Not Just a Cycle: Three *gab* Genes Enable the Non-Cyclic Flux Toward Succinate via GABA Shunt in ‘*Candidatus Liberibacter asiaticus*’–Infected Citrus

Yasser Nehela^{1,2} and Nabil Killiny^{1,†}

¹ Department of Plant Pathology, Citrus Research and Education Center, University of Florida, 700 Experiment Station Rd., Lake Alfred, FL 33850, U.S.A.

² Department of Agricultural Botany, Faculty of Agriculture, Tanta University, Tanta, Egypt

Accepted 11 November 2021.

Although the mitochondria retain all required enzymes for an intact tricarboxylic acid (TCA) cycle, plants might shift the cyclic flux from the TCA cycle to an alternative noncyclic pathway via γ -aminobutyric acid (GABA) shunt under specific physiological conditions. We hypothesize that several genes may ease this noncyclic flux and contribute to the citrus response to the phytopathogenic bacterium ‘*Candidatus Liberibacter asiaticus*’, the causal agent of Huanglongbing in citrus. To test this hypothesis, we used multiomics techniques (metabolomics, fluxomics, and transcriptomics) to investigate the potential roles of putative *gab* homologies from Valencia sweet orange (*Citrus sinensis*). Our findings showed that ‘*Ca. L. asiaticus*’ significantly increased the endogenous GABA and succinate content but decreased ketoglutarate in infected citrus plants. Citrus genome harbors three putative *gab* genes, including amino-acid permease (also known as GABA permease; *CsgabP*), GABA transaminase (*CsgabT*), and succinate-semialdehyde dehydrogenase (also known as GABA dehydrogenase; *CsgabD*). The transcript levels of *CsgabP*, *CsgabT*, and *CsgabD* were upregulated in citrus leaves upon the infection with ‘*Ca. L. asiaticus*’ and after the exogenous application of GABA or deuterium-labeled GABA isotope (GABA-D₆). Moreover, our finding showed that exogenously applied GABA is quickly converted to succinate and fed into the TCA cycle. Likewise, the fluxomics study showed that GABA-D₆ is rapidly metabolized to succinate-D₄. Our work proved that GABA shunt and three predicated *gab* genes from citrus, support the upstream noncyclic flux toward succinate rather than an intact TCA cycle and contribute to citrus defense responses to ‘*Ca. L. asiaticus*’.

Keywords: citrus, GABA, *gab* genes, GABA permease (*gabP*), GABA transaminase (*gabT*), GABA dehydrogenase (*gabD*), Huanglongbing, *Liberibacter*, TCA cycle

More than 700 destructive plant diseases are caused by vector-borne phytopathogenic microorganisms that cause high-impact losses in economic crops worldwide (Killiny 2016; Nehela and Killiny 2020c; Nicaise 2014; Strange and Scott 2005). Huanglongbing (HLB), also known as citrus greening disease, is the most devastating vector-borne bacterial disease in citrus across several geographical regions worldwide (Gottwald 2010; Nehela and Killiny 2020c). HLB is associated with the plant-fastidious, phloem-limited, gram-negative α -proteobacterium ‘*Candidatus Liberibacter* spp.’ (Bové 2006; Garnier et al. 2000; Gottwald 2010; Jagoueix et al. 1994), however, the Koch’s postulates have not yet been fulfilled due to the difficulty in culturing this putative pathogen. Based on the 16S rhizobial DNA sequence of ‘*Ca. Liberibacter* spp.’ and their geographical distribution, three species have been suggested to be associated with HLB: ‘*Ca. L. asiaticus*’ in Asia and the Americas (Bové 2006; Gottwald et al. 2010), ‘*Ca. L. africanus*’ in Africa (da Graça 1991), and ‘*Ca. L. americanus*’ in Brazil (Teixeira et al. 2005). However, circumstantial evidence indicates that ‘*Ca. L. asiaticus*’ is the most dominant *Liberibacter* species (Bové 2006; Gottwald et al. 2010).

As a vector-borne disease, HLB is widely spread by two psyllid vectors, the Asian citrus psyllid *Diaphorina citri* Kuwayama (Hemiptera: Liviidae) that transmits ‘*Ca. L. asiaticus*’ and ‘*Ca. L. americanus*’ and the African psyllid *Trioza erytreae* Del Guercio (Hemiptera: Triozidae) that transmits ‘*Ca. L. africanus*’ (Bové 2006; da Graça 1991; Gottwald 2010). Nevertheless, the three HLB forms (Asian, African, and American form) might produce similar symptoms that are usually more noticeable during cooler seasons than warmer ones (McCollum and Baldwin 2016; Nehela and Killiny 2020c). Unfortunately, HLB infects most, if not all, citrus varieties without a known sustainable cure; though, some varieties have been described to be more susceptible than others (Cevallos-Cevallos et al. 2012; Folimonova et al. 2009; Hijaz et al. 2016; Killiny 2017; Killiny and Hijaz 2016; Killiny et al. 2017, 2018a). However, the molecular mechanisms behind the pathogenicity of ‘*Ca. L. asiaticus*’ and the internal factors behind the susceptibility or tolerance of the host plant are poorly investigated. We believe that plant metabolites are key players in the host-pathogen-vector interactions during the progression of HLB.

It has been proposed that plant metabolites are responsible for securing the energy requirements for plant responses (Bolton 2009; Kangasjärvi et al. 2012); nevertheless, this is not the main role. The alteration in plant metabolites is directly involved in plant defense responses. Previously, we showed that infection with ‘*Ca. L. asiaticus*’ causes numerous biochemical and metabolic changes in citrus plants (Nehela and Killiny 2020c) as well as its insect vector (Nehela and Killiny 2018a, 2021;

[†]Corresponding author: N. Killiny; nabillkilliny@ufl.edu

Funding: This work was generously supported by a grant to N. Killiny from the University of Florida Citrus Initiative Program.

The author(s) declare no conflict of interest.

Copyright © 2022 The Author(s). This is an open access article distributed under the CC BY-NC-ND 4.0 International license.

Killiny et al. 2018b). These changes include but are not limited to volatile organic compounds (Hijaz et al. 2013), carboxylic compounds (Killiny and Nehela 2017a), leaf pigments (Killiny and Nehela 2017b), phytohormones (Nehela and Killiny 2018b; Nehela et al. 2018), nonproteinogenic amino acids (Nehela and Killiny 2019), polyamines (Killiny and Nehela 2020; Nehela and Killiny 2019, 2020d), melatonin (Nehela and Killiny 2020a, b), and the tricarboxylic acid (TCA)-associated compounds (Nehela and Killiny 2019).

The TCA (also known as the citric acid cycle or Krebs cycle) is a ubiquitous iconic metabolic hub of the metabolism of aerobic organisms to ensure the required cellular energy via the oxidation of carbohydrates, lipids, and amino acids under aerobic conditions (Sweetlove et al. 2010). Even though the mitochondria retain all required enzymes for an intact TCA cycle, metabolic network modeling studies suggest that the TCA cycle in plants may work as independent or partial sets of reactions based on the metabolic and physiological requirements of the cells (Sweetlove et al. 2010). Therefore, plants might have one or more alternative pathways that may contribute to this noncyclic flux mode. These alternative fluxes might occur in plant cells under specific physiological circumstances such as biotic and abiotic stress (Che-Othman et al. 2020; Michaeli et al. 2011; Nehela and Killiny 2019; Sweetlove et al. 2010).

Gamma-aminobutyric acid (GABA) shunt is a key alternative route that supports the noncyclic flux toward succinate outside the TCA cycle (Fait et al. 2008) and it has been reported previously that the GABA shunt and the TCA cycle are functionally linked (Michaeli et al. 2011; Nehela and Killiny 2019) and several *gab* genes play a key regulatory role in this connection. For instance, in bacteria, the *gab* operon handles the conversion of GABA to succinate. The well-characterized *gab* operon of *Escherichia coli* (*gabDTPC*) comprised of three structural genes that encode for a succinate semialdehyde dehydrogenase (*gabD*), GABA transaminase (*gabT*), and a GABA-specific permease (*gabP*) (Schneider et al. 2002).

In plants, a GABA-TCA connection requires the activity of several evolutionarily conserved enzymes that bypass two steps of α -ketoglutarate-to-succinate conversion outside the mitochondrial TCA cycle (Bouché et al. 2003b; Che-Othman et al. 2020; Fait et al. 2008; Michaeli et al. 2011; Nehela and Killiny 2019; Shelp et al. 1999). Briefly, citrate is catalyzed to α -ketoglutarate via the activity of aconitase (*ACO*) and isocitrate dehydrogenase (*IDH*), then to glutamate via glutamate dehydrogenase (*GDH*) and glutamate synthase (*GS*) activity. Then, glutamate is decarboxylated into GABA via irreversible reaction by the activity of the cytosolic enzyme glutamate decarboxylase (*GAD*), which consumes a proton and releases CO_2 , causing an accumulation of GABA in the cytosol. Subsequently, cytosolic GABA is transported into the mitochondria via the activity of the mitochondrial *gabP* (Michaeli et al. 2011; Nehela and Killiny 2019), then catabolized to succinic semialdehyde via the activity of GABA aminotransferase (*gabT*, also known as GABA transaminase). The later step paves the way for succinate formation using succinate semialdehyde dehydrogenase (*SSADH*; also known as *gabD*).

Although there is indirect evidence from *in folio* isotopic fluxomics and metabolic network modeling studies for the presence of mitochondrial GABA transporters in eukaryotes, none have yet been identified in land plants. Recently, we proved that both the GABA shunt and the TCA cycle in the economically important nonmodel citrus plants are functionally linked and GABA permease plays a key role in this connection (Nehela and Killiny 2019). Previously, we showed that '*Ca. L. asiaticus*' and its vector, *D. citri*, augment the TCA cycle of their host plant via the GABA shunt and polyamines pathway (Nehela and Killiny 2019). Moreover, using *in silico* analysis, we roughly identified a GABA permease protein from *Citrus sinensis* (*CsgabP*) encoded by the

locus LOC102610833 (amino-acid permease BAT1-like isoform X1; accession number XP_006468761.1; 521 aa), which was relatively high-homology with *AtGABP* from *A. thaliana* and was upregulated in '*Ca. L. asiaticus*'-infected plants (Nehela and Killiny 2019). However, our knowledge about other *gab* proteins (*gabT* and *gabD*) is limited and none of these enzymes have been previously identified from citrus yet.

