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Regulatory Effect of Exogenous γ-Aminobutyric Acid on Respiratory Rate through the γ-Aminobutyric Acid Shunt in *Malus baccata* (L.) Borkh. Roots under Suboptimal Low Root-Zone Temperature

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Abstract: *Malus baccata* (L.) Borkh. is one of the most widely used rootstocks in the apple-producing region of Northern China. However, in the early growing season, apple roots are often subjected to suboptimal low root-zone temperatures. The regulatory effects of exogenous γ-aminobutyric acid (GABA) on both the γ-aminobutyric acid shunt (GABA shunt) and the respiratory activity of roots under suboptimal low root-zone temperatures remain unknown. To explore the physiological basis for GABA alleviation of low-temperature stress in *M. baccata* Borkh. roots, the following treatments were examined: suboptimal low root-zone temperature (potted parts of the seedlings were maintained at 5 ± 0.5 °C; L); suboptimal low root-zone temperature + GABA (LG); and suboptimal low root-zone temperature + vigabatrin (VGB; LV), which is a specific active inhibitor of γ-aminobutyric acid transaminase (GABA-T). Each treatment was matched with a control (18 °C/8 °C day/night; CK) for comparison. Our results showed that the L treatment reduced the root vitality, increased malondialdehyde (MDA) content, promoted the accumulation of GABA, activated the GABA shunt, and inhibited the total root respiration rate (V_{Total}) by decreasing the respiratory rates of Embden–Meyerhof pathway (V_{EMP}) and tricarboxylic acid cycle (V_{TCAC}). The LG treatment significantly increased the content of endogenous GABA, accelerated the metabolism of the GABA shunt, enhanced root respiratory activity by increasing V_{Total}, V_{EMP}, V_{TCAC}, and increased the cytochrome pathway respiratory rate (V_{CP}), thus alleviating the damage of low root-zone temperature stress. Meanwhile, contrasting results were observed in the LV treatment. These findings revealed that exogenous GABA improved the tolerance of apple rootstocks to suboptimal low temperatures in early spring by regulating the GABA shunt and root respiratory activity.

Keywords: *Malus baccata* (L.) Borkh.; apple rootstock; suboptimal low root-zone temperature; GABA shunt; tricarboxylic acid cycle; root respiration

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1. Introduction

Low temperature is a major environmental stressor which limits plant growth, development, and yield due to a variety of physiological and metabolic disproportions such as nutritional disorders, membrane dysfunction, reactive oxygen species (ROS) accumulation, and protein degradation [1,2]. In addition, low temperatures can lead to energy shortages in plants as a result of mitochondrial dysfunction and the inhibition of respiratory metabolism [3]. Adjustments in respiratory metabolism are critical for plant growth and adaptation to environmental stresses [4], such as chilling (above 0 °C) stress which can cause growth retardation and reversible damage in plants [5]. The types and pathways of plant metabolism are mutable, with changes occurring in response to environmental

shifts [6]. These metabolic changes regulate physiological functions, and, in turn, functional changes can impact metabolism [7].

Recently, inconsistencies between the characteristics of soil temperature and air temperature have become a growing concern. Soil temperature is affected by season, diurnal temperature variation, soil depth, and other factors (e.g., mulching in orchards); however, it is resistant to change, and temperature adjustments are often delayed compared to similar changes in air [8]. A suboptimal low root-zone temperature is one which is below the lower limit of the optimal temperature range of plant growth and development, but not low enough to cause lethal damage to the plant [9]. While low air temperature can cause damage to a plant, suboptimal low root-zone temperatures directly impose a variety of negative effects on root development, absorption and transport of water and mineral nutrients, and accumulation of plant hormones, all of which inhibit the growth of the whole plant [10–12]. In their study, Li et al. [9] found that suboptimal low root-zone temperatures can cause oxidative stress in plant roots, but an exogenous jasmonic acid treatment can alleviate this damage by regulating the activity of the ascorbate–glutathione cycle. Many other studies corroborated this effect, showing that the addition of exogenous substances can effectively alleviate stress-induced damage by regulating plant metabolism components, such as γ-aminobutyric acid (GABA) [13], spermidine [14], and putrescine [15].

GABA is a ubiquitous, four-carbon, non-protein amino acid found in plants [16]. It is formed from glutamic acid (Glu) in a reaction catalyzed by glutamate decarboxylase (GAD) and catabolized into succinate (Suc) by γ-aminobutyric acid transaminase (GABA-T) and succinic semialdehyde dehydrogenase (SSADH) [17]. With a molecular structure similar to that of proline, GABA can act as an osmotic regulator to maintain balance under stress [18]. Stress can cause plants to quickly accumulate GABA, and some studies have reported that this acts as a signal for various stress-relieving plant responses [19–22].

Glu and Suc are important components of nitrogen metabolism and the tricarboxylic acid cycle (TCAC), respectively. The γ-aminobutyric acid shunt (GABA shunt) uses GABA to convert Glu to Suc for use in respiration [16]. Therefore, under stressful conditions, the GABA shunt is believed to serve as the hub of carbon and nitrogen metabolism [17,23]. Under salt stress, the inhibition of the TCAC in wheat can be overcome by enhancing the GABA shunt activity, which provides an alternative carbon source for the mitochondria that bypasses salt-sensitive enzymes and facilitates leaf respiration [24]. Furthermore, in response to UV stress, the GABA shunt pathway and the accumulation of GABA in *Arabidopsis* seedlings have been identified as major components of the antioxidant machinery associated with ROS scavenging, H_2O_2 equilibrium, cellular redox state balance, and cellular signals in acquiring tolerance [25]. The application of exogenous GABA has been confirmed to have a positive role in the activity of the GABA shunt when responding to stresses. Specifically, it has been found that exogenous GABA can effectively regulate seed germination, root growth, photosynthesis, and fruit development in response to stresses such as chilling, drought, hypoxia, and salinity [26–28]. A previous study found that, in the Mazzard F12/1 genotype of Prunus species, exogenous GABA induces the transcriptional activities of *GAD2* and *GAD4* in the roots; improves the photosynthetic rate, stomatal conductance, and total chlorophyll content; affects leaf H_2O_2 levels; and affects the contents of endogenous GABA, Glu, and alanine in a genotype- and organ-specific manner, thus, alleviating the deleterious effects of oxygen deficiency on the roots [29]. Exogenous GABA can also relieve stress by modulating other metabolic processes. For example, in *Malus huphehensis*, exogenous GABA can activate the GABA shunt to significantly increase the contents of malate, citric acid, and Suc, as well as the activity of malate dehydrogenase, citrate synthase, isocitrate dehydrogenase, and aconitase. This contributes to alleviating the decrease in biomass, inhibition of root growth, and oxidative stress caused by alkaline stress [30]. Many researchers have sought to clarify the GABA regulatory mechanisms in plants under stress. However, this is difficult since plant species exhibit different tolerances to low temperatures, and even within one plant the response mechanisms vary between

different parts. Therefore, additional experiments are needed to clarify the physiological mechanisms of the GABA shunt in a plant response to suboptimal temperatures.

Malus baccata (L.) Borkh., a widely used rootstock variety in the apple-producing region of Northern China, faces difficulties in early spring due to suboptimal low temperatures in the root zone. Our previous studies showed that exogenous application of GABA can help alleviate oxidative stress caused by these low temperatures [31]. In the present study, we applied GABA and vigabatrin (VGB), a specific active GABA-T inhibitor, to further investigate the characteristics of the GABA shunt in response to suboptimal low root-zone temperature and its effects on respiration in root cells. Our results provide a theoretical foundation for further studies on the adaptation of apple rootstock roots to suboptimal low root-zone temperatures. The applications outlined in this study have the potential to improve the cold tolerance of apple rootstocks in early spring.

