 
especially as described elsewhere15. RNA integrity was 
verified using formaldehyde RNA gel electrophoresis. 
RNA (1 μg) was treated with DNase I using the Turbo 
DNA-free kit (Applied Biosystems) according to the 
manufacturer’s protocol. For first strand cDNA synthesis, 
10–100 ng total RNA was incubated with oligo(dT)20 and 
Superscript III RT (Invitrogen) at 50 °C, followed by 55 °C 
for 30 min.

Identification of apple genes

The apple genes for three GADs, two GABA-Ts, two 
GLYRs, five AOs and two ALDH10As have been reported 
elsewhere5,7,15,24. Methods for identifying the putative
apple SSADH, ALANINE TRANSAMINASE and POLY-AMINE OXIDASE genes are described in Supplementary Information Materials and Methods S2.

Quantitative real-time PCR

Primers used for quantitative polymerase chain reaction (qPCR) were designed using Primer Express 3 software (Applied Biosystems) with the following default conditions: 60°C primer melting temperature; 50–80 bp amplicon length; and, 40–60% primer GC content. The list of primers used here is provided in Supplementary Information Table S3. It was not possible to design primers that enabled separate monitoring of the two distinct apple GABA-T genes. Quantitative PCR was performed in a 96-well plate iQ5 Multicolor Real-Time PCR Detection System (BioRad) as previously described. Dissociation curve analysis was performed after 40 cycles of qPCR to ensure the presence of a single PCR product. Efficiency of the primer pairs ranged from 90 to 105%. The data were analyzed and relative expression calculated using the $2^{-\Delta\Delta C_t}$ method. The expression of each target gene was normalized to the housekeeping apple ELONGATION FACTOR-1a (EF-1a) gene (MD0000294265). Each treatment replicate was analyzed in duplicate.

Statistical analysis

The data were analyzed as a completely randomized design with a split-plot design using ANOVAs (Proc Mixed method of SAS® software). Since there was no block effect for the incidence of external and internal disorders, time course data for levels of metabolites and transcripts are presented as means of four and three treatment replicates, respectively. Four apples were subsampled periodically from each treatment replicate; these were considered as repeated measures. Assumptions of randomness, homogeneity, and independence of errors were confirmed using plots of residuals, as well as a Shapiro–Wilk test for normality. Treatment means were compared within and across weeks using the Tukey’s Least Significance Difference method for multiple comparisons at the 95% confidence level.

The relationships among metabolites and transcripts were assessed by Pearson’s correlation test using R and corrected for false discovery rate. The relationships among metabolites and transcripts was also assessed by principal component analysis (PCA) using R. Replicates with missing subsample values for a variable/treatment were removed from the analysis. In some cases, the data set for each variable was not normally distributed when expressed on their original scale; therefore, the Shapiro–Wilk test for normality was performed on each variable individually. If a variable deviated from normality, boxcox transformation was used to identify transformed variables that approximate normality. Then the variables were scaled to a mean of 0 and a variance of 1 prior to PCA.

Results

Levels and ratios of pyridine dinucleotides

The major non-phosphorylated and phosphorylated pyridine dinucleotides in both freshly harvested and stored ‘Empire’ fruit were the reduced forms. The concentrations of NAD$^+$, NADH and NADP$^+$ in both control and CO$_2$-treated fruit generally decreased with storage time, whereas NADPH increased, resulting in lower levels of NAD(H) and higher levels of NADP(H) (Fig. 1a–f). Overall, there was an approximately 60% decrease in the concentrations of total pyridine dinucleotides (Fig. 1g), a 50% decrease in the NADH/NAD$^+$ ratio (Fig. 1i), and a two-fold increase in NADPH/NADP$^+$ ratio (Fig. 1h). The NADH/NAD$^+$ and NADPH/NADP$^+$ ratios ranged from 20–70 and 60–150, respectively.