We hypothesize that *gab* genes (*gabP*, *gabT*, and *gabD*) facilitate shifting the cyclic flux of the TCA cycle to an alternative noncyclic pathway via GABA shunt and might contribute to the citrus response to '*Ca. L. asiaticus*'. To test this hypothesis, we comprehensively used multiomics techniques including metabolomics, fluxomics, and transcriptomics to quantify the endogenous levels of GABA shunt-related compounds (α -ketoglutarate, GABA, and succinate) and evaluate the expression patterns of the predicted genes (*gabP*, *gabT*, and *gabD*) within the healthy and '*Ca. L. asiaticus*'-infected Valencia sweet orange (*C. sinensis*) trees after the treatment with exogenous GABA and the nonradioactive isotope, deuterium-labeled GABA (GABA-D₆). The importance of the current study is not only to decipher the role of *gab* genes in citrus response to '*Ca. L. asiaticus*' infection but to possibly explicate the molecular and functional connection between the GABA shunt and the TCA cycle. Ultimately, dissecting the metabolic changes and their associated genes in citrus plants after '*Ca. L. asiaticus*' infection could lead to a comprehensive picture of defense responses to HLB, which is critical to find novel and sustainable management strategies for HLB.

RESULTS

The citrus genome possesses three putative *gab* genes.

To better understand the regulatory role or roles of *gab* genes in the functional connection between ketoglutarate and succinate outside the TCA cycle via GABA shunt, the predicted *gab* genes in sweet orange (*C. sinensis*) were dissected using a comparative *in silico* analysis. Putative candidate *gab* genes are presented in Table 1 as the top-matched sequences with *gab* genes from the model plant *Arabidopsis thaliana*. Briefly, the citrus genome harbors three putative *gab* genes, including GABA permease (*CsgabP*), GABA transaminase (*CsgabT*), and GABA dehydrogenase (*CsgabD*) (Table 1; Fig. 1). i) *CsgabP* was identified in two different loci (LOC102610833 and LOC102627227), which are located on chromosome 2, NC_023047.1. Locus LOC102610833 encodes for amino-acid permease BAT1-like isoform X1 (GenBank accession number XP_006468761.1; 521 aa) and isoform X2 (GenBank accession number XP_006468762.1; 419 aa), whereas locus LOC102627227 encodes for one amino-acid permease, BAT1 (GenBank accession number XP_006469954.1; 482 aa) (Fig. 1A and B). ii) *CsgabT* was identified in only one locus (LOC102624895), which is located on chromosome 6, NC_023051.1 (Fig. 1A), and encodes for GABA transaminase 3, chloroplastic (GenBank accession number XP_006481368.1; 514 aa) (Fig. 1C). iii) *CsgabD* was found in a separated locus (LOC102625593), which is located on an uncharacterized chromosome, NW_006257118.1 (Fig. 1A), and encodes for three copies of SSADH, mitochondria include isoform X1 (GenBank accession numbers XP_024949454.1 and XP_015381305.1; 535 aa), isoform X2 (GenBank accession number XP_006493749.1; 530 aa), and isoform X3 (GenBank accession number XP_024949455.1; 495 aa) (Fig. 1D).

'*Ca. L. asiaticus*' infection alters the GABA shunt-associated compounds and upregulated the expression of predicated *gab* genes.

The impact of '*Ca. L. asiaticus*' infection on the endogenous levels of ketoglutarate, succinate, and GABA, as well as the expression of predicated *gab* genes in the leaves of 20-month-old

Valencia sweet orange was studied under greenhouse conditions (Fig. 2). Targeted metabolomics showed that ketoglutarate was significantly decreased in ‘*Ca. L. asiaticus*’-infected leaves at 8 months postinoculation compared with healthy (control) plants ($P < 0.0001$) (Fig. 2A and B). However, the endogenous GABA content in ‘*Ca. L. asiaticus*’-infected leaves was significantly higher than healthy ones ($P < 0.0001$) (Fig. 2C and D). Likewise, the endogenous succinate level was also increased significantly in the presence of ‘*Ca. L. asiaticus*’ compared with healthy leaves ($P < 0.0001$) (Fig. 2E and F).

Furthermore, real-time quantitative PCR (qPCR) was used to quantify gene expression of the three predicated *gab* genes (*CsgabP*, *CsgabT*, and *CsgabD*) from citrus leaves after challenging with ‘*Ca. L. asiaticus*’ infection (Fig. 2G). Briefly, the transcript level of *CsgabP* was increased significantly in ‘*Ca. L. asiaticus*’-infected leaves compared with healthy (control) ones (Fig. 2G). Likewise, *CsgabT* was upregulated (almost threefold) in ‘*Ca. L. asiaticus*’-infected leaves (Fig. 2G), whereas the expression level of *CsgabD* was increased more than fivefold in ‘*Ca. L. asiaticus*’-infected leaves compared with healthy (control) ones (Fig. 2G).

GABA supplementation increased the accumulation of GABA and succinate but not ketoglutarate in treated citrus leaves.

To better understand the impact of GABA accumulation on GABA shunt-related compounds (ketoglutarate, GABA, and succinate), we investigated the effect of exogenous GABA supplementation on the endogenous levels of these compounds, in the leaves of 20-month-old healthy and ‘*Ca. L. asiaticus*’-infected Valencia sweet orange under greenhouse conditions (Fig. 3). Generally, exogenous GABA supplementation significantly decreased the endogenous levels of ketoglutarate in both healthy and ‘*Ca. L. asiaticus*’-infected leaves ($P = 0.0193$ and $P = 0.0046$, respectively) (Fig. 3A). However, the presence of ‘*Ca. L. asiaticus*’ considerably stimulated the reduction of ketoglutarate in GABA-treated ‘*Ca. L. asiaticus*’-infected leaves compared with GABA-treated healthy ones ($P = 0.0030$) (Fig. 3A and B). On the other hand, a significant increase in endogenous GABA level was observed in both GABA-treated healthy and ‘*Ca. L. asiaticus*’-infected citrus leaves (Fig. 3C and D). It is worth noting that the presence of ‘*Ca. L. asiaticus*’ facilitated the accumulation of endogenous GABA in GABA-treated infected leaves compared

Table 1. Top matched sequences of *gab* genes from *Citrus sinensis* produce significant alignments with query sequences from *Arabidopsis thaliana*

Gene description	NCBI database ^a			Citrus sinensis genome database ^b	
	Gene ID	Accession no.		Gene	Protein accession no.
		mRNA	Protein		
<i>γ</i> -Aminobutyric acid (GABA) permease (<i>CsgabP</i>) ^c					
Amino-acid permease BAT1-like isoform X1 (<i>CsgabP</i> -1)	LOC102610833	XM_006468698.3	XP_006468761.1	<i>BAT1</i>	orange1.1g011598m
Amino-acid permease BAT1-like isoform X2 (<i>CsgabP</i> -2)	LOC102610833	XM_006468699.3	XP_006468762.1	<i>BAT1</i>	orange1.1g011598m
Amino-acid permease BAT1 (<i>CsgabP</i> -3)	LOC102627227	XM_006469891.3	XP_006469954.1	<i>BAT1</i>	orange1.1g011598m
GABA transaminase (<i>CsgabT</i>) ^d					
GABA transaminase 3, chloroplastic	LOC102624895	XM_006481305.3	XP_006481368.1	<i>POP2</i>	orange1.1g010250m
<i>GABA dehydrogenase (CsgabD)</i> ^e					
Succinate-semialdehyde dehydrogenase, mitochondrial isoform X1	LOC102625593	XM_015525819.2	XP_015381305.1	<i>ALDH5F1</i> <i>ALDH5F1</i> <i>ALDH5F1</i>	orange1.1g009618m orange1.1g009516m orange1.1g010482m
Succinate-semialdehyde dehydrogenase, mitochondrial isoform X2	LOC102625593	XM_006493686.3	XP_006493749.1	<i>ALDH5F1</i> <i>ALDH5F1</i> <i>ALDH5F1</i>	orange1.1g009618m orange1.1g009516m orange1.1g010482m
Succinate-semialdehyde dehydrogenase, mitochondrial isoform X3	LOC102625593	XM_025093687.1	XP_024949455.1	<i>ALDH5F1</i> <i>ALDH5F1</i> <i>ALDH5F1</i>	orange1.1g009618m orange1.1g009516m orange1.1g010482m

^a The listed putative gene candidates were identified based on the top matches of recently available data in the National Center for Biotechnology Information (NCBI) GenBank database, with the query sequences using the nucleotide-nucleotide BLAST (BLASTn) and protein-protein BLAST (BLASTp) tools (Altschul et al. 1997, 2005). The shortlist of top matches was generated based on the phylogenetic trees, identity more than 90%, and excluding all the hypothetical and low-quality proteins that have these characteristics.

^b The listed genes are the top matched sequences of the “*Citrus sinensis* CDS, phytozome 154 v1.1” and “*Citrus sinensis* proteins, phytozome 154 v1.1” BLAST datasets available on the Citrus Greening Solutions website (Flores-Gonzalez et al. 2019), using the BLASTp tool (Altschul et al. 1997, 2005) using the NCBI query sequences. *BAT1* = Bidirectional amino acid transporter 1, *POP2* = PLP-dependent transferases superfamily protein, and *ALDH5F1* = aldehyde dehydrogenase 5F1.

^c The listed putative gene candidates were identified using bidirectional amino acid transporter 1 (*BAT1*; GenBank accession number NP_565254.1, 516 aa) from *Arabidopsis thaliana* (also known as GABA permease [*AtgabP*]) (Michaeli et al. 2011) as a query sequence against the *Citrus sinensis* genome available in NCBI’s GenBank and the Citrus Greening Solutions websites (Flores-Gonzalez et al. 2019).

^d The listed putative gene candidates were identified using BLASTp, using pyridoxal phosphate (PLP)-dependent transferases superfamily protein (*POP2*; GenBank accession number NP_001189947.1, 513 aa) from *Arabidopsis thaliana* (also known as GABA transaminase [*AtgabT*]) (Uzma Jalil et al. 2019) as a query sequence against the *Citrus sinensis* genome available in NCBI’s GenBank and the Citrus Greening Solutions websites (Flores-Gonzalez et al. 2019).

^e The listed putative gene candidates were identified using BLASTp, using succinic semialdehyde dehydrogenase (*SSADH*; GenBank accession number AAF23590.1; 528 aa) from *Arabidopsis thaliana* (also known as GABA dehydrogenase [*AtgabD*]) (Bouché et al. 2003a; Busch and Fromm 1999) as a query sequence against the *Citrus sinensis* genome available NCBI’s GenBank and Citrus Greening Solutions websites (Flores-Gonzalez et al. 2019).

with GABA-treated healthy ones ($P = 0.0486$) (Fig. 3C and D). Similarly, the application of 10 mM GABA induced the accumulation of succinate in both healthy and ‘*Ca. L. asiaticus*’-infected leaves ($P < 0.0001$ and $P = 0.0327$, respectively) (Fig. 3E and F). Nevertheless, no significant differences were observed between GABA-treated healthy and ‘*Ca. L. asiaticus*’-infected leaves ($P = 0.1972$) (Fig. 3F). Taken together, these findings suggest that the presence of ‘*Ca. L. asiaticus*’ enables greater metabolic changes in GABA shunt-related compounds in GABA-treated plants.