2. Materials and Methods

2.1. Plant Materials and Experimental Treatments

Malus baccata (L.) Borkh. seedlings were used as the experimental plant material. Seedlings with five leaves were transplanted into plastic pots (13 × 12 cm) containing garden soil, river sand, and substrate (*v:v:v* = 2:1:1). The potted seedlings were cultivated in a greenhouse. When seedlings had 15 leaves and were determined to be free of diseases and pests, they were selected for experimental treatment. The experiment was conducted in an artificial climate chamber with a day/night temperature regime of 18 °C/8 °C and a light/dark photoperiod of 14 h/10 h. After 2 days, the seedlings were grouped into four sets and subjected to four different treatments: (1) CK: control treatment in which seedling roots were not subjected to any treatments with an ambient temperature of 18 °C (day)/8 °C (night); (2) L: potted parts of the seedlings were placed in a thermostatic bath maintained at 5 ± 0.5 °C; (3) LG: each seedling was watered with 100 mL of γ-aminobutyric acid (GABA) solution (10 mmol·L^{-1}) (Solarbio, Beijing, China) and subsequently placed in a thermostatic bath maintained at 5 ± 0.5 °C; and (4) LV: each seedling was watered with 100 mL of vigabatrin (VGB) solution (0.1 mmol·L^{-1}) (MedChemExpress, Monmouth, NJ, USA) and then placed in a thermostatic bath maintained at 5 ± 0.5 °C. The roots of *M. baccata* Borkh. seedlings were sampled on days 0, 1, 2, 4, and 6 of treatment and washed with distilled water (dH_2O). A portion of each root sample was immediately used to measure root respiration, and the remainder was frozen in liquid nitrogen and stored at −80 °C until further analysis.

2.2. Determination of Root Vitality and Malondialdehyde (MDA) Content

Root vitality and malondialdehyde (MDA) content were measured according to the methods of Su et al. [32]. Fresh roots (500 mg) were homogenized in a 10 mL solution containing 5 mL triphenyl tetrazolium chloride (TTC) and 5 mL Na_2HPO_4-KH_2PO_4 (pH 7.0). Root vitalities were calculated by reducing TTC. Approximately 500 mg of root tissue was homogenized in a solution containing 2-thiobarbituric acid and trichloroacetic acid, and the MDA contents were determined spectrophotometrically at 450, 532, and 600 nm.

2.3. Extraction and Determination of Glutamic Acid (Glu) and γ-Aminobutyric Acid (GABA) Content

Glutamic acid (Glu) and endogenous GABA were extracted according to the methods of Baum et al. [33], with slight modifications. Briefly, approximately 500 mg of fresh root tissue was homogenized in 2 mL of a solution containing chloroform, methanol, and water (*v:v:v* = 5:12:3). The mixture was ground on ice and then centrifuged at 12,000× *g* for 5 min at 4 °C. A total of 750 µL of chloroform and 1250 µL of dH_2O were added to the collected supernatant. The resulting mixture was vortexed and centrifuged at 12,000× *g* for 5 min at 4 °C. The upper portion containing Glu and GABA was collected, dried in a speed-vac (Eppendorf GmbH, Hamburg, Germany), and redissolved in 200 µL of dH_2O. The sample derivatization method was applied as described by Liu et al. [34]. This solution and an equal volume of dansyl chloride solution (4 mg·mL^{-1}) were added to a 2 mL centrifuge tube and shaken, placed in a water bath (55 °C) in the dark for 1 h, cooled to room temperature, and diluted with methanol to a volume of 1.0 mL. The mixture was filtered through a 0.22 µm membrane. The Glu and GABA contents were analyzed using high-performance liquid chromatography (VWD, Angilent1260, Santa Clara, CA, USA) with an ODS C18 chromatographic column (4.6 × 250 mm, 5 µm). The injection volume was 10 µL, the column temperature was 35 °C, the flow rate was 1 mL·min^{-1}, and the maximum absorption peak was observed at 254 nm. The gradient elution procedure was used with methanol as the mobile phase A, and a methanol solution containing 85% sodium acetate as the mobile phase B. Standard samples were purchased from Sigma-Aldrich (St. Louis, MO, USA). The standard curve had a linear relationship between 0.001–0.1 mmol·mL^{-1}. The Glu and GABA contents in the roots were calculated based on the standard curve and peak area.

2.4. Extraction and Determination of Succinate (Suc) Content

Succinate (Suc) was extracted using double-distilled water (ddH_2O). Approximately 200 mg of root tissue was deactivated with enzymes at 110 °C for 10 min, then oven-dried at 80 °C until a constant weight was reached. The sample was homogenized in 1 mL of ddH_2O, and the resulting mixture was shocked at 1200 rpm for 2 h at room temperature then centrifuged for 30 min at 10,000× *g*. The supernatant was collected and filtered through a 0.22 µm water syringe filter [35]. The samples were then analyzed using a high-performance liquid chromatograph (Angilent1260, Santa Clara, CA, USA) combined with a VWD detector (210 nm) according to the methods of Arnetoll et al. [36]. For separation, an Agilent SB-Aq column (4.6 × 250 mm, 5 µm) was used, with KH_2PO_4 (20 mM, pH 2.2) and acetonitrile serving as the mobile phases at a flow rate of 0.8 mL·min^{-1}. The standard was obtained from Sigma-Aldrich (St. Louis, MO, USA) and standard curves were linear from 0.25–25 µmol·mL^{-1}. The Suc content was calculated using the standard curve and peak areas.

2.5. Extraction and Determination of Enzyme Activities in GABA Shunt

Fresh roots (200 mg) were homogenized in 1 mL of ice-cold protease extraction buffer containing 50 mM HEPES-KOH, 5 mM magnesium acetate, 15% glycerol, 1 mM EDTA, 1 mM EGTA, and 5 mM mercaptoethanol (pH 6.8). The samples were then transferred to 1.5 mL tubes and centrifuged at 12,000× *g* for 15 min at 4 °C, and the resulting supernatant was collected [37]. The activities of glutamate decarboxylase (GAD), γ-aminobutyric acid transaminase (GABA-T), and succinic semialdehyde dehydrogenase (SSADH) were measured using ELISA detection kits (Mlbio, Enzyme-linked Biotechnology Co., Shanghai, China) according to the manufacturer's instructions.

2.6. Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR) Analysis

Total RNA was extracted from all samples using a Plant RNA Kit (R6827, Omega Biotek, Inc., Norcross, GA, USA), and the DNA was removed according to the manufacturer's instructions. The RNA concentration and quality were determined using a NanoDrop1000 spectrophotometer. Total RNA (1 μg) was used for cDNA synthesis by reverse transcription using a PrimeScript RT-PCR Kit (DRR037A, Takara, Dalian, China), which was then diluted 10-fold. qRT-PCR was performed using an ABI StepOnePlus Real-Time PCR system using SYBR Premix Ex Taq II (DRR820A, Takara). The qRT-PCR procedure was as follows: initial denaturation for 5 min at 95 °C, 40 cycles of amplification with a denaturation step for 30 s at 95 °C, and extension for 30 s at 55 °C. The results were analyzed with reference to published pipelines [38], and the $2^{-\Delta\Delta CT}$ method was used to calculate relative expression levels. Primer sets were designed using Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA), and are listed in Table 1.

Table 1. Primer sequences of the GABA shunt genes used for quantitative real time PCR reactions.

Genes	Sequence ID	Primer Sequence (5′-3′)
Actin	-	F: GGCTGGATTTGCTGGTGATG R: TGCTCACTATGCCGTGCTCA
GAD1	NM_001294057.1	F: TCAGCCCACTTTCACCCT R: CATCCGAACTTCCTCAAACTAT
GAD2	NM_001293835.1	F: GGAGCCAATGTCCAGGTG R: GCCGAGGATAGCAGCAAC
GAD3	NM_001294084.1	F: CGGTGGGACAGACACAGAGA R: CACTCCGACTAGTAGCATTTTGCA
GABA-T1	NM_001328897.1	F: CTATTTATTGCCGACGAG R: ACAAGAACAGCACCGATT
GABA-T2	NM_001293859.1	F: ACACTGACTGCCCACATT R: TCACAAGAACAGCACCAA
SSADH1	XM_008357890.3	F: CAGTGGCACCCCTTTTGC R: GCAGCTAACCCTGCATTGGT
SSADH2	XM_029110078.1	F: TCATACTTTGATACCTCATCCTCCAT R: GAGCAGCAGGATAGAAATTTGAATG

F, forward primer; R, reverse primer.