Levels of GABA and closely related metabolites and gene transcripts

The major amino acids in both freshly harvested and stored fruit were aspartate, asparagine and glutamate (Supplementary Information Table S4). In control fruit, the concentrations of total amino acids (TAA), GABA and GABA-related amino acids (i.e., glutamate and alanine) increased to a maximum within 2–4 weeks, and then declined to their original levels after 8–16 weeks (Fig. 2a–d). There was also a transient peak in GHB, but this was delayed in comparison to the amino acids (Fig. 2e). The concentration of succinate was low and steady over the entire storage period (Fig. 2f). Storage with 5 kPa CO$_2$ significantly increased the concentrations of alanine, GHB and succinate early during storage, and GHB and GABA later in the storage period. Notably, the concentration of GHB is much lower than succinate and also increasing with high CO$_2$ when succinate is decreasing.

The transcript abundance of ALA-T, which could be considered as a reliable indicator of hypoxic conditions, reached a maximum in the control within 2 wk of storage, and then levelled off for the remaining storage time (Fig. 3i). Treatment with CO$_2$ further enhanced ALA-T transcript abundance at 4–8 weeks. Of the GAD transcripts in the control, GAD1 was most abundant, GAD2 was moderately abundant, and GAD3 was lowly abundant (Fig. 3a–c). The abundance of GAD1 and GAD2 transcripts increased linearly up to 4–8 weeks and then levelled off, whereas the abundance of GAD3 transcript declined after a transient peak. Treatment with CO$_2$ delayed the increase in GAD1 transcript abundance, and increased the maximal abundance of GAD2 transcript late in the storage period. GABA-T1,2 (Fig. 3d) and SSADH1...
Fig. 1 (See legend on next page.)
(Fig. 3g) transcripts were moderately abundant in the control and displayed similar CO2 responses and patterns as GAD1, whereas the SSADH 2 (Fig. 3h) transcript was much less abundant and rapidly declined in a CO2-independent manner with storage time. The transcripts for GLYR1 and GLYR2 were moderately abundant and transiently increased by ~1-fold early in the storage period (Fig. 3e–f). Treatment with CO2 decreased abundance of the GLYR1 and GLYR2 transcripts during mid and early storage, respectively.
In silico analysis of putative apple PAOs

In silico analysis revealed that the six putative apple PAO genes encode proteins ranging from 488 to 533 amino acids and from 24% (PAO1 and PAO6) to 91% (PAO3 and PAO4) sequence identity (Supplementary Information Table S5). Apple PAO1 is 72% identical to Arabidopsis PAO1, apple PAO2 is 79% identical to Arabidopsis PAO2, and apple PAO3 is 60% identical to Arabidopsis PAO3. In particular, apple PAO5 and apple PAO6 have a high degree of identity (90%) to each other, as well as Arabidopsis PAO5.

Sequence comparison and phylogenetic analysis of the six putative apple PAO genes to known Arabidopsis PAOs reveal that they can be divided into three distinct groups as described for Arabidopsis.35 Apple PAO2, PAO3 and PAO4, together with Arabidopsis PAO2, PAO3 and PAO4 form a cluster possessing a peroxisome targeting signal 1 (Supplementary Information Figure S1a–b). Arabidopsis members of this group are localized in the peroxisome4, and similar subcellular localization is predicted for apple members (PAO2–4) of this group. Moreover, apple PAO1 clusters with apple PAO1, whereas apple PAO5, apple PAO6 and Arabidopsis PAO5 cluster separately. Arabidopsis PAO1 and PAO5 appear to encode cytosolic proteins; therefore, apple PAO5 and PAO6 are predicted to be cytosolic.

Levels of polyamines and expression of genes associated with their catabolism to GABA

The major polyamines in both freshly harvested and stored fruit were putrescine and spermidine, with minor concentrations of spermine. The concentrations of putrescine and spermidine were relatively steady in the control over the storage period, whereas spermine slowly declined (Fig. 4a–d). Treatment with CO2 increased the concentrations of all polyamines early in the storage period, but only spermine was higher than the control over the entire period. In general, concentrations of the polyamines were much lower than those for the GABA-related amino acids (Fig. 2b–d).