Exogenous GABA treatment induced the expression of predicted *gab* genes.

To better understand the effect of GABA accumulation on the transcript levels of the three predicated *gab* genes (*CsgabP*, *CsgabT*, and *CsgabD*), qPCR-based gene expression of these genes was investigated in the leaves of healthy and ‘*Ca. L. asiaticus*’-infected Valencia sweet orange after treatment with 10 mM GABA under greenhouse conditions. Generally, the three *gab* candidate genes (*CsgabP*, *CsgabT*, and *CsgabD*) were up-regulated (up to sixfold) in GABA-treated healthy and ‘*Ca. L. asiaticus*’-infected leaves compared with nontreated control trees. Briefly, the transcript levels of *CsgabP* were elevated in GABA-treated plants (healthy and infected), however, it was expressed at higher levels in the presence of ‘*Ca. L. asiaticus*’ (Fig. 3G).

Likewise, *CsgabT* was highly expressed after GABA supplementation of healthy plants ($P = 0.0007$) but not ‘*Ca. L. asiaticus*’-infected ones ($P = 0.0832$) (Fig. 3H), and no significant differences were observed in the expression of *CsgabT* between GABA-treated healthy and ‘*Ca. L. asiaticus*’-infected leaves ($P = 0.9958$) (Fig. 3H). Furthermore, *CsgabD* was expressed at higher levels after the treatment of 10 mM GABA in both healthy ($P < 0.0001$) and ‘*Ca. L. asiaticus*’-infected leaves ($P = 0.0125$) (Fig. 3I). However, the effect of GABA supplementation was boosted in the presence of ‘*Ca. L. asiaticus*’ ($P = 0.0032$) (Fig. 3I). Collectively, these findings suggest that the presence of ‘*Ca. L. asiaticus*’ enhances the expression of predicted *gab* genes under GABA-accumulated conditions.

Exogenous GABA-D₆ application confirms the metabolism of GABA to succinate.

Due to the presence of endogenous GABA in citrus plants, studying the metabolism of GABA supplementation is challenging. Therefore, we used GABA-D₆ to examine the metabolism of exogenous GABA in healthy and ‘*Ca. L. asiaticus*’-infected sweet orange. A characteristic total ion current chromatogram, the mass spectrum of methyl chloroformate (MCF)-derivatized authentic standards (100 ppm each), and the mass spectrum of MCF-derivatized citrus leaves are presented in Figure 4. Although

Fig. 1. Genomic context, regions, transcripts, and products of putative *gab* genes from Valencia sweet orange (*Citrus sinensis*). **A**, Genomic context and localization of three putative *gab* genes from *C. sinensis*, including *CsgabP*, *CsgabT*, and *CsgabD*, which encode for γ -aminobutyric acid (GABA)-specific permease, GABA transaminase, succinate semialdehyde dehydrogenase, respectively. **B**, Exon and domain structure of selected *CsgabP* genes from *C. sinensis*. *CsgabP*-1 (XP_006468761.1), *CsgabP*-2 (XP_006468762.1), and *CsgabP*-3 (XP_006469954.1) exhibit 521, 419, and 482 amino acids, respectively. Yellow boxes represent the exons, whereas black lines represent the introns. **C**, Exon and domain structure of *CsgabT* from *C. sinensis*. *CsgabT* (XP_006481368.1) exhibit 514, 419, and 482 amino acids. Green boxes represent the exons, whereas black lines represent the introns. **D**, Exon and domain structure of selected *CsgabDs* from *C. sinensis*. *CsgabD*-1 (XP_015381305.1), *CsgabD*-2 (XP_006493749.1), and *CsgabD*-3 (XP_024949455.1) exhibit 535, 530, and 495 amino acids, respectively. Blue boxes represent the exons, whereas black lines represent the introns.

ketoglutarate was detected in sweet orange (*C. sinensis*) leaves after derivatization with MCF (Fig. 4A), no evidence for the formation of deuterated ketoglutarate was found in GABA-D₆-treated plants. Moreover, The gas chromatography-mass spectrometry (GC-MS) analysis showed that endogenous GABA (Fig. 4B, nonlabeled) could be easily distinguished from exogenous GABA-D₆ (Fig. 4C) after derivatization with MCF. On the other hand, MCF derivatization resulted in highly reproducible chromatograms of succinate (Fig. 4D) and its deuterated form (succinate-D₄) (Fig. 4E) in GABA-D₆-treated plants even without the treatment with succinate-D₄. These findings suggest that GABA-D₆ is rapidly metabolized to succinate-D₄.

GABA-D₆ supplementation enhanced the accumulation of GABA and succinate but decreased the ketoglutarate content in treated citrus plants.

We investigated the effect of supplementary GABA-D₆ on the endogenous levels of three GABA shunt-related compounds, including ketoglutarate, GABA, and succinate in healthy and ‘*Ca. L. asiaticus*’-infected leaves (Fig. 5). Briefly, exogenous application of GABA-D₆ markedly decreased the endogenous ketoglutarate levels in healthy (control) leaves ($P = 0.0044$), but it did not significantly affect the ketoglutarate levels in ‘*Ca. L.*

asiaticus’-infected leaves ($P = 0.5565$) (Fig. 5A and B). Moreover, no significant differences were observed between GABA-D₆-treated healthy and infected citrus leaves ($P = 0.2005$) (Fig. 5A and B). Conversely, GABA-D₆ supplementation enhanced the endogenous GABA content in both healthy ($P = 0.0240$) and ‘*Ca. L. asiaticus*’-infected leaves ($P = 0.0111$) (Fig. 5C and D). Noteworthy, the presence of ‘*Ca. L. asiaticus*’ significantly increased the endogenous GABA content in GABA-D₆-treated infected leaves compared with GABA-treated healthy trees ($P = 0.0334$) (Fig. 5C and D). Likewise, exogenous application of GABA-D₆ significantly increased the endogenous succinate levels in healthy ($P = 0.0293$) and infected leaves ($P = 0.0002$) (Fig. 5E and F). Interestingly, ‘*Ca. L. asiaticus*’-infected trees, treated with 10 mM GABA-D₆, had the highest succinate content during the experiment over the nontreated ($P < 0.0001$) and GABA-D₆-treated healthy plants ($P = 0.0001$) (Fig. 5E and F). These findings indicate that the presence of ‘*Ca. L. asiaticus*’ empowers greater metabolic alterations in GABA shunt-related compounds in GABA-D₆-treated plants.

Exogenous GABA-D₆ treatment increased the levels of deuterated GABA and succinate.

Although there is no evidence for the formation of deuterated ketoglutarate in GABA-D₆-treated plants, our findings showed

Fig. 2. Effect of ‘*Candidatus Liberibacter asiaticus*’ infection on the endogenous contents of γ -aminobutyric acid (GABA), α -ketoglutarate, and succinate and the relative gene expression of putative *gab* genes in Valencia sweet orange (*Citrus sinensis*) leaves. **A, C, and E,** Representative chromatograms of α -ketoglutarate, GABA, and succinate, respectively. **B, D, and F,** Endogenous content of α -ketoglutarate (in nanograms per gram of fresh weight [ng g⁻¹ FW]), GABA (ng g⁻¹ FW), and succinate (μg g⁻¹ FW), respectively, from healthy (control) and ‘*Ca. L. asiaticus*’-infected (infected) Valencia sweet orange leaves. Whiskers show the minimum and the maximum values, horizontal thick lines indicate the medians (50th percentile of the data), boxes signify the interquartile ranges (25th to 75th percentile of the data), white dots specify the raw data ($n = 6$), black dots indicate potential outliers, and the gray shading represents the corresponding violin plot. **G,** Relative gene expression (mean $\pm$ standard deviation; $n = 6$) of *CsgabP*, *CsgabT*, and *CsgabD* from healthy (control) or after the infection with ‘*Ca. L. asiaticus*’ (infected). In F and G, presented P values are based on a two-tailed t -test to statistically compare between treatments (healthy versus infected). Statistical significance was established as $P < 0.05$.

that exogenous GABA-D₆ is rapidly metabolized to succinate-D₄ by the activity of *CsgabT* and *CsgabD* (Fig. 6A). Briefly, GABA-D₆ was not detected in nontreated control plants, however, considerable levels of GABA-D₆ were detected in both GABA-D₆-treated healthy and infected leaves (Fig. 6B and C). The level of GABA-D₆ was higher in ‘*Ca. L. asiaticus*’-infected leaves (731.63 ± 85.36 ng per gram of fresh weight [FW]) than healthy ones (555.10 ± 117.88 ng g⁻¹ FW, $P = 0.0155$) (Fig. 6C). In parallel, no deuterated succinate-D₄ was detected in nontreated control plants (healthy and infected), though, a notable amount of succinate-D₄ was detected in both GABA-D₆-treated healthy and ‘*Ca. L. asiaticus*’-infected leaves (Fig. 6D and E). Similar to GABA-D₆, succinate-D₄ was found at higher levels in ‘*Ca. L. asiaticus*’-infected trees (946.25 ± 79.13 ng g⁻¹ FW) than healthy ones (615.24 ± 177.96 ng g⁻¹ FW, $P = 0.0044$) (Fig. 6E). This

result indicated that GABA-D₆ was metabolized to succinate-D₄ in GABA-D₆-treated trees.

Application of GABA-D₆ upregulated the expression of predicted *gab* genes.