2.7. Respiratory Rate Determination

The root respiration was measured as the oxygen consumption rate by using an Oxytherm^{+} oxygen electrode (Hansatech, King's Lynn, Norfolk, UK) according to a modification of the procedure described by Bouma et al. [39]. Fresh roots (500 mg) of each replicate were used to measure the root respiration rate. NaF, malonic acid, Na3PO4, hydroxyzine salicylic acid, and sodium cyanide were used as the inhibitors of the Embden–Meyerhof pathway (EMP), tricarboxylic acid cycle (TCAC), pentose phosphate pathway (PPP), cytochrome pathway (CP), and alternate pathway (AP), respectively. The difference in oxygen consumption of the roots, with and without a specific inhibitor, was considered to be the respiration rate of each pathway [40].

2.8. Statistical Analysis

Statgraphics XVII software (STN, St. Louis, MO, USA) was used for statistical analysis. All data were tested for normality and then subjected to a one-way analysis of variance (ANOVA). Differences were considered significant at $p < 0.05$. Significant differences between the treatments and between full data were marked with different lowercase letters and capital letters, respectively. The values are presented as the mean ± standard error (SE). For the principal component analysis (PCA), the data were standardized and computed in SPSS software.

3. Results

3.1. Root Vitality and Malondialdehyde (MDA) Content

To evaluate the damage of suboptimal low root-zone temperature to *Malus baccata* (L.) Borkh. roots, we determined the root vitality (Figure 1a) and malondialdehyde (MDA) content (Figure 1b). Compared with the control (CK), the low temperature (L) treatment significantly inhibited the root vitality and increased the MDA content. Compared with the L treatment, exogenous GABA application (LG) significantly enhanced the root vitality and significantly decreased the MDA content. Compared with the LG treatment, plants pretreated with VGB (LV) had an opposite influence on root vitality and MDA content.

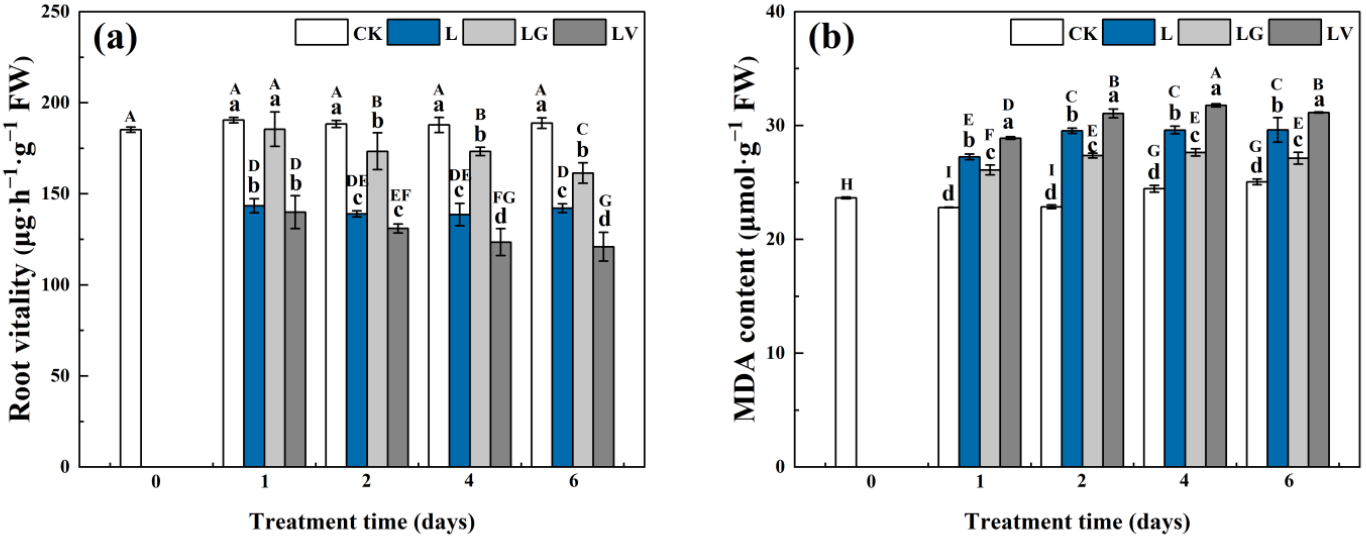

Figure 1. Changes in root vitality (**a**) and malondialdehyde (MDA) content (**b**) in *Malus baccata* (L.) Borkh. roots pre-treated with GABA or VGB under suboptimal low root-zone temperatures from days 0 to 6. Means ± SE were calculated from three replicates for each treatment. Different lowercase letters above the bars indicate significant differences between treatments ($p < 0.05$). Different capital letters above the bars indicate significant differences between full data ($p < 0.05$). CK: control; L: suboptimal low root-zone temperature (potted parts of the seedlings were maintained at 5 ± 0.5 °C); LG: suboptimal low root-zone temperature + 10 mmol L^{-1} GABA; LV: suboptimal low root-zone temperature + 0.1 mmol·L^{-1} VGB.

3.2. Effect on the GABA Shunt

To investigate the response of the GABA shunt to suboptimal low root-zone temperature and the physiological role of exogenous GABA and VGB in this response, the metabolite contents (Figure 2), enzyme activities (Figure 3), and gene expressions (Figure 4) of the GABA shunt were measured.