Of the six apple PAO genes identified, only the transcripts for PAO2 and PAO4 genes were readily detected in fruit (Fig. 5a, b). The PAO2 transcript was slightly more abundant than the PAO4 transcript, although both peaked midway through the storage period. The AO2 transcript was the most abundant of the five apple AO genes, and peaked late in storage (Fig. 5c–e). The AO1 transcript was moderately abundant and peaked within 2–4 weeks. Any significant responses of these aforementioned PAO and AO transcripts to elevated CO2 seemed to be transient. The AO3–5 transcripts were present in low abundance and peaked transiently at 4–8 weeks; AO4 appeared to show CO2-dependent stimulation during late storage (Fig. 5e–g). The ALDH10A transcripts were moderately abundant and peaked by 2–4 weeks, although a positive response to elevated CO2 tended to be delayed (Fig. 5h–i).

Correlation and principal component analyses of metabolite and gene transcript levels

Correlation analysis was performed on the entire data set of metabolites and gene transcripts from apples subjected to low-temperature/low-O2 storage at two CO2 levels by calculation of the Pearson’s correlation coefficient for each metabolite/metabolite or metabolite/trascript pair (Fig. 6a). Notably, GABA and GHB were not significantly correlated with each other, nor with any of the other metabolites measured, including glutamate and NADPH/NADP+. Positive correlations were found among various metabolites and transcripts: glutamate with alanine; alanine with glutamate, TAA, succinate and GAD1; succinate with alanine, putrescine, spermine and total polyamines; total polyamines with succinate, putrescine, spermidine and spermine; putrescine with succinate, spermine and total polyamines; spermidine with total polyamines; spermine with succinate, putrescine, spermidine and total polyamines; and, spermine with putrescine, spermidine and total polyamines. NADPH/NADP+ was positively correlated with NADPH, NADP+ and NADP (H), and negatively correlated with NAD+. Also, there were significant positive correlations among various transcripts: GAD1 with GABA-T, SSADH1, AO2, ALDH10A8 and ALDH10A9; GABA-T with GAD1, SSADH1, AO2 and ALDH10A8, ALDH10A9 and PAO4; GAD1 with SSADH1, AO4 and ALDH10A9; SSADH1 with GADI, GABA-T, GLYR2, AO2, AO4, ALDH10A9, ALDH10A8 and PAO2; AO2 with GADI, GABA-T, SSADH1, ALDH10A8 and ALDH10A9; AO4 with SSADH1, AO5, GLYR2 and ALDH10A9; ALDH10A9 with GAD1, GABA-T, GLYR2, SSADH1, AO2 and AO4, ALDHA8 and PAO4; ALDH10A8 with GADI, GABA-T, SSADH1, AO2, ALDH10A9 and PAO4; PAO2 with SSADH1; PAO4 with GABA-T, ALDH10A9 and ALDH10A8; and, SSADH2 with GAD3.

The metabolite and transcript data set was also examined by PCA. The score plot suggests that PC1 captures the variability between early and late storage periods, and PC2 captures the variability between CO2 treatments (Fig. 6b). Hence, the biological variables in the loading plot can be discussed in these terms (Fig. 6c). GABA responded slightly to CO2, but not storage, whereas GHB responded slightly and moderately to storage and CO2, respectively. The pyridine dinucleotides and the NAD(P)H/NAD(P)+ ratio moderately responded to storage, and NADP+ in particular strongly responded to CO2. Several metabolites (i.e., succinate, spermine, putrescine, alanine and glutamate), including TAA, moderately to strongly responded to storage, and succinate, putrescine and
Fig. 3 Impact of elevated CO₂ on the expression of genes associated with the biosynthesis and catabolism of GABA from glutamate in 'Empire' apple frui under low-temperature/low-O₂ storage for up to 16 weeks. Panels a-i represent time-course profiles for GAD1, GAD2, GAD3, GABA-T₁,₂, GLYR₁, GLYR₂, SSADH₁, SSADH₂ and ALA-T, respectively. Storage conditions: 0 °C, 2.5 kPa O₂ and 5 kPa (●) or 0.03 kPa (○) CO₂. All the data represent the mean of three treatment replicates, each being the average of three subsamples. The error bar above 0 weeks represents the least significant difference at the P ≤ 0.05 level. Note that the y-axis varies among the panels. ALA-T alanine dehydrogenase, GABA-T GABA transaminase, GAD glutamate decarboxylase, GLYR glyoxylate reductase, SSADH succinic semialdehyde dehydrogenase.
spermine strongly responded to CO2. Many gene transcripts (i.e., GAD2, PAO2, AO4, GLYR2, PAO4, SSADH1, ALDH10A8, AO2, GAD1, GABA-T and ALDH10A9) moderately to strongly responded to storage, and several of these (i.e., GAD2, PAO2, ALDH10A8, AO2) moderately responded to CO2. Other transcripts strongly responded to CO2, but only slightly to storage (i.e., ALA-T, AO1, SSADH2 and GAD3).