The transcript levels of *CsgabP* (Fig. 6F), *CsgabT* (Fig. 6G), and *CsgabD* (Fig. 6H) were investigated in healthy (control) and ‘*Ca. L. asiaticus*’-infected sweet orange leaves after treatment with exogenous GABA-D₆. Generally, exogenous application of GABA-D₆ upregulated the expression of all predicted *gab* genes in both healthy and ‘*Ca. L. asiaticus*’-infected leaves. Briefly, relative *CsgabP* expression was increased up to 5.6-fold in GABA-D₆-treated healthy plants and 12.9-fold in GABA-D₆-treated infected leaves compared with nontreated healthy ones ($P = 0.0344$ and $P < 0.0001$, respectively) (Fig. 6F). Likewise,

Fig. 3. Effect of exogenous γ -aminobutyric acid (GABA) supplementation on the endogenous contents of GABA shunt-related compounds and the relative gene expression of putative *gab* genes in Valencia sweet orange (*Citrus sinensis*) leaves. **A, C, and E,** Representative chromatograms of α -ketoglutarate, GABA, and succinate, respectively, from healthy (control) and ‘*Candidatus Liberibacter asiaticus*’-infected (infected) Valencia sweet orange leaves after the treatment with 10 mM GABA. **B, D, and F,** Endogenous content of α -ketoglutarate (nanograms per gram of fresh weight [ng g⁻¹ FW]), GABA (ng g⁻¹ FW), and succinate (μg g⁻¹ FW), respectively, from healthy (control) and ‘*Ca. L. asiaticus*’-infected (infected) Valencia sweet orange leaves after the treatment with 10 mM GABA. Whiskers show the minimum and the maximum values, horizontal thick lines indicate the medians (50th percentile of the data), boxes signify the interquartile ranges (25th to 75th percentile of the data), white dots specify the raw data ($n = 6$), black dots indicate potential outliers, and the gray shading represents the corresponding violin plot. **G, H, and I,** Relative gene expression (mean $\pm$ standard deviation; $n = 6$) of *CsgabP*, *CsgabT*, and *CsgabD*, respectively, from healthy (control) and ‘*Ca. L. asiaticus*’-infected (infected) Valencia sweet orange leaves after the treatment with 10 mM GABA. Presented P values are based on a two-tailed t -test to statistically compare between each pair of treatments. Statistical significance was established as $P < 0.05$.

the relative transcript levels of *CsgabT* were enhanced in GABA- D_6 -treated healthy (about 5.2-fold) and infected leaves (approximately 6.7-fold) compared with nontreated healthy ones ($P = 0.0004$ and $P < 0.0001$, respectively) (Fig. 6G). Although the expression levels of *CsgabD* were induced in healthy (control) and ‘*Ca. L. asiaticus*’-infected sweet orange leaves after the treatment with exogenous GABA- D_6 , its transcript levels did

not reach as high as those of *CsgabP* and *CsgabT* (Fig. 6H). The transcript levels of *CsgabT* were doubled in GABA- D_6 -treated healthy (just about 2.2-fold) and significantly increased in GABA- D_6 -treated infected leaves (approximately 3.6-fold) compared with nontreated healthy ones ($P = 0.0244$ and $P < 0.0001$, respectively) (Fig. 6H). It is worth mentioning that the presence of ‘*Ca. L. asiaticus*’ enhanced the expression levels of

Fig. 4. Chromatographic characteristics of α -ketoglutarate, γ -aminobutyric acid (GABA), succinate, and their deuterium-labeled derivatives, using gas chromatography-mass spectrometry selected ion monitoring (GC-MS-SIM)-based targeted metabolomic analysis. Representative total ion current GC-MS-SIM chromatograms, the mass spectrum of authentic standards (100-ppm), and the mass spectrum of Valencia sweet orange (*Citrus sinensis*) leaf samples of **A**, α -ketoglutarate, **B**, GABA, **C**, GABA- D_6 , **D**, succinate, and **E**, succinate- D_4 after derivatization with methyl chloroformate (MCF) using GC-MS running in the SIM mode.

all predicted *gab* genes, included *CsgabP* (Fig. 6F), *CsgabT* (Fig. 6G), and *CsgabD* (Fig. 6H), regardless of treatment with GABA- D_6 .

DISCUSSION

A four-carbon nonproteinogenic amino acid, GABA, is ubiquitously distributed among all life domains, including both prokaryotes and eukaryotes (Bouché et al. 2003b; Fromm 2020; Li et al. 2021). Although GABA was discovered first in plants (Steward et al. 1949), it has been extensively investigated in animals as a signaling molecule in neurotransmission (Buddhala et al. 2009; Medina and Chudotvorova 2006). In land plants, GABA is metabolized through a short metabolic route called the GABA shunt (Bouché et al. 2003b; Fromm 2020; Li et al. 2021). GABA shunt plays a critical role in the central carbon and nitrogen metabolism in many prokaryotic and eukaryotic species (Fait et al. 2008; Shelp et al. 1999). In addition, it plays a key role in plant response to several environmental stressors, such as heavy metals, salinity, drought, and wounding (Fromm 2020; Kinnersley and Turano 2000; Li et al. 2021; Seifikalhor et al. 2019; Tarkowski et al. 2020). Moreover, GABA is accumulated in response to numerous insect herbivory (Killiny and Nehela 2017a; Nehela and Killiny 2019; Scholz et al. 2015; Tarkowski et al. 2020), fungal pathogens (Takahashi et al. 2008; Seifi et al. 2013), and bacterial pathogens (Killiny and Nehela 2017a; Lang et al. 2016; Nehela and Killiny 2019; O'Leary et al. 2016; Wang et al. 2019).

Our findings showed that endogenous GABA content was dramatically increased in citrus leaves after the '*Ca. L. asiaticus*' infection. The GABA accumulation in infected leaves might be

a part of citrus defense responses to negatively regulate and interfere with the quorum sensing (QS) of '*Ca. L. asiaticus*'. It has been suggested that QS is essential for the growth and multiplication of '*Ca. L. asiaticus*' (Yan et al. 2013). Interestingly, accumulated GABA negatively regulates the level of QS signal in *Agrobacterium tumefaciens* in tobacco plants (Chevrot et al. 2006). Moreover, GABA-rich transgenic *GAD* tobacco lines (expressing a full-length tobacco *GAD* enzyme) and *GADΔC* lines (expressing a truncated *GAD* enzyme) were more tolerant to *Agrobacterium tumefaciens* than wild-type plants (Chevrot et al. 2006). Collectively, a hypothesis in line with these findings is that elevated GABA levels play a key defensive role against '*Ca. L. asiaticus*' via regulating its QS signaling system. However, due to the difficulty in culturing '*Ca. L. asiaticus*', the molecular mechanism or mechanisms behind the GABA-mediated QS interference during host-pathogen interactions is poorly understood and requires further research.

GABA metabolism requires the activity of several evolutionarily conserved enzymes that bypass two steps of α -ketoglutarate-to-succinate conversion outside the mitochondrial TCA cycle (Bouché et al. 2003b; Che-Othman et al. 2020; Fait et al. 2008; Michaeli et al. 2011; Nehela and Killiny 2019; Shelp et al. 1999). Briefly, citrate is catalyzed to α -ketoglutarate via the activity of *ACO* and *IDH*, then to glutamate via *GDH* and *GS* activity. Then, glutamate is decarboxylated into GABA via irreversible reaction by the activity of the cytosolic enzyme *GAD*, which consumes a proton and releases CO_2 , causing an accumulation of GABA in the cytosol. Subsequently, cytosolic GABA is transported into the mitochondria via the activity of the mitochondrial *gabP* (Michaeli et al. 2011; Nehela and Killiny 2019), then catabolized to succinic

Fig. 5. Effect of exogenous application of deuterium-labeled γ -aminobutyric acid (GABA- D_6) on the endogenous contents of GABA shunt-related compounds in Valencia sweet orange (*Citrus sinensis*) leaves. **A, C, and E,** Representative chromatograms of α -ketoglutarate, GABA, and succinate, respectively, from healthy (control) and '*Candidatus Liberibacter asiaticus*'-infected (infected) Valencia sweet orange leaves after the treatment with 10 mM GABA- D_6 . **B, D, and F,** Endogenous content of α -ketoglutarate (nanograms per gram of fresh weight [$ng\ g^{-1}\ FW$]), GABA ($ng\ g^{-1}\ FW$), and succinate ($\mu g\ g^{-1}\ FW$), respectively, from healthy (control) and '*Ca. L. asiaticus*'-infected (infected) Valencia sweet orange leaves after the treatment with 10 mM GABA- D_6 . Whiskers show the minimum and the maximum values, horizontal thick lines indicate the medians (50th percentile of the data), boxes signify the interquartile ranges (25th to 75th percentile of the data), white dots specify the raw data ($n = 6$), black dots indicate potential outliers, and the gray shading represents the corresponding violin plot. Presented *P* values are based on a two-tailed *t*-test to statistically compare between each pair of treatments. Statistical significance was established as $P < 0.05$.

semialdehyde via the activity of *gabT* (also known as GABA transaminase). The later step paves the way for succinate formation using *SSADH* (also known as *gabD*).

Interestingly, our findings showed that the citrus genome harbors three putative *gab* genes including GABA permease (*CsgabP*), GABA transaminase (*CsgabT*), and GABA dehydrogenase (*CsgabD*). *CsgabP* was identified as three proteins in two different loci. The amino acid sequences of these three proteins were highly similar to each other. Our findings showed that gene expression of *CsgabP* was upregulated after '*Ca. L. asiaticus*' infection and after the exogenous GABA application. Likewise, the gene encoding for *gabP* in *A. thaliana* (*AtgabP*) was highly coexpressed in response to limited carbon availability on artificial media and in soil under low light intensity

(Michaeli et al. 2011). Moreover, *gabP* deficiency mutants of *A. thaliana* negatively affected the GABA uptake into mitochondria and induced the TCA cycle activity (Michaeli et al. 2011).

Taken together, these findings suggest that *gabP* might slightly contribute to GABA transit into the mitochondria under normal growth conditions (Fig. 7A and B). However, under specific physiological circumstances such as '*Ca. L. asiaticus*' infection, *gabP* becomes more active and transports more cytosolic GABA into the mitochondria to support the succinate biosynthesis via the GABA shunt rather than from α -ketoglutarate directly via the TCA cycle (Fig. 7C and D). From our perspective, *GABP* is a critical enzyme that plays a key role within the GABA shunt to control the cytosolic GABA transit, regulate the central carbon and nitrogen metabolism, maintain the endogenous glutamate

Fig. 6. Effect of exogenous application of deuterium-labeled γ -aminobutyric acid (GABA-D₆) on the fluxomic of deuterated derivatives of GABA (GABA-D₆) and succinate (succinate-D₄) and the relative gene expression of putative *gab* genes in Valencia sweet orange (*Citrus sinensis*) leaves. **A**, A schematic diagram for the conversion of D₆-GABA to succinate-D₄ and their reaction with methyl chloroformate (MCF). **B and D**, Representative chromatograms of GABA-D₆ and succinate-D₄, respectively, from healthy (control) and '*Candidatus Liberibacter asiaticus*'-infected (infected) Valencia sweet orange leaves after the treatment with 10 mM GABA-D₆. **C and E**, Endogenous content of GABA-D₆ (nanograms per gram of fresh weight [ng g⁻¹ FW]) and succinate-D₄ (ng g⁻¹ FW), respectively, from healthy (control) and '*Ca. L. asiaticus*'-infected (infected) Valencia sweet orange leaves after the treatment with 10 mM GABA-D₆. Whiskers show the minimum and the maximum values, horizontal thick lines indicate the medians (50th percentile of the data), boxes signify the interquartile ranges (25th to 75th percentile of the data), white dots specify the raw data (*n* = 6), black dots indicate potential outliers, and the gray shading represents the corresponding violin plot. **F, G, and H**, Relative gene expression (mean $\pm$ standard deviation; *n* = 6) of *CsgabP*, *CsgabT*, and *CsgabD*, respectively, from healthy (control) and '*Ca. L. asiaticus*'-infected (infected) Valencia sweet orange leaves after the treatment with 10 mM GABA-D₆. Presented *P* values are based on a two-tailed *t*-test to statistically compare between each pair of treatments. Statistical significance was established as *P* < 0.05.

homeostasis, or bypass steps in the TCA cycle to secure more energy that is required for plant adaptation and resilience to biotic and abiotic stress.