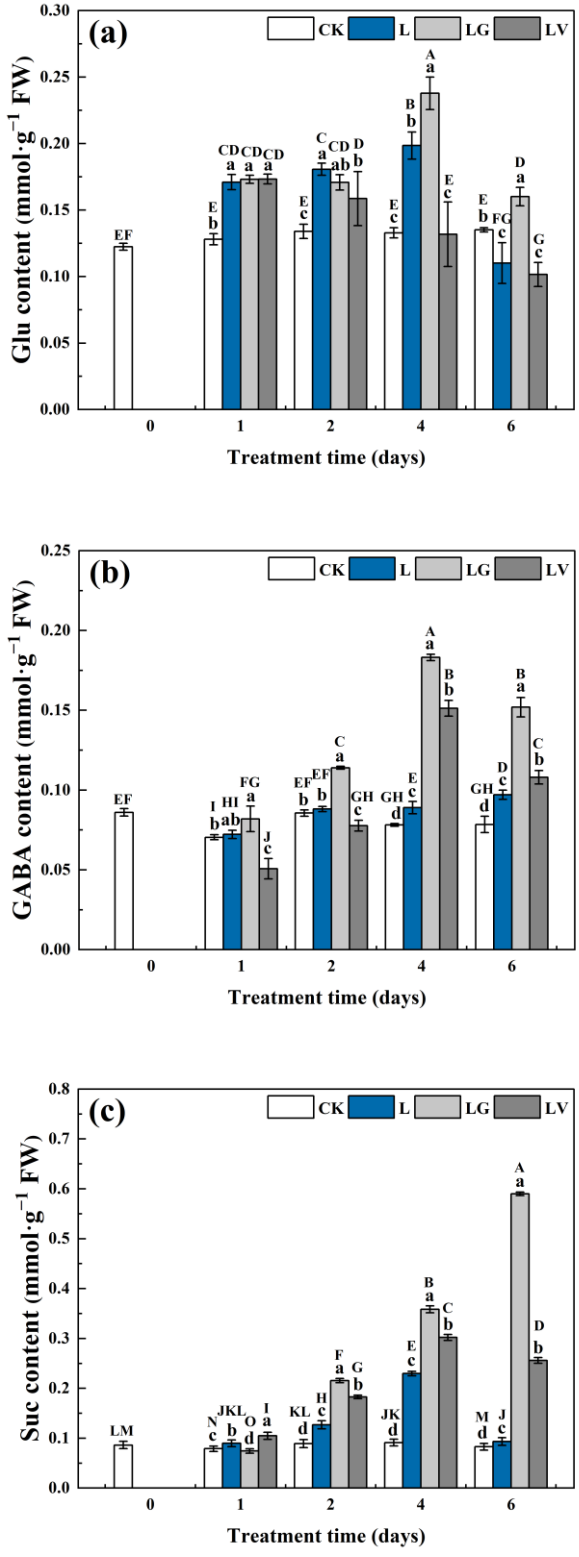

Figure 2. Effects of GABA or VGB application on the contents of (**a**) glutamic acid (Glu), (**b**) γ-aminobutyric acid (GABA), and (**c**) succinate (Suc) in *Malus baccata* (L.) Borkh. roots under suboptimal low root-zone temperatures from days 0 to 6. Means ± SE were calculated from three replicates for each treatment. Different lowercase letters above the bars indicate significant differences between treatments ($p < 0.05$). Different capital letters above the bars indicate significant differences between full data ($p < 0.05$). Treatments legend as in Figure 1.

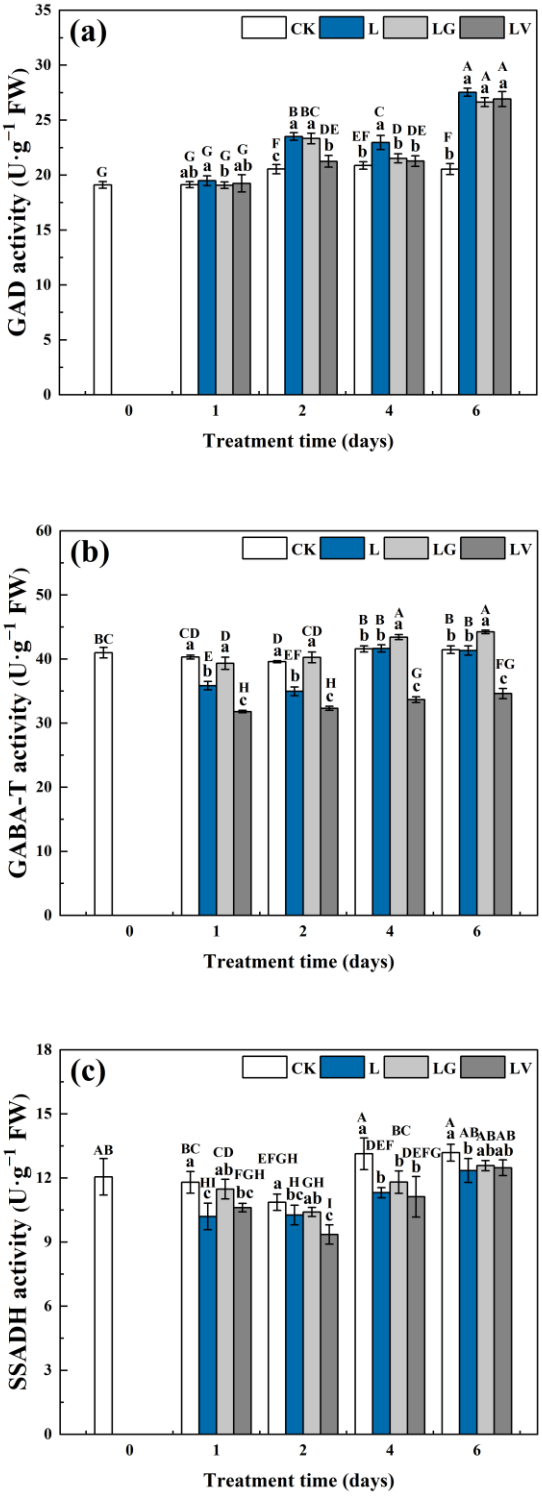

Figure 3. Effects of GABA or VGB application on the activities of (**a**) glutamate decarboxylase (GAD), (**b**) GABA transaminase (GABA-T), and (**c**) succinic semialdehyde dehydrogenase (SSADH) in *Malus baccata* (L.) Borkh. roots under suboptimal low root-zone temperatures from days 0 to 6. Means ± SE were calculated from three replicates for each treatment. Different lowercase letters above the bars indicate significant differences between treatments ($p < 0.05$). Different capital letters above the bars indicate significant differences between full data ($p < 0.05$). Treatments legend as in Figure 1.