**Discussion**

Metabolite relationships

The ratios of reduced to oxidized pyridine dinucleotides in freshly harvested and low-temperature CA-stored ‘Empire’ apples (Fig. 1) were much higher than in photosynthesizing unstressed Arabidopsis leaves13,33, and ALA-T expression was induced and maintained during storage (Fig. 3). Furthermore, the NADH/NAD\(^+\) ratio declined over the storage period, and the NADPH/NADP\(^+\) ratio increased, particularly during late storage, regardless of the treatment regimen (Fig. 1). These findings are consistent with the idea that intact ‘Empire’ apples are already in a reduced state and metabolism is O\(_2\)-limited (i.e., hypoxic) at harvest, and that this becomes increasingly so with storage1,16,17,19,20,31.

The major amino acids in both freshly harvested and stored fruit were aspartate, asparagine and glutamate (Fig. 2; Supporting Information Table S4), whereas polyamines consisted of similar levels of putrescine and spermidine, together with minor levels of spermine (Fig. 4). TAA and GABA-related amino acids transiently accumulated early in storage, regardless of the CO2 regimen (Fig. 2). This was accompanied by relatively stable levels of polyamines, succinate and GHB with ambient CO2, but transient accumulation of these same metabolites with elevated CO2 (Figs. 2 and 4). Notably, the succinate level further declined thereafter, whereas GABA and GHB increased over the longer term. These findings are in general agreement with previous reports of dynamic changes in proteins, proteolytic activity, amino acids and polyamines in apple fruit stored under low-temperature conditions, in the absence or presence of low O\(_2\) and elevated CO234–37. Thus, it can be suggested that the low temperature stimulated protein hydrolysis during early storage, thereby temporarily increasing the pools of amino acids available for various metabolic processes. The elevated glutamate level in particular seemed to influence the relative level of GABA much more than polyamines, despite lower concentrations of the polyamines. Further
Fig. 5 Impact of elevated CO₂ on the expression of genes associated with the catabolism of polyamines to GABA in 'Empire' apple fruit under low-temperature/low-O₂ storage for up to 16 wk. Panels a-i represent time-course profiles for PAO2, PAO4, AO1, AO2, AO3, AO4, AO5, ALDH10A9 and ALDH10A8, respectively. Storage conditions: 0 °C, 2.5 kPa O₂ and 5 kPa (●) or 0.03 kPa (○) CO₂. All the data represent the mean of three treatment replicates, each being the average of three subsamples. The error bar above 0 wk represents the least significant difference at the p ≤ 0.05 level. Note that the y-axis varies among the panels. AO Amine Oxidase, ALDH Aldehyde dehydrogenase, PAO Polyamine oxidase.
research is required to establish whether this result can be explained by differences in the utilization (e.g., substrate affinity and/or localization) of glutamate by GAD or biosynthetic enzymes for arginine, the primary precursor for polyamines. Also, it can be suggested that elevated CO$_2$ had distinct effects on the production of polyamines, as well as succinate and GHB, during early storage, and on GABA and GHB production over the longer term (see “Metabolite-transcript relationships”). Overall, a complex pattern of GABA-related metabolites could be recognized in ‘Empire’ apple exposed to a combination of low temperature/low O$_2$ and elevated CO$_2$. 