Moreover, our findings showed that GABA transaminase (*CsgabT*) was identified in only one locus (LOC102624895) and encodes for GABA transaminase 3, chloroplastic. *gabT*, a class III pyridoxal-phosphate-dependent aminotransferase, is ubiquitously found in bacteria, fungi, plants, and animals (Cao et al. 2013; Fait et al. 2008; Shelp et al. 2017; Tunnicliff 1986). *gabT* employs one cofactor, pyridoxal phosphate, to catalyze GABA via facilitating a reaction that transfers an amine group ($R-NH_2$) from GABA to pyruvate or glyoxylate and a ketone group from pyruvate/glyoxylate to GABA to produce succinate semialdehyde and L-glutamate (Cao et al. 2013; Shelp et al. 2017; Tunnicliff 1986). It is worth mentioning that GABA shunt-generated succinate constitutes a significant amount of the succinate required by the mitochondrion (Fait et al. 2008).

Our findings showed that the expression of *gabT* as well as the endogenous succinate content were enhanced after the infection with ‘*Ca. L. asiaticus*’ and after the exogenous GABA application. Previous studies showed that knocking-down of *gabT* using virus-induced gene silencing enhances the disease susceptibility of tomato plants to the necrotrophic fungal pathogen *Rhizoctonia solani* (Rani and Jha 2021). On the other hand, some phytopathogenic bacteria, such as *Pseudomonas syringae* pv. *tomato* DC3000, harbor *gabT* genes (Park et al. 2010). Bacterial mutants lacking *gabT* genes were incapable of using GABA as a sole carbon and nitrogen source and demonstrated reduced bacterial virulence (Park et al. 2010). Taken together, these findings suggest that *gabT* might be a double-edged sword in host-pathogen interactions. Moreover, the results from exogenous GABA supplementation and gene expression analyses suggest that *gabT* is positively correlated with succinate levels and might contribute to one or both plant defense responses and ‘*Ca. L. asiaticus*’ virulence.

GABA dehydrogenases (*gabD*) is located within a unidirectionally transcribed gene cluster and might be cotranscribed with *gabT* from a mutual promoter upstream of *gabD* in several bacterial species, including *E. coli* (Niegemann et al. 1993), *Ralstonia eutropha* (Hidalgo et al. 1991), and *Bacillus subtilis*. *gabD* is a member of the aldehyde dehydrogenase family, and it has the same evolutionary origin and enzymatic mechanism of lactaldehyde dehydrogenase (Ramos et al. 1985). *gabD* catalyzes the final step in the GABA shunt via an NAD^+ -dependent reaction that converts succinic semialdehyde to succinate (Chambliss et al. 1995). Interestingly, our findings showed that *CsgabD* shared high sequence homology to the well-characterized aldehyde dehydrogenase from *Brucella melitensis* and displayed conserved cofactor binding residues and putative active sites.

gabD plays a key role in plant responses to several abiotic stresses via preventing the accumulation of reactive oxygen species (ROS). For instance, T-DNA knock-out mutants of SSADH (*gabD*) of *A. thaliana* were sensitive to heat and UV light and displayed HLB-like symptoms including bleached and smaller leaves, reduced chlorophyll content, developed necrotic lesions, and enhanced cell death due to the rapid surge in the ROS levels (Bouché et al. 2003a). Initially, it was suggested that the stunted phenotype and the hyperaccumulation of reactive oxygen intermediates (ROI) in *ssadh* mutants might be due to GABA accumulation and the shortage of succinate and NADH allocation to the TCA cycle. However, double *pop2ssadh* mutant proved that GABA transaminase (*gabT*; also known as POP2) is epistatic to SSADH (Ludewig et al. 2008). Moreover, *pop2ssadh* mutants were more sensitive to succinic semialdehyde and γ -hydroxybutyrate than wild-type plants (Ludewig et al. 2008). Together, these findings indicate that the high levels of succinic semialdehyde and γ -hydroxybutyrate emitted downstream of the *gabT* transamination step, which is toxic to the plant tissues, increases the ROI levels, and diminishes the plant growth. Interestingly, the transcript levels of both genes (*CsgabT* and *CsgabD*) were upregulated in

Fig. 7. Hypothetical model of the potential roles of putative *gab* genes in the connection between γ -aminobutyric acid (GABA) shunt and tricarboxylic acid (TCA) cycle in Valencia sweet orange (*Citrus sinensis*). **A and B.** Briefly, under normal growth conditions, *CsgabP* might slightly contribute to GABA transit into the mitochondria. **C and D.** However, under specific physiological circumstances such as ‘*Candidatus Liberibacter asiaticus*’ infection, *CsgabP* becomes more active and transports more cytosolic GABA into the mitochondria to support the succinate biosynthesis via the GABA shunt rather than from α -ketoglutarate directly via the TCA cycle.

citrus leaves upon the infection with the bacterial pathogen '*Ca. L. asiaticus*' and the exogenous application of GABA and GABA-D₆.

Because our study showed that '*Ca. L. asiaticus*' infection significantly enhanced the accumulation of GABA in citrus leaves, we hypothesized that the GABA accumulation is associated with the upstream noncyclic flux toward succinate via regulating the activity of the three *gab* genes (*CsgabP*, *CsgabD*, and *CsgabT*). To test this hypothesis, we enhanced the endogenous GABA levels via exogenous GABA application to mimic the GABA accumulation caused by '*Ca. L. asiaticus*' infection. Our findings showed that GABA supplementation increased the accumulation of GABA and succinate but not the ketoglutarate levels in GABA-treated leaves. Additionally, exogenous GABA treatment significantly induced the transcript levels of the three predicated *gab* genes (*CsgabP*, *CsgabT*, and *CsgabD*) in GABA-treated leaves. These findings prove that the effect of exogenously induced GABA accumulation is comparable with the '*Ca. L. asiaticus*'-induced GABA accumulation and support our hypothesis that GABA accumulation is associated with the upstream noncyclic flux toward succinate via regulating the activity of the three *gab* genes (*CsgabP*, *CsgabD*, and *CsgabT*).

Furthermore, our previous study showed that exogenously applied GABA is quickly metabolized to succinate and fed into the TCA cycle (Hijaz and Killiny 2019). Recently, using GABA-D₆, we showed that D₄-labeled succinate was detected in citrus plants only after treatment with GABA-D₆ (Hijaz and Killiny 2020). Additionally, we reported the half-life of GABA-D₆ in citrus to be approximately 1.3 h (Hijaz and Killiny 2020). Collectively, these findings support the idea that GABA is quickly converted to succinate. Additionally, our findings showed that '*Ca. L. asiaticus*' infection accelerates the conversion of α -ketoglutarate, which was reduced in our study, to GABA, then to succinate in sequential reactions, both of which accumulated in citrus leaves (Nehela and Killiny 2019). Our work provides evidence that three predicated *gab* genes from citrus (*CsgabP*, *CsgabT*, and *CsgabD*) support the upstream noncyclic flux toward succinate via GABA shunt.

However, it is not clear yet whether these metabolic alterations are for the benefit of '*Ca. L. asiaticus*' itself or if they are for the benefit of the host plant to boost metabolites-based defense responses. For instance, the GABA accumulation in infected leaves might be a part of citrus defense responses to negatively regulate and interfere with the QS of '*Ca. L. asiaticus*'. It has been suggested that QS is essential for the growth and multiplication of '*Ca. L. asiaticus*' (Yan et al. 2013). Moreover, the TCA cycle is an important metabolic hub that directly or indirectly supports many defense-associated metabolic pathways, such as carboxylic compounds (Killiny and Nehela 2017a), phytohormones (Nehela and Killiny 2018b; Nehela et al. 2018), and polyamines (Nehela and Killiny 2019, 2020d; Killiny and Nehela 2020) to mediate the effect of '*Ca. L. asiaticus*' infection.

On the other hand, the metabolic changes in the TCA-associated compounds might be for benefit of the bacterial pathogen '*Ca. L. asiaticus*'. For example, previous studies showed that '*Ca. L. asiaticus*' can manipulate the TCA cycle of its vector, *D. citri*, and exploits the energy metabolism for its benefit, to fulfill its nutritional needs or to neutralize the host defense responses (Lu and Killiny 2017; Killiny et al. 2016). Furthermore, several TCA-associated intermediates, particularly citrate, were reported as energy sources for the growth of the only culturable *Liberibacter* species, *L. crescens*, on chemically defined media (Cruz-Munoz et al. 2018). Citrate accumulation was reported previously in citrus leaves and fruits upon '*Ca. L. asiaticus*' infection (Cevallos-Cevallos et al. 2011; Killiny and Nehela 2017a; Nehela and Killiny 2019; Slisz et al. 2012). The accumulation of TCA-associated intermediates might be due to the catabolism of some proteinogenic amino acids (Stipanuk 2006).

Moreover, the cellular and molecular mechanisms behind how various stressors impede the TCA cycle and why the GABA shunt is a suitable alternative route toward succinate biosynthesis remain unclear. Moreover, the mechanism(s) of how *gab* genes respond to stresses and how they retain the balance between cyclic and noncyclic flux modes in the TCA cycle is not well-understood. Further studies are required to clarify the functional or regulatory roles of *gab* genes in citrus particularly, and in other plant species in general.

MATERIALS AND METHODS

Plant materials and growth conditions.

All experiments were carried out at the Citrus Research and Education Center, University of Florida, Lake Alfred, U.S.A. Valencia sweet orange [*C. sinensis* (L.) Osbeck] was used as an experimental plant in all experiments throughout this study. All trees were maintained in an approved United States Department of Agriculture APHIS/CDC secured greenhouse, at $28 \pm 2^\circ\text{C}$, with $65 \pm 5\%$ relative humidity and 16-h-light and 8-h-dark photoperiod. Trees were irrigated twice weekly and were fertilized monthly with 20:10:20 NPK water-soluble fertilizer (Allentown, PA, U.S.A.). At the time of sampling, citrus trees were approximately 95 ± 5 cm tall and around 20 months old. To obtain the '*Ca. L. asiaticus*'-infected plants, 12-month-old, healthy ('*Ca. L. asiaticus*'-free) Valencia plants were inoculated using budwoods grafting with a '*Ca. L. asiaticus*'-positive plant materials and were maintained in the same conditions as described above. Six to seven months later, the initial symptom of HLB started to develop, and the presence of '*Ca. L. asiaticus*' was confirmed, using PCR according to Tatineni et al. (2008). In all experiments, we tested healthy ('*Ca. L. asiaticus*'-free) versus '*Ca. L. asiaticus*'-infected, with six biological replicates each. From each biological replicate, three leaves were collected, chopped, and mixed and were immediately frozen on ice. Plant materials were kept at -80°C until further analysis. Each biological replicate was analyzed in duplicate (two technical replicates for each). The technical replicates were used to test the reproducibility and variability of our extraction and derivatization protocols as well as the reproducibility of the GC-MS instrument but were not used for statistical analysis.