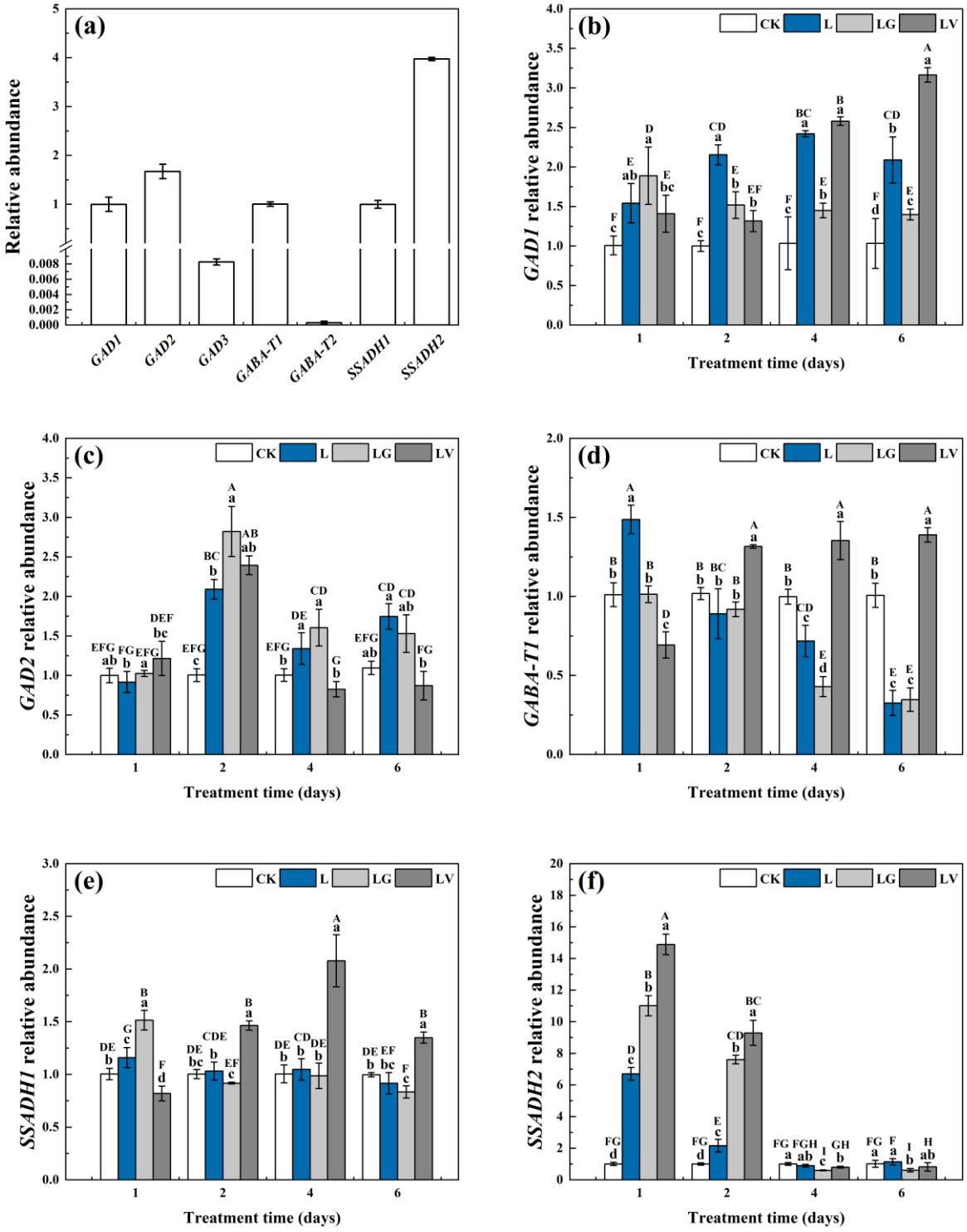

Figure 4. (**a**) mRNA levels of *GAD*, *GABA-T*, and *SSADH* gene family members in control *Malus baccata* (L.) Borkh. roots. Means were calculated from six replicates for each gene. (**b**–**f**) Effects of GABA or VGB application on the mRNA levels of *GAD1* (**b**), *GAD2* (**c**), *GABA-T1* (**d**), *SSADH1* (**e**), and *SSADH2* (**f**) in *M. baccata* Borkh. roots under suboptimal low root-zone temperatures. Means ± SE were calculated from six replicates for each treatment. Different lowercase letters above the bars indicate significant differences between treatments ($p < 0.05$). Different capital letters above the bars indicate significant differences between full data ($p < 0.05$). Treatments legend as in Figure 1.

3.2.1. Metabolite Contents in the GABA Shunt

Figure 2a shows that the L treatment was characterized by an increasing Glu content from days 1 to 4, reaching a significant increase of 49.4% compared with the CK treatment. After day 4, the glutamic acid (Glu) content in the L treatment began to decline, reaching a significantly lower value on day 6 compared with the CK treatment. Compared with the L treatment, the application of GABA significantly increased the Glu content on days 4 and 6 by 19.8% and 45.4%, respectively. In the LV treatment, the Glu content was significantly increased on day 1 relative to CK, which was similar to the results of the L and LG treatments. Subsequently, the Glu content decreased over the following days of temperature treatment.

In the L treatment, the endogenous GABA content gradually increased and was significantly higher than that of the CK from days 4 to 6. Compared with the L treatment, the LG treatment significantly increased endogenous GABA content, peaking on day 4 and increasing by 105.7%. Compared with that in L treatment, the endogenous GABA content in the LV treatment was significantly reduced on days 1 and 2, and significantly increased on days 4 and 6, peaking on day 4 with an increase of 69.9%; however, it was still significantly lower than that of the LG treatment (Figure 2b).

Compared with the CK treatment, there was a significant increase in succinate (Suc) content in the L treatment. This followed a similar trend to Glu content. The Suc content peaked on day 4 with an increase of 151.9% compared to that of CK. In the LG treatment, the Suc content greatly increased from days 2 to 6, and was 5.23-fold higher on day 6 than that in the L treatment. The Suc content in the LV treatment was significantly higher on day 1 than that in all other treatments. However, from days 2 to 6, the Suc content was significantly lower in the LV treatment than in the LG treatment, though it was still significantly higher than the L treatment (Figure 2c).

3.2.2. Key Enzyme Activities of GABA Shunt

Glutamate decarboxylase (GAD) activity increased continuously during the L treatment, with an increase of 31.4% on day 6 compared with that in the CK treatment. The LG treatment significantly inhibited GAD activity on days 1 and 4 compared with the L treatment; however, no significant differences were observed on days 2 and 6. Meanwhile, GAD activity in the LG treatment was significantly higher than that in the CK treatment on days 2 and 6. In the LV treatment, the trend in GAD activity was similar to that of the L treatment. GAD activity in the LV treatment was consistently lower than in that of the L treatment, and the difference was most significant from days 2 to 4 (Figure 3a).

Compared with the CK treatment, the L treatment resulted in significantly inhibited GABA transaminase (GABA-T) activity from days 1 to 2. Then, GABA-T activity slowly increased, which was similar to its trend in the CK treatment on days 4 and 6. The LG treatment significantly enhanced GABA-T activity, which was maintained at a high level throughout the entire processing period. This was consistent with its trend in the L treatment. The GABA-T activity in the LV treatment was significantly inhibited and consistently lower than that of the other treatments (Figure 3b).

Succinic semialdehyde dehydrogenase (SSADH) activity was significantly inhibited by the L treatment, which is consistent with the change in GABA-T activity under the L treatment. SSADH activity was higher in the LG treatment compared with the L treatment; however, the difference was only significant on day 1. The LV treatment had no significant effect on SSADH activity compared with the L treatment (Figure 3c).

3.2.3. Gene Expressions of Key Enzymes in the GABA Shunt

The gene family members for key enzymes in the GABA shunt, *GADs*, *GABA-Ts*, and *SSADHs*, were quantitatively analyzed using qRT-PCR. As shown in Figure 4a, the gene family members exhibited different expression levels in the control *M. baccata* Borkh. roots. Expression levels of *GAD1*, *GAD2*, *GABA-T1*, *SSADH1*, and *SSADH2* were relatively high, while *GAD3* and *GABA-T2* were negligibly expressed. To investigate the changing

characteristics of key enzyme genes in the GABA shunt under suboptimal temperatures, we further analyzed genes that were highly expressed at the mRNA level.

Compared with the CK treatment, *GAD1* expression was upregulated following L treatment and peaked on day 4 at a 2.42-fold increase. Compared with the L treatment, the expression of *GAD1* in the LG treatment was upregulated on day 1, and significantly downregulated from days 2 to 6. In the LV treatment, *GAD1* expression tended to gradually increase over time, and was 1.7-fold higher than that in the L treatment on day 6 (Figure 4b).

GAD2 expression in the L treatment was also significantly higher from days 2 to 4 than that in the CK, but no significant differences were observed on days 1 and 6. Compared with the L treatment, *GAD2* expression in the LG treatment was significantly upregulated from days 1 to 4, peaking on day 2. The LV treatment significantly upregulated *GAD2* expression on day 1, after which it was sharply downregulated with a reduction of 50% on day 6 compared with that of the L treatment (Figure 4c).

Under the L treatment, *GABA-T1* expression significantly increased and was 1.49-fold greater than that of the CK on day 1. On day 6, *GABA-T1* expression gradually decreased to 68% less than that of the CK. *GABA-T1* expression in the LG treatment was significantly lower than that in the L treatment on day 1, and then it continuously decreased, with a similar trend to that described for the L treatment. Meanwhile, the trend of *GABA-T1* expression in the LV treatment directly opposed that in the L treatment; that is, *GABA-T1* expression in the LV treatment was significantly downregulated on day 1, and significantly upregulated on day 6, with a 4.21-fold increase compared to the L treatment (Figure 4d).

Compared with the CK treatment, *SSADH1* expression in the L treatment was significantly upregulated on day 1, after which it decreased gradually and no significant differences were observed. The change in *SSADH1* expression in the LG treatment was similar to that in the L treatment. *SSADH1* expression on day 1 was 1.5 times greater than that of the L treatment. However, it gradually decreased after day 1, becoming lower than in that of the L treatment. Compared with the L treatment, the LV treatment significantly downregulated *SSADH1* expression on day 1, after which it became significantly upregulated. It peaked on day 4 in the LV treatment, at which point it was significantly higher than that in all other treatments (Figure 4e).

Compared with the CK, *SSADH2* expression in the L treatment was significantly upregulated on days 1 and 2 by 6.71-fold and 2.17-fold, respectively. It then decreased to a level that was not significantly different from that of the CK treatment. The trends of *SSADH2* expression in the LG and LV treatments were similar to those in the L treatment. *SSADH2* expression in the LG treatment was 1.64-fold and 3.51-fold higher than that in the L treatment on days 1 and 2, respectively. After day 2, *SSADH2* expression dropped sharply and was significantly lower than that in the L treatment. Compared with the L treatment, *SSADH2* expression following LV treatment was significantly increased on days 1 and 2 by 1.22-fold and 3.92-fold, respectively, and then declined sharply, similar to that of the L treatment (Figure 4f).