Fig. 6 (See legend on next page.)
Transcript relationships

Figure 7 contextualizes our current understanding of stress-induced GABA production in apple fruit from the decarboxylation of glutamate and the catabolism of polyamines. The route from glutamate to GABA is probably catalyzed by two of the three cytosolic MdGADs (MdGAD1 and MdGAD2), which are abundant and interact with Ca²⁺/calmodulin²⁴. Putrescine and spermidine also represent potential sources of GABA via the metabolite 4-aminobutanal. Spermidine and spermine, respectively, are known to be back-converted to putrescine and/or spermidine in dicotyledonous plants¹⁴, and preliminary assessment here, based on in silico analysis, suggests that specific apple FAD-polyamine oxidases (PAO2,4) are peroxisomal (Supplementary Information Figure S1). Five copper AOs are present in ‘Empire’ apple fruit, but only one of the two most abundant forms (i.e., AO1) is peroxisomal and prefers diamines as

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**Fig. 7 Model for the induction and subcellular localization of enzymes associated with GABA and GHB metabolism in ‘Empire’ apple fruit stored under low temperature, low O₂, and elevated CO₂ conditions.** Bold blue lettering represents known biochemically characterized enzymes, whereas bold white lettering and dashed arrows represent putative polyamine oxidases, which were localized to the peroxisome using in silico comparisons with Arabidopsis orthologs (see Supplementary Information Fig. S1). 4-ABAL 4-aminobutanal, DAP 1,3-diaminopropane, SSA succinic semialdehyde, TCA tricarboxylic acid. For other abbreviations, see Fig. 6.
substrates\(^5\). Two putative peroxisomally-located, NAD\(^+\)-dependent MdALDH10As can convert 4-aminobutanal to GABA\(^7\). Therefore, AO1 and the ALDH10As represent a likely path for putrescine oxidation to GABA in apple fruit.

In the present study, the apple fruit genes associated with GABA anabolism from both glutamate and polyamines, and with GABA catabolism to succinate were all co-ordinately upregulated by low temperature/low O\(_2\) with ambient CO\(_2\) (Figs 3, 5, 6, 7). Furthermore, there was evidence for CO\(_2\)-upregulated expression of GAD2, AO1, ALDH10A8 and PAO2 with mid- to long-term storage (Fig. 6). Previous studies have attempted to directly link transcriptome changes to stress-induced metabolite pool sizes are not by themselves very informative in addressing mechanisms\(^{44}\). For example, the first peak of GHB is difficult to reconcile on the basis of redox balance alone. Previous studies have reported that succinate accumulates continuously for up to 50 h in rice germinating under anoxia, with GHB accumulating prior to GABA\(^1\), and a lack of correlation between GLYR expression and GHB accumulation in submerged Arabidopsis\(^{18,19}\). Another study has demonstrated that a transient increase in GHB level in shoots of chilled Arabidopsis plants follows a transient increase in GABA and it is independent of GLYR expression\(^1\). However, there is a concomitant and sustained accumulation of succinate, which can be interpreted as support for the operation of a non-conventional TCA cycle\(^1\). Additional research is required to determine if stress-induced peroxidation of phospholipids containing 4-hydroxybutyryl chains generates GHB in apple fruit, as it does in mammals\(^{49–53}\), or if the prolonged storage period alters the carbon/nitrogen balance, resulting in carbon limitation of the TCA cycle and diversion of GABA carbon from succinate to GHB\(^1,20,54\).

Together, these findings suggest that both transcriptional and biochemical mechanisms are associated with GABA and GHB metabolism in apple fruit stored under multiple abiotic stress conditions. Exploration of the function of GHB in model plants such as Arabidopsis during exposure to elevated CO\(_2\), low O\(_2\) and/or low temperature, using single and double overexpression or knockout mutants of GABA-T and GLYR, is warranted.

**Disclaimer**

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Author contributions

B.J.S. conceived the idea, and B.J.S. and G.G.B. supervised the work. J.R.D. supplied the apples, and J.L., G.R.B. and B.J.S. conducted the experiment; C.J.B., A.Z., G.Z.C., K.L.D. and G.H. conducted the metabolite and transcript analyses and related ANOVAs; A.Z. and C.P.T. identified the novel apple genes; and S.S. conducted the correlation analyses. J.R.D. discussed the project. B.J.S., C.J.B., A.Z., G.Z.C., K.L.D., G.G.B. and S.S. wrote and/or edited the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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Supplementary Information

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