In silico analysis.

The putative *gab* genes candidates were identified based on the recently available data in the National Center for Biotechnology Information (NCBI) GenBank database, with the query sequences and the recently available data on the "*Citrus sinensis* CDS, phytozome 154 v1.1" and "*Citrus sinensis* proteins, phytozome 154 v1.1" BLAST datasets available on the Citrus Greening Solutions website (Flores-Gonzalez et al. 2019), using the nucleotide-nucleotide BLAST (BLASTn) and protein-protein BLAST (BLASTp) tools (Altschul et al. 1997, 2005). Putative *CsgabP* candidates were identified using bidirectional amino acid transporter 1 (BAT1; GenBank accession number NP_565254.1; 516 aa) from *Arabidopsis thaliana* (also known as GABA permease [*ArgabP*]) (Michaeli et al. 2011) as a query sequence. Putative *CsgabT* candidates were identified using pyridoxal phosphate-dependent transferases superfamily protein (POP2; GenBank accession number NP_001189947.1; 513 aa) from *A. thaliana* (also known as GABA transaminase [*AtgabT*]) (Uzma Jalil et al. 2019) as a query sequence. Whereas putative *CsgabD* candidates were identified using SSADH (GenBank accession number AAF23590.1; 528 aa) from *A. thaliana* (also known as GABA dehydrogenase [*AtgabD*]) (Bouché et al. 2003a; Busch and Fromm 1999), as a query sequence against the *Citrus sinensis* genome available in the NCBI GenBank database and on the Citrus Greening Solutions website (Flores-Gonzalez et al. 2019),

using the compositionally adjusted substitution matrices (Altschul et al. 1997, 2005). Subsequently, a shortlist of the top matched proteins was generated (Table 1) based on the evolutionary relationships within the phylogenetic trees, identity more than 50%, and excluding all the hypothetical, partial, and low-quality proteins that have these characteristics.

Treatment of citrus plants with exogenous GABA and GABA-D₆.

In two separate experiments, 20-month-old Valencia sweet orange (*C. sinensis*) trees (healthy versus ‘*Ca. L. asiaticus*’-infected) were treated with 250 ml of aqueous GABA or GABA-D₆ (Sigma-Aldrich, St. Louis) solutions at a final concentration of 10 mM that were previously reported to induce the endogenous GABA content at 7 days posttreatment (Hijaz and Killiny 2020; Hijaz et al. 2018). GABA-D₆ was reported previously as a valuable tool to study the uptake, translocation, and metabolism of GABA in citrus plants (Hijaz and Killiny 2020). The water that exceeded the soil capacity and drained from the bottom of the pot was collected, using a plastic plate, and was reapplied to the soil. On the other hand, distilled water was used as a negative control. After the treatment, all plants (six replicates per treatment) were maintained under the same growth condition described above. From each biological replicate, three leaves were collected, chopped, and pooled together. The collected samples were chopped and kept at –80°C until analysis.

Targeted metabolomic analysis of α-ketoglutarate, GABA, and succinate.

Targeted compounds (α-ketoglutarate, GABA, and succinate) as well as GABA-D₆ and succinate-D₄ from GABA-D₆-treated plants were extracted from frozen leaf tissue using acidic methanol 80%, as described in our previous studies (Killiny and Nehela 2017a; Nehela and Killiny 2018b, 2019; Nehela et al. 2016, 2018). Each biological sample was extracted in triplicate, and the supernatants were pooled together and were then concentrated to 50 µl under a nitrogen stream. Subsequently, samples were derivatized with MCF following the protocol of (Hijaz and Killiny 2014) with slight modifications, as described in our previous studies (Killiny and Nehela 2017a; Nehela and Killiny 2018b, 2019; Nehela et al. 2016, 2018). Before derivatization, 5 µl of heptadecanoic acid (200 ppm) was added to each sample as an internal standard.

After derivatization, approximately 40 µl of the organic layer was collected and was dehydrated using four to five crystals of sodium sulfate. Then, 1 µl of the derivatized samples was injected into a GC-MS system (model Clarus 680; Perkin Elmer,

Waltham, MA, U.S.A.) running in the selected ion monitoring mode. ZB-5MS GC column (5% phenyl-arylene 95% dimethylpolysiloxane; low bleed, 30 m × 0.25 mm × 0.25 µm film thickness) (Phenomenex, Torrance, CA, U.S.A.) was used as a separation column and helium was used as the carrier gas (1 ml min^{–1} flow rate). The GC temperature program, MS ion identification, data acquisition, and chromatogram analysis were carried out as described in our previous studies (Killiny and Nehela 2017a; Nehela and Killiny 2019).

Briefly, α-ketoglutarate, GABA, and succinate were first identified by comparing their mass spectra with spectra from our published work and other literature (Killiny and Nehela 2017a; Nehela and Killiny 2019). The identification was verified using library entries of the ninth edition of Wiley mass spectral database (John Wiley and Sons, Inc., Hoboken, NJ, U.S.A.), NIST 2011 mass spectral database (National Institute of Standards and Technology, Gaithersburg, MA, U.S.A.), and the Golm Metabolome database. Finally, the identification of targeted compounds (α-ketoglutarate, GABA, GABA-D₆, succinate, and succinate-D₄) was further confirmed by comparing their retention times, linear retention indices, and mass spectra with those of authentic reference standards (Sigma-Aldrich) treated identically to samples. Calibration curves, using a series of reference standards (0, 5, 10, 25, and 50 ppm) derivatized and treated identically to samples, were used to quantify targeted metabolites.

Gene expression analysis.

The transcript levels of the three predicted *gab* genes (*gabP*, *gabT*, and *gabD*) were determined during three separate experiments: i) after challenging with ‘*Ca. L. asiaticus*’, ii) after treatment with exogenous GABA (healthy versus ‘*Ca. L. asiaticus*’-infected), or iii) after treatment with exogenous GABA-D₆ (healthy versus ‘*Ca. L. asiaticus*’-infected). Briefly, the total RNA was extracted using TriZol reagent (Ambion, Life Technologies, Grand Island, NY, U.S.A.) as described in our previous studies (Killiny and Nehela 2017a; Nehela and Killiny 2019). Then, a SuperScript first-strand synthesis system (Invitrogen, Carlsbad, CA, U.S.A.) with random hexamer primers was used to synthesize cDNA as described by the manufacturer instructions. SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, U.S.A.) was used to perform the qPCR on an ABI 7500 Fast-Time PCR system (Applied Biosystems). Samples were analyzed in triplicate for each biological replicate for each treatment. Primers of the three predicted *gab* genes (*gabP*, *gabT*, and *gabD*) were used to measure gene expression (Table 2). Although both *CsgabP* and *CsgabT* were identified as three potential copies of each gene, the multiple sequence alignment of the three *CsgabP* genes or three *CsgabD*

Table 2. Primer used for gene expression analysis of putative *gab* genes from Valencia sweet orange (*Citrus sinensis*) leaves using real-time reverse transcription-PCR^a

Gene	Accession ID	Primer (forward and reverse)	Melting temperature (°C)	Product size (bp)
<i>CsgabP</i>	XM_006468698.3	F: TTCATCCCCGGACCTTTCAA R: CGAGCACTGAAGATCCAAGC	58.93 58.99	197
<i>CsgabT</i>	XM_006481305.2	F: CTCTGAATGGGGGATAGGT R: TGCTGGGACTTGAGTTCCTT	60.15 59.84	196
<i>CsgabD</i>	XM_006493686.2	F: CAACGTACATGGCGTGTCTC R: CTGGCCCATCATTTTCTGTT	60.18 59.93	200
<i>CsEF1^b</i>	AY498567.1	F: GGAAGTTCGAGACCACCAAG R: ACACCAAGGGTGAAAGCAAG	59.70 60.15	202
<i>CsF-box^b</i>	XM_006482390.1	F: ACTTGACAGATGGGCTGTCC R: CAGCAACCAATACCCGTCT	60.12 59.99	197
<i>CsGAPC1^b</i>	XM_006483974.2	F: ACTCCAGAGGGATGATGTGG R: ATGGGATCTCCTCTGGGTTC	59.92 60.28	200
<i>CsSAND^b</i>	XM_006488024.2	F: GCATCAGCTGCACAGAAGAG R: GGAATGTAGCTGGGTTCCTCAA	59.89 59.93	204

^a The listed genes were identified based on recently available data in the National Center for Biotechnology Information GenBank website.

^b Genes have been used as reference/housekeeping genes for data normalization according to (Mafra et al. 2012; Wei et al. 2014).

proteins, when aligned separately, showed high similarity and conserved sequences between them; therefore, a one-fits-all primer (forward and reverse) that matched up in the consensus region between every three sequences was designed to determine the gene expression of *CsgabP* and *CsgabT* as a total of their three copies. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative changes in gene expression among PCR products (Livak and Schmittgen 2001). Four housekeeping (reference genes) genes were used for the normalization of gene expression including elongation factor-1 alpha (*CsEF-1 α*), F-box/kelch-repeat protein (*CsF-box*), glyceraldehyde-3-phosphate dehydrogenase, cytosolic (*CsGAPC1*, also known as GAPDH), and SAND family protein (*CsSAND*) (Mafra et al. 2012; Wei et al. 2014).

Statistical analysis.

All experiments were designed in a completely randomized design using six biological replicates per treatment. A two-tailed *t* test was used for statistical comparison between every two treatments including healthy versus ‘*Ca. L. asiaticus*’-infected, non-treated versus GABA-treated, and nontreated versus GABA-D₆-treated, and statistical significance was established as *P* < 0.05.

ACKNOWLEDGMENTS

The authors acknowledge S. Jones and F. Hijaz, Citrus Research and Education Center, University of Florida for their technical assistance with GC-MS. Additionally, we thank the members of our laboratory for helpful discussions and comments.