3.3. Effect on the Respiratory Rate in the Roots

3.3.1. Total Respiration Rate (V_{Total})

Compared with the CK, the L treatment significantly inhibited the total root respiration rate (V_{Total}) which gradually decreased over time. After exogenous GABA application, V_{Total} was significantly increased, peaking on day 2 and reaching a significant increase of 37.8% compared with that in the L treatment. In the LV treatment, V_{Total} was significantly lower than that of the other treatments during the entire processing time; however, it tended to steadily increase over time (Figure 5a). To further study the effects of GABA or VGB application on the rate of each respiratory pathway under suboptimal low root-zone temperatures, the respiratory rates of the basic biochemical pathways and electron transport pathways were measured.

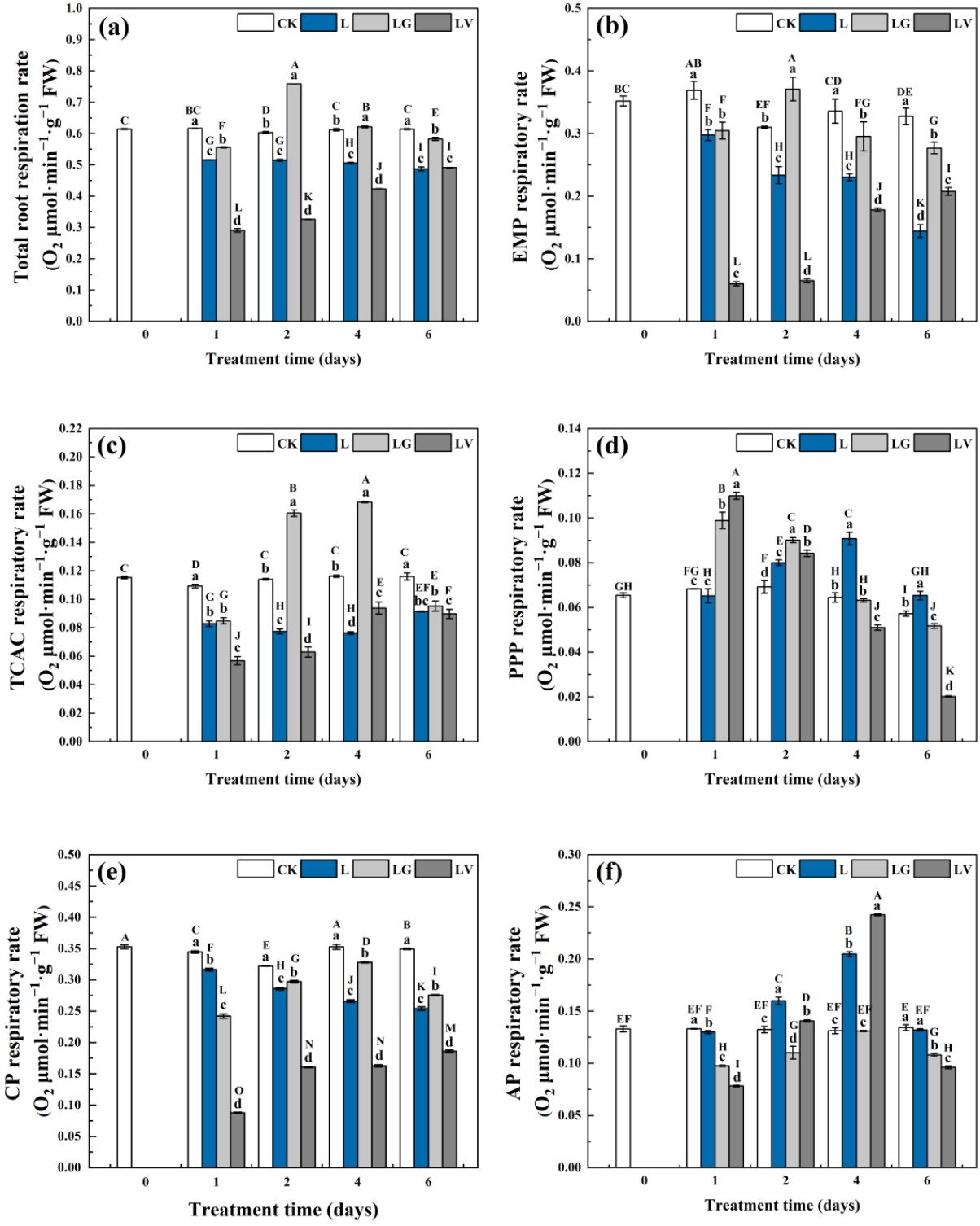

Figure 5. Effects of GABA or VGB application on the root respiratory rate of *Malus baccata* (L.) Borkh. under suboptimal low root-zone temperatures from days 0 to 6. (**a**) Total root respiration rate (V_{Total}); (**b**) Embden–Meyerhof pathway (EMP, V_{EMP}); (**c**) tricarboxylic acid cycle (TCAC, V_{TCAC}); (**d**) pentose phosphate pathway (PPP, V_{PPP}); (**e**) cytochrome pathway (CP, V_{CP}), and (**f**) alternate pathway (AP, V_{AP}). Means ± SE were calculated from three replicates for each treatment. Different lowercase letters above the bars indicate significant differences between treatments ($p < 0.05$). Different capital letters above the bars indicate significant differences between full data ($p < 0.05$). Treatments legend as in Figure 1.

3.3.2. Respiration Rate of Basic Biochemical Pathways

Basic biochemical pathways, including the Embden–Meyerhof pathway (EMP; Figure 5b), tricarboxylic acid cycle (TCAC; Figure 5c), and pentose phosphate pathway (PPP; Figure 5d), maintained stable levels in the CK. Compared with the CK, the L treatment significantly inhibited the EMP (V_{EMP}) and TCAC (V_{TCAC}) respiratory rates during the whole treatment. In contrast, the L treatment significantly increased the PPP respiratory rate (V_{PPP}) from days 2 to 6. In the LG treatment, V_{EMP} and V_{TCAC} were significantly increased from days 1 to 6 compared with the L treatment; V_{EMP} reached its peak on day 2 with an increase of 58.9%, and V_{TCAC} peaked on day 4 with an increase of 120.5% compared with the L treatment. V_{PPP} was significantly increased on days 1 and 2, and it continued to decrease with time. Compared with the L treatment, the LV treatment significantly inhibited V_{EMP} from days 1 to 4, V_{TCAC} on days 1 and 2, and V_{PPP} from days 4 to 6. However, the LV treatment enhanced V_{TCAC} on day 4 and V_{PPP} on days 1 and 2.

3.3.3. Respiratory Rate of Electron Transport Pathways in Mitochondria

The electron transport pathways, including the cytochrome pathway (CP) and alternate pathway (AP), had minimal changes and were maintained at relatively stable states under the CK treatment. However, the CP respiratory rate (V_{CP}; Figure 5e) was higher than the AP respiratory rate (V_{AP}; Figure 5f), indicating that the CP was the dominant pathway in the CK treatment. Compared with the CK, V_{CP} in the L treatment was significantly decreased from days 1 to 6, decreasing by 30.1% on day 6; in the L treatment, V_{AP} significantly increased from days 2 to 4, reaching a peak on day 4 with a 57.8% increase. Following LG treatment, V_{CP} was higher than that of the L treatment from days 2 to 6, increasing by 21.2% on day 4, and V_{AP} was significantly inhibited compared with the L treatment. After the VGB application, V_{CP} was significantly reduced compared with that of the L treatment. The trend of V_{AP} was similar to that in the L treatment; V_{AP} was significantly lower except for on day 4, at which point it was 12.2% higher.

3.4. Principal Component Analysis (PCA)

To comprehensively evaluate the effects of GABA and VGB application on *M. baccata* Borkh. roots under suboptimal low root-zone temperatures, the correlation and correlation intensity of the data were analyzed by a principal component analysis (PCA) which explained 89.95% of the data variability (Figure 6a). The biplot showed that PC1 separated the effects of exogenous GABA application under suboptimal low root-zone temperature and accounted for 70.5% of the observed variance. The V_{Total}, V_{EMP}, V_{TCAC}, V_{CP}, and root vitality (RV) from days 1 to 6 were the main positive contributors to PC1; the V_{PPP} (days 1 and 2), V_{AP} (days 2 and 4), and MDA content (days 1 to 6) were the main negative contributors to PC1. PC2 separated the effects of the L treatment and accounted for 19.5% of the observed variance. The V_{PPP} (days 4 and 6), V_{AP} (days 1, 2, and 6), and V_{CP} (day 1) were the main positive contributors to PC2. In addition, Figure 6b showed the comprehensive scores rank was CK > LG > L > LV. In conclusion, the improvement of root respiratory activity including an enhancement of V_{Total}, V_{EMP}, V_{TCAC}, V_{CP}, and RV were important factors in the efficacy of GABA application in response to suboptimal low root-zone stress. However, the application of VGB was a negative factor.

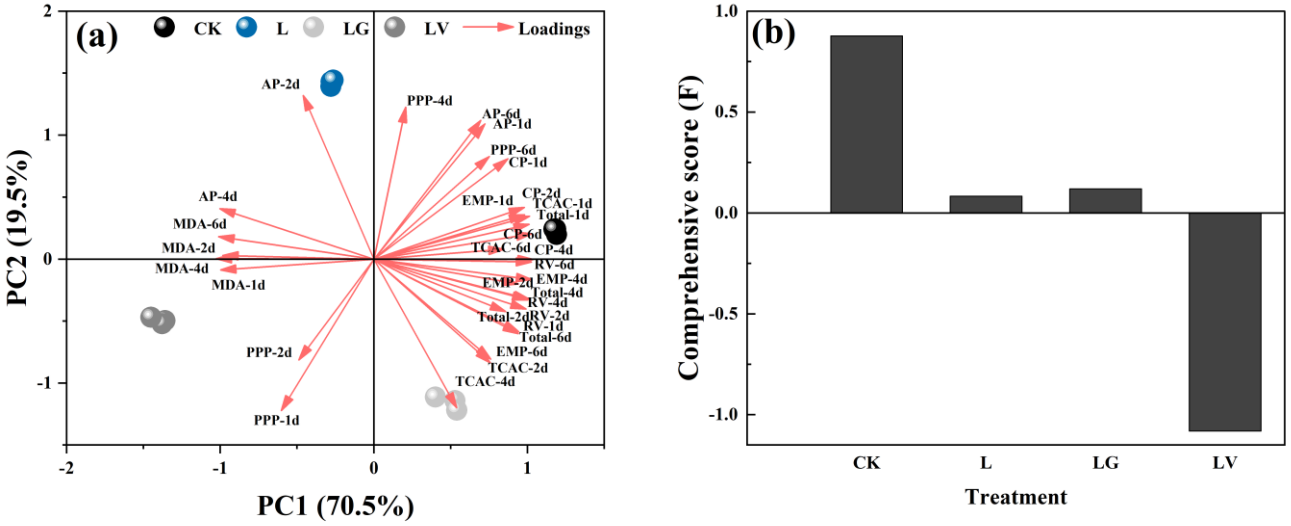

Figure 6. (**a**) Two-dimensional analysis diagram of eigenvectors of PC1 and PC2. The direction and length of the arrow represent the correlation and correlation intensity of the parameters, respectively. The biplot by principal component analysis (PCA) illustrates factors involved in root respiratory activity, root vitality, and MDA content in *Malus baccata* (L.) Borkh. roots after GABA or VGB application under suboptimal low root-zone temperatures from days 1 to 6. (**b**) The comprehensive score (F) and ranking of four treatments. F = F1 × 70.50% + F2 × 19.50%; F1, PC1 score; F2, PC2 score; F1 = α1 (Root vitality on day 1) × Z Root vitality on day 1 + α1 (MDA on day 1) ×Z MDA on day 1 + + α1 (PPP on day 6) × Z PPP on day 6; F2 = α2 (Root vitality on day 1) × Z Root vitality on day 1 + α2 (MDA on day 1) × Z (MDA on day 1) + + α2 (PPP on day 6) × Z PPP on day 6. Z-standardized values calculated in SPSS software. Treatments legend as in Figure 1. The PCA loadings and scores are presented in Supplementary Materials.

4. Discussion

The malondialdehyde (MDA) level is an important parameter reflecting the potential antioxidant capacity of the plant, which can reflect the rate and intensity of lipid peroxidation and also indirectly reflect the degree of tissue peroxidation damage [41]. Evaluation by MDA content has shown that low-temperature stress can inhibit root vitality and aggravate lipid peroxidation [42]. In this study, it was found that suboptimal low root-zone temperature (L treatment) significantly reduced root vitality and increased MDA content (Figure 1). We found that the exogenous application of GABA (LG treatment) could alleviate the negative effects of low-temperature stress; however, it was found that negative effects were aggravated by the vigabatrin treatment (LV treatment). Previous studies have indicated that exogenous GABA could promote the upregulation of antioxidant activity, and decrease the contents of ROS under stresses [43,44]. This may be the cause of the enhancement in root vitality and the reduction in MDA content after GABA treatment.

As a nonproteinogenic amino acid and a product of glutamic acid (Glu) by GAD in response to stress, GABA has recently been attracting more attention [45,46]. In this study, the L treatment increased the Glu content (days 1 to 4) and the endogenous GABA content (Figure 2a). Glu is the precursor of GABA synthesis, so its accumulation likely causes an increase in GABA, as suggested by Wang et al. [47]. At the same time, GABA accumulation may also be related to the increased activity of GAD under the L treatment (Figure 3a). Moreover, GABA content under L treatment was significantly higher than that of the CK, while Glu content decreased on day 6, indicating that the L treatment promoted the conversion of Glu to GABA (Figure 2a,b). Some studies have found that GABA accumulation is proportional to the severity of the stress [48], and is involved in the regulation of cytosolic pH balance, osmoregulation, and ROS levels in response to drought [49], hypoxia [50], salinity, and alkalinity [26]. Possible explanations for this variability could be the depletion of a proton in GABA synthesis and its structural similarity to the osmotic regulatory

substance proline. Previous research has shown that exogenous GABA plays an important role in improving stress tolerance [51]. For example, Wang, X. [46] found that GABA treatment strengthened the resistance of wheat seedlings to heat stress by reducing the production of ROS and coordinated amino acid homeostasis. Similarly, our study showed that exogenous GABA further increased the endogenous GABA content. At the same time point, the LV treatment increased endogenous GABA content and decreased Glu content on days 4 and 6 compared with L treatment (Figure 2a,b). According to Hijaz et al. [52], exogenous GABA could be taken up by the roots in plants. Thus, we speculated that the increase in endogenous GABA content may be partly due to the synthesis of GABA and the absorption of exogenous GABA from the soil in the LG treatments. In the LG treatment, the GAD activity decreased slightly and Glu content increased (days 4 and 6) when compared with the L treatment (Figures 2a and 3a), which is consistent with recent studies [43,53]. A reasonable explanation is that exogenous GABA has a feedback inhibition effect on the conversion of Glu to GABA by decreasing GAD activity.