AUTHOR-RECOMMENDED INTERNET RESOURCES

Citrus Genome database:

https://citrusgreening.org/organism/Citrus_sinensis/genome

Golm Metabolome database: <http://gmd.mpimp-golm.mpg.de>

NCBI GenBank database: <https://www.ncbi.nlm.nih.gov/gene>

LITERATURE CITED

- Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. 1997. Gapped BLAST and PSI-BLAST: A new generation of protein database search programs. *Nucleic Acids Res.* 25:3389-3402.
- Altschul, S. F., Wootton, J. C., Gertz, E. M., Agarwala, R., Morgulis, A., Schäffer, A. A., and Yu, Y.-K. 2005. Protein database searches using compositionally adjusted substitution matrices. *FEBS J.* 272:5101-5109.
- Bolton, M. D. 2009. Primary metabolism and plant defense—Fuel for the fire. *Mol. Plant-Microbe Interact.* 22:487-497.
- Bouché, N., Fait, A., Bouchez, D., Möller, S. G., and Fromm, H. 2003a. Mitochondrial succinic-semialdehyde dehydrogenase of the γ -aminobutyrate shunt is required to restrict levels of reactive oxygen intermediates in plants. *Proc. Natl. Acad. Sci. U.S.A.* 100:6843-6848.
- Bouché, N., Lacombe, B., and Fromm, H. 2003b. GABA signaling: A conserved and ubiquitous mechanism. *Trends Cell Biol.* 13:607-610.
- Bové, J. J. M. 2006. Huanglongbing: A destructive, newly emerging, century-old disease of citrus. *J. Plant Pathol.* 88:7-37.
- Buddhala, C., Hsu, C. C., and Wu, J. Y. 2009. A novel mechanism for GABA synthesis and packaging into synaptic vesicles. *Neurochem. Int.* 55:9-12.
- Busch, K. B., and Fromm, H. 1999. Plant succinic semialdehyde dehydrogenase. Cloning, purification, localization in mitochondria, and regulation by adenine nucleotides. *Plant Physiol.* 121:589-598.
- Cao, J., Barbosa, J. M., Singh, N., and Locy, R. D. 2013. GABA transaminases from *Saccharomyces cerevisiae* and *Arabidopsis thaliana* complement function in cytosol and mitochondria. *Yeast* 30:279-289.
- Cevallos-Cevallos, J. M., Futch, D. B., Shilts, T., Folimonova, S. Y., and Reyes-De-Corcuera, J. I. 2012. GC-MS metabolomic differentiation of selected citrus varieties with different sensitivity to citrus huanglongbing. *Plant Physiol. Biochem.* 53:69-76.
- Cevallos-Cevallos, J. M., García-Torres, R., Etxeberria, E., and Reyes-De-Corcuera, J. I. 2011. GC-MS analysis of headspace and liquid extracts for metabolomic differentiation of citrus Huanglongbing and zinc deficiency in leaves of ‘Valencia’ sweet orange from commercial groves. *Phytochem. Anal.* 22:236-246.
- Chambliss, K. L., Caudle, D. L., Hinson, D. D., Moomaw, C. R., Slaughter, C. A., Jakobs, C., and Gibson, K. M. 1995. Molecular cloning of the mature NAD⁺-dependent succinic semialdehyde dehydrogenase from rat and human. cDNA isolation, evolutionary homology, and tissue expression. *J. Biol. Chem.* 270:461-467.
- Che-Othman, M. H., Jacoby, R. P., Millar, A. H., and Taylor, N. L. 2020. Wheat mitochondrial respiration shifts from the tricarboxylic acid cycle to the GABA shunt under salt stress. *New Phytol.* 225:1166-1180.
- Chevrot, R., Rosen, R., Haudecoeur, E., Cirou, A., Shelp, B. J., Ron, E., and Faure, D. 2006. GABA controls the level of quorum-sensing signal in *Agrobacterium tumefaciens*. *Proc. Natl. Acad. Sci. U.S.A.* 103:7460-7464.
- Cruz-Munoz, M., Petrone, J. R., Cohn, A. R., Munoz-Beristain, A., Killiny, N., Drew, J. C., and Triplett, E. W. 2018. Development of chemically defined media reveals citrate as preferred carbon source for *Liberibacter* growth. *Front. Microbiol.* 9:668.
- da Graça, J. V. V. 1991. Citrus greening disease. *Annu. Rev. Phytopathol.* 29:109-136.
- Fait, A., Fromm, H., Walter, D., Galili, G., and Fernie, A. R. 2008. Highway or byway: The metabolic role of the GABA shunt in plants. *Trends Plant Sci.* 13:14-19.
- Flores-Gonzalez, M., Hosmani, P. S., Fernandez-Pozo, N., Mann, M., Humann, J. L., Main, D., Heck, M., Brown, S., Mueller, L. A., and Saha, S. 2019. Citrusgreening.org: An open access and integrated systems biology portal for the Huanglongbing (HLB) disease complex. *bioRxiv* 868364.
- Folimonova, S. Y., Robertson, C. J., Garnsey, S. M., Gowda, S., and Dawson, W. O. 2009. Examination of the responses of different genotypes of citrus to huanglongbing (citrus greening) under different conditions. *Phytopathology* 99:1346-1354.
- Fromm, H. 2020. GABA signaling in plants: Targeting the missing pieces of the puzzle. *J. Exp. Bot.* 71:6238-6245.
- Garnier, M., Jagoueix-Eveillard, S., Cronje, P. R., Le Roux, H. F., and Bové, J. M. 2000. Genomic characterization of a liberibacter present in an ornamental rutaceous tree, *Calodendrum capense*, in the Western Cape Province of South Africa. Proposal of ‘*Candidatus Liberibacter africanus* subsp. *capensis*’. *Int. J. Syst. Evol. Microbiol.* 50:2119-2125.
- Gottwald, T. R. 2010. Current epidemiological understanding of citrus Huanglongbing. *Annu. Rev. Phytopathol.* 48:119-139.
- Hidalgo, E., Chen, Y. M., Lin, E. C. C., and Aguilar, J. 1991. Molecular cloning and DNA sequencing of the *Escherichia coli* K-12 *ald* gene encoding aldehyde dehydrogenase. *J. Bacteriol.* 173:6118-6123.
- Hijaz, F., El-Shesheny, I., and Killiny, N. 2013. Herbivory by the insect *diaphorina citri* induces greater change in citrus plant volatile profile than does infection by the bacterium, *Candidatus Liberibacter asiaticus*. *Plant Signal. Behav.* 8:e25677.
- Hijaz, F., and Killiny, N. 2014. Collection and chemical composition of phloem sap from *Citrus sinensis* L. Osbeck (sweet orange). *PLoS One* 9:1-11.
- Hijaz, F., and Killiny, N. 2019. Exogenous GABA is quickly metabolized to succinic acid and fed into the plant TCA cycle. *Plant Signal. Behav.* 14:e1573096.
- Hijaz, F., and Killiny, N. 2020. The use of deuterium-labeled γ -aminobutyric (D₆-GABA) to study uptake, translocation, and metabolism of exogenous GABA in plants. *Plant Methods* 16:24.
- Hijaz, F., Nehela, Y., and Killiny, N. 2016. Possible role of plant volatiles in tolerance against huanglongbing in citrus. *Plant Signal. Behav.* 11:e1138193.
- Hijaz, F., Nehela, Y., and Killiny, N. 2018. Application of γ -aminobutyric acid increased the level of phytohormones in *Citrus sinensis*. *Planta* 248:909-918.
- Jagoueix, S., Bové, J. M., and Garnier, M. 1994. The phloem-limited bacterium of greening disease of citrus is a member of the alpha subdivision of the Proteobacteria. *Int. J. Syst. Bacteriol.* 44:379-386.
- Kangasjärvi, S., Neukermans, J., Li, S., Aro, E.-M., and Noctor, G. 2012. Photosynthesis, photorespiration, and light signalling in defence responses. *J. Exp. Bot.* 63:1619-1636.
- Killiny, N. 2016. Generous hosts: What makes Madagascar periwinkle (*Catharanthus roseus*) the perfect experimental host plant for fastidious bacteria? *Plant Physiol. Biochem.* 109:28-35.
- Killiny, N. 2017. Metabolite signature of the phloem sap of fourteen citrus varieties with different degrees of tolerance to *Candidatus Liberibacter asiaticus*. *Physiol. Mol. Plant Pathol.* 97:20-29.
- Killiny, N., and Hijaz, F. 2016. Amino acids implicated in plant defense are higher in *Candidatus Liberibacter asiaticus*-tolerant citrus varieties. *Plant Signal. Behav.* 11:e1171449.

- Killiny, N., Hijaz, F., Ebert, T. A., and Rogers, M. E. 2016. Plant bacterial pathogen manipulates the energy metabolism of its insect vector. *Appl. Environ. Microbiol.* 83:e03005-16.
- Killiny, N., Jones, S. E., Nehela, Y., Hijaz, F., Dutt, M., Gmitter, F. G., and Grosser, J. W. 2018a. All roads lead to Rome: Towards understanding different avenues of tolerance to Huanglongbing in citrus cultivars. *Plant Physiol. Biochem.* 129:1-10.
- Killiny, N., and Nehela, Y. 2017a. Metabolomic response to Huanglongbing: Role of carboxylic compounds in *Citrus sinensis* response to 'Candidatus Liberibacter asiaticus' and its vector, *Diaphorina citri*. *Mol. Plant-Microbe Interact.* 30:666-678.
- Killiny, N., and Nehela, Y. 2017b. One target, two mechanisms: The impact of 'Candidatus Liberibacter asiaticus' and its vector, *Diaphorina citri*, on citrus leaf pigments. *Mol. Plant-Microbe Interact.* 30:543-556.
- Killiny, N., and Nehela, Y. 2020. Citrus polyamines: Structure, biosynthesis, and physiological functions. *Plants Basel* 9:426.
- Killiny, N., Nehela, Y., Hijaz, F., and Vincent, C. I. 2018b. A plant pathogenic bacterium exploits the tricarboxylic acid cycle metabolic pathway of its insect vector. *Virulence* 9:99-109.
- Killiny, N., Valim, M. F., Jones, S. E., Omar, A. A., Hijaz, F., Gmitter, F. G., Jr., and Grosser, J. W. 2017. Metabolically speaking: Possible reasons behind the tolerance of 'Sugar Belle' mandarin hybrid to Huanglongbing. *Plant Physiol. Biochem.* 116:36-47.
- Kinnersley, A. M., and Turano, F. J. 2000. Gamma aminobutyric acid (GABA) and plant responses to stress. *Crit. Rev. Plant Sci.* 19:479-509.
- Lang, J., Gonzalez-Mula, A., Taconnat, L., Clement, G., and Faure, D. 2016. The plant GABA signaling downregulates horizontal transfer of the *Agrobacterium tumefaciens* virulence plasmid. *New Phytol.* 210:974-983.
- Li, L., Dou, N., Zhang, H., and Wu, C. 2021. The versatile GABA in plants. *Plant Signal. Behav.* 16:1862565.
- Livak, K. J., and Schmittgen, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ Method. *Methods* 25:402-408.
- Lu, Z., and Killiny, N. 2017. Huanglongbing pathogen *Candidatus Liberibacter asiaticus* exploits the energy metabolism and host defence responses of its vector *Diaphorina citri*. *Physiol. Entomol.* 42:319-335.
- Ludewig, F., Hüser, A., Fromm, H., Beauclair, L., and Bouché, N. 2008. Mutants of GABA transaminase (POP2) suppress the severe phenotype of succinic semialdehyde dehydrogenase (*ssadh*) mutants in Arabidopsis. *PLoS One* 3:e3383.
- Mafra, V., Kubo, K. S., Alves-Ferreira, M., Ribeiro-Alves, M., Stuart, R. M., Boava, L. P., Rodrigues, C. M., and Machado, M. A. 2012. Reference genes for accurate transcript normalization in citrus genotypes under different experimental conditions. *PLoS One* 7:e31263.
- McCollum, G., and Baldwin, E. 2016. Huanglongbing: Devastating disease of citrus. *Hortic. Rev. (Am. Soc. Hortic. Sci.)* 44:315-361.
- Medina, I., and Chudotvorova, I. 2006. GABA neurotransmission and neural cation-chloride co-transporters: Actions beyond ion transport. *Crit. Rev. Neurobiol.* 18:105-112.
- Michaeli, S., Fait, A., Lagor, K., Nunes-Nesi, A., Grillich, N., Yellin, A., Bar, D., Khan, M., Fernie, A. R., Turano, F. J., and Fromm, H. 2011. A mitochondrial GABA permease connects the GABA shunt and the TCA cycle, and is essential for normal carbon metabolism. *Plant J.* 67:485-498.
- Nehela, Y., Hijaz, F., Elzaawely, A. A., El-Zahaby, H. M., and Killiny, N. 2016. Phytohormone profiling of the sweet orange (*Citrus sinensis* (L.) Osbeck) leaves and roots using GC-MS-based method. *J. Plant Physiol.* 199:12-17.
- Nehela, Y., Hijaz, F., Elzaawely, A. A., El-Zahaby, H. M., and Killiny, N. 2018. Citrus phytohormonal response to *Candidatus Liberibacter asiaticus* and its vector *Diaphorina citri*. *Physiol. Mol. Plant Pathol.* 102:24-35.
- Nehela, Y., and Killiny, N. 2018a. Infection with phytopathogenic bacterium inhibits melatonin biosynthesis, decreases longevity of its vector, and suppresses the free radical-defense. *J. Pineal Res.* 65:e12511.
- Nehela, Y., and Killiny, N. 2018b. Multiple phytohormonal signaling mediates citrus response to the bacterial pathogen *Candidatus Liberibacter asiaticus*. *Phytopathology* 108:1092-1101.
- Nehela, Y., and Killiny, N. 2019. 'Candidatus Liberibacter asiaticus' and its vector, *Diaphorina citri*, augment the tricarboxylic acid cycle of their host via the γ -aminobutyric acid shunt and polyamines pathway. *Mol. Plant-Microbe Interact.* 32:413-427.
- Nehela, Y., and Killiny, N. 2020a. Detection of phytomelatonin in Huanglongbing-infected sweet orange (*Citrus sinensis*) plants and its correlation with stress-associated phytohormones. *Abstr. Phytopathology* 110:S2.46.
- Nehela, Y., and Killiny, N. 2020b. Melatonin is involved in citrus response to the pathogen Huanglongbing via modulation of phytohormonal biosynthesis. *Plant Physiol.* 184:2216-2239.
- Nehela, Y., and Killiny, N. 2020c. Revisiting the complex pathosystem of Huanglongbing: Deciphering the role of citrus metabolites in symptom development. *Metabolites* 10:409.
- Nehela, Y., and Killiny, N. 2020d. The unknown soldier in citrus plants: Polyamines-based defensive mechanisms against biotic and abiotic stresses and their relationship with other stress-associated metabolites. *Plant Signal. Behav.* 15:1761080.
- Nehela, Y., and Killiny, N. 2021. *Diaphorina citri* genome possesses a complete melatonin biosynthesis pathway differentially expressed under the influence of the phytopathogenic bacterium, *Candidatus liberibacter asiaticus*. *Insects* 12:317.
- Nicaise, V. 2014. Crop immunity against viruses: Outcomes and future challenges. *Front. Plant Sci.* 5:660.
- Niegemann, E., Schulz, A., and Bartsch, K. 1993. Molecular organization of the *Escherichia coli* gab cluster: Nucleotide sequence of the structural genes *gabD* and *gabP* and expression of the GABA permease gene. *Arch. Microbiol.* 160:454-460.
- O'Leary, B. M., Neale, H. C., Geilfus, C. M., Jackson, R. W., Arnold, D. L., and Preston, G. M. 2016. Early changes in apoplast composition associated with defence and disease in interactions between *Phaseolus vulgaris* and the halo blight pathogen *Pseudomonas syringae* pv. *phaseolicola*. *Plant Cell Environ.* 39:2172-2184.
- Park, D. H., Mirabella, R., Bronstein, P. A., Preston, G. M., Haring, M. A., Lim, C. K., Collmer, A., and Schuurink, R. C. 2010. Mutations in γ -aminobutyric acid (GABA) transaminase genes in plants or *Pseudomonas syringae* reduce bacterial virulence. *Plant J.* 64:318-330.
- Ramos, F., el Guezzar, M., Grenson, M., and Wiame, J.-M. 1985. Mutations affecting the enzymes involved in the utilization of 4-aminobutyric acid as nitrogen source by the yeast *Saccharomyces cerevisiae*. *Eur. J. Biochem.* 149:401-404.
- Rani, M., and Jha, G. 2021. Host gamma aminobutyric acid metabolic pathway is involved in resistance against *Rhizoctonia solani*. *Phytopathology* 111:1207-1218.
- Schneider, B. L., Ruback, S., Kiupakis, A. K., Kasbarian, H., Pybus, C., and Reitzer, L. 2002. The *Escherichia coli* gabDTPC operon: Specific γ -aminobutyrate catabolism and nonspecific induction. *J. Bacteriol.* 184:6976-6986.
- Scholz, S. S., Reichelt, M., Mekonnen, D. W., Ludewig, F., and Mithöfer, A. 2015. Insect herbivory-elicited GABA accumulation in plants is a wound-induced, direct, systemic, and jasmonate-independent defense response. *Front. Plant Sci.* 6:1128.
- Seifi, H. S., Curvers, K., De Vleeschauwer, D., Delaere, I., Aziz, A., and Höfte, M. 2013. Concurrent overactivation of the cytosolic glutamine synthetase and the GABA shunt in the ABA-deficient sitiens mutant of tomato leads to resistance against *Botrytis cinerea*. *New Phytol.* 199:490-504.
- Seifkhalhor, M., Aliniaefard, S., Hassani, B., Niknam, V., and Lastochkina, O. 2019. Diverse role of γ -aminobutyric acid in dynamic plant cell responses. *Plant Cell Rep.* 38:847-867.
- Shelp, B. J., Bown, A. W., and McLean, M. D. 1999. Metabolism and functions of gamma-aminobutyric acid. *Trends Plant Sci.* 4:446-452.
- Shelp, B. J., Bown, A. W., and Zarei, A. 2017. 4-Aminobutyrate (GABA): A metabolite and signal with practical significance. *Botany* 95:1015-1032.
- Slisz, A. M., Breksa, A. P., 3rd, Mishchuk, D. O., McCollum, G., and Slupsky, C. M. 2012. Metabolomic analysis of citrus infection by 'Candidatus Liberibacter' reveals insight into pathogenicity. *J. Proteome Res.* 11:4223-4230.
- Steward, F. C., Thompson, J. F., and Dent, C. E. 1949. γ -Aminobutyric acid: A constituent of the potato tuber? *Science* 110:439-440.
- Stipanuk, M. H. 2006. *Biochemical, Physiological, & Molecular Aspects of Human Nutrition*, 2nd ed. Elsevier Inc., St Louis.
- Strange, R. N., and Scott, P. R. 2005. Plant disease: A threat to global food security. *Annu. Rev. Phytopathol.* 43:83-116.
- Sweetlove, L. J., Beard, K. F. M., Nunes-Nesi, A., Fernie, A. R., and Ratcliffe, R. G. 2010. Not just a circle: Flux modes in the plant TCA cycle. *Trends Plant Sci.* 15:462-470.
- Takahashi, H., Matsumura, H., Kawai-Yamada, M., and Uchimiya, H. 2008. The cell death factor, cell wall elicitor of rice blast fungus (*Magnaporthe grisea*) causes metabolic alterations including GABA shunt in rice cultured cells. *Plant Signal. Behav.* 3:945-953.
- Tarkowski, L. P., Signorelli, S., and Höfte, M. 2020. γ -Aminobutyric acid and related amino acids in plant immune responses: Emerging mechanisms of action. *Plant Cell Environ.* 43:1103-1116.

- Tatineni, S., Sagaram, U. S., Gowda, S., Robertson, C. J., Dawson, W. O., Iwanami, T., and Wang, N. 2008. In planta distribution of '*Candidatus Liberibacter asiaticus*' as revealed by polymerase chain reaction (PCR) and real-time PCR. *Phytopathology* 98:592-599.
- Teixeira, D. C., Luc Danet, J., Eveillard, S., Cristina Martins, E., de Jesus Junior, W. C., Takao Yamamoto, P., Aparecido Lopes, S., Beozzo Bassanezi, R., Juliano Ayres, A., Saillard, C., and Bové, J. M. 2005. Citrus huanglongbing in São Paulo State, Brazil: PCR detection of the '*Candidatus*' Liberibacter species associated with the disease. *Mol. Cell. Probes* 19:173-179.
- Tunncliffe, G. 1986. 4-Aminobutyrate transaminase. Pages 389-420 in: *Neurotransmitter Enzymes. Neuromethods (Series 1: Neurochemistry)*, Vol 5. A. A. Boulton, G. B. Baker, and P. H. Yu, eds. Humana Press, Totowa, NJ, U.S.A.
- Uzma Jalil, S., Khan, M. I. R., and Ansari, M. I. 2019. Role of GABA transaminase in the regulation of development and senescence in *Arabidopsis thaliana*. *Curr. Plant Biol.* 19:100119.
- Wang, G., Kong, J., Cui, D., Zhao, H., Niu, Y., Xu, M., Jiang, G., Zhao, Y., and Wang, W. 2019. Resistance against *Ralstonia solanacearum* in tomato depends on the methionine cycle and the γ -aminobutyric acid metabolic pathway. *Plant J.* 97:1032-1047.
- Wei, X., Chen, C., Yu, Q., Gady, A., Yu, Y., Liang, G., and Gmitter, F. G. 2014. Novel expression patterns of carotenoid pathway-related genes in citrus leaves and maturing fruits. *Tree Genet. Genomes* 10:439-448.
- Yan, Q., Sreedharan, A., Wei, S., Wang, J., Pelz-Stelinski, K., Folimonova, S., and Wang, N. 2013. Global gene expression changes in *Candidatus Liberibacter asiaticus* during the transmission in distinct hosts between plant and insect. *Mol. Plant Pathol.* 14:391-404.