GAD, as a cytosolic enzyme, could catalyze Glu to GABA. It is reported that GAD gene expression levels differ depending on the plant species and plant part, and can be inconsistent in response to different abiotic stresses [19,54–56]. Trobacher et al. [57] has reported three *GADs* in apple, that is, *GAD1*, *GAD2* and *GAD3*. In *M. baccata* Borkh. roots, we found that *GAD1* and *GAD2* expressions were nearly 200-fold higher than that of *GAD3* (Figure 4a). Meanwhile, Compared with CK, *GAD1* and *GAD2* expression increased under the L treatment. In addition, *GAD1* and *GAD2* presented different characteristics in response to GABA or VGB application. Overall, *GAD1* was more sensitive to exogenous VGB and its expression was significantly enhanced on days 4 and 6. *GAD2* was more sensitive to exogenous GABA and its expression was significantly enhanced in the LG treatment on days 2, 4, and 6 (Figure 4b,c). Combined with the results that GAD activity under the LG or LV treatment was more inhibited compared with L treatment, it was speculated that GAD activity could be stimulated by other factors.

In plants, the conversion of GABA to succinate (Suc) is catalyzed by GABA-T and SSADH [58]. As is known, Suc is an intermediate, which would have been converted to citrate and 2-oxoglutaric acid in the TCAC; Suc can contribute to the production of C skeletons and NADH via the TCAC as well as the generation of ATP via the mitochondrial electron transport chains, which in turn prevents the accumulation of ROS. These functions provided evidence that the GABA shunt plays a pivotal role in alleviating stress-induced damage by supplementing the TCAC [46,59]. Under the L treatment, GABA-T activity and SSADH activity were inhibited compared with the CK (Figure 3b,c). There are two possible reasons for this result. One reason is that enzyme activity was inhibited by low temperatures, and the other is that GABA accumulation by promoting GABA synthesis and reducing GABA catabolism may be an important survival reaction in response to the L treatment, similar to the response of muskmelon seedlings under $Ca(NO_3)_2$ stress [60]. Furthermore, Suc content in the L treatment was significantly higher than that of CK (Figure 2c). Previous studies have shown that, due to their modifying effect on enzyme activity, low temperatures inhibit primary metabolism, secondary metabolism, and the transportation of metabolites; however, organic acid accumulation can improve plant tolerance in response to stress [30,61,62]. These findings provided a reasonable explanation for Suc accumulation under stresses. Interestingly, the activities of GABA-T and SSADH continuously increased on days 4 to 6, indicating that the flow of GABA to Suc could be promoted with an extended L treatment time (Figure 3b,c). Thus, it is speculated that partial Suc accumulation might have been derived from GABA via GABA shunt [63]. Fortunately, this is confirmed by the results of the LG and LV treatments. Exogenous GABA application enhanced GABA-T activity and SSADH activity, and significantly increased the Suc content (days 2 to 6). In contrast, Suc content in the LV treatment was lower than that of the LG treatment due to the VGB inhibiting GABA-T activity and SSADH activity (Figures 2c and 3b,c). Therefore, these results further suggest that the GABA shunt

could be activated by suboptimal low root-zone temperatures, and that exogenous GABA application further accelerated the flow of GABA to Suc.

VGB is an irreversible, and specific, inhibitor of GABA-T that is generally applicable to animals [64,65]. Deleu et al. [66] showed that the activity of GABA-T decreased after treatment with VGB, while the endogenous GABA content increased; similar results were found in this study (Figures 2b and 3b). GABA-T is specific for GABA as an amino donor, and appears to be a homodimer [19,67]. *GABA-Ts* were ubiquitously expressed in all plant organs with different expression levels. In *M. baccata* Borkh. roots, *GABA-T1* expression was higher than *GABA-T2* expression (Figure 4d), similar to that reported for *Oryza sativa* [58]. It is generally accepted that GABA-T activity depends on pyruvate and glyoxylate as amino acceptors; however, there is an evidence that 2-oxoglutarate is preferred over pyruvate in tomato [46]. Therefore, GABA-T activity was effectively inhibited by the substrate feedback. In addition, disparities between mRNA abundance and enzyme activity can occur; the increases in enzyme activity can exceed the corresponding mRNA abundance, or large increases in the levels of gene expression can be incongruent with changes in enzyme activity [68,69]. The completely different responses of *SSADH1* and *SSADH2* expressions suggest that these genes were sensitive to suboptimal low root-zone temperatures and that *SSADH2* responds more quickly to this type of stress than *SSADH1* (Figure 4e,f).

Root respiration provides the necessary energy and substrates for ion absorption and material synthesis, and it also plays a pivotal role in maintaining the balance between carbon and nitrogen metabolism [70]. Many studies have indicated that root respiration is sensitive to soil temperature and that respiratory flux is likely to be limited under low temperatures [32]. Similar results were found in this study, as the L treatment significantly decreased V_{Total} (Figure 5a). This occurred because of the inhibiting effect of low temperatures on potential enzyme activity (both in soluble and membrane-bound compartments) and/or because of the limitations on the function of enzymes that are embedded in membranes due to temperatures below the transition temperature [64]. Root respiration can also be influenced by substrate-dependent factors. Previous studies have indicated that the addition of exogenous glucose can increase root respiration under stress, and there is a positive relationship between soluble sugar concentration and root respiration. In this study, it was also found that exogenous GABA application significantly increased V_{Total}. Combined with the results of the GABA shunt in the LG treatment, it was speculated that exogenous GABA effectively increased V_{Total} by enhancing V_{TCAC}, and that this was related to an increase in Suc content via GABA shunt. This was further confirmed by the LV treatment causing a significant decrease in V_{Total} by inhibiting the activity of the GABA shunt (Figure 5a,c).

To verify the responses of root respiration to suboptimal low root-zone temperatures, we measured the rates of respiratory pathways. In general, basic biochemical pathways included EMP, TCAC, and PPP. Glucose is decomposed by the EMP to form pyruvate, which then enters the TCAC and produces high-energy materials. Compared with the PPP, the EMP and TCAC are dominant. In this study, the L treatment significantly inhibited V_{EMP} and V_{TCAC}, which was why V_{Total} was reduced. However, the L treatment significantly enhanced V_{PPP} (days 2 to 6) compared with CK (Figure 5a–d). The PPP is a major source of reductants for the biosynthetic processes that occur in non-photosynthetic cells, and it also maintains the redox potential necessary to protect against oxidative stress [71]. Under low temperatures, the metabolic flow of glucose-6-phosphate to the PPP is increased, and related intermediate products provide substrates for many important physiological and biochemical reactions [64]. Therefore, the increase in V_{PPP} is beneficial for alleviating low-temperature stress. Interestingly, the LG treatment significantly increased the V_{TCAC}, especially on days 2 to 4 in this study (Figure 5c). In the pre-*Arabidopsis* era, researchers developed a model of the intracellular compartmentation of the GABA shunt, which indicated that the catabolism of the GABA shunt occurs in the mitochondria, linking with the TCAC; due to that, the Suc produced by the GABA shunt was an important intermediate product of the TCAC when the plant is under stress [72,73]. Thus, in this

study, the strengthening of V_{Total} after exogenous GABA application could be attributed to the increase in V_{TCAC}. Moreover, it was also observed that V_{EMP} and V_{PPP} (days 1 and 2) were increased with LG treatment (Figure 5a–d). The respiration of higher plants is not a simple combination of biochemical processes [7]. Therefore, further studies are needed to determine how plant roots coordinate the operation of multiple respiratory metabolic pathways to maintain the balance between energy demand and physiological functioning under suboptimal low root-zone temperatures.

It is well known that mitochondria are the main organelles responsible for material metabolism and energy transformation in plant cells [74]. The TCAC produces NADH and other high-energy materials that eventually enter the mitochondrial electron transport chain that drives ATP synthesis [75]. In addition to the CP in plant mitochondria, there is another pathway from ubiquinone called the cyanide-resistant pathway or the AP. The AP is a regulatory mechanism that enables some plants to adapt in response to environmental stresses such as low temperature, drought, salinity, alkalinity, and nutrient deficiency [64,76]. Many previous studies have found similar results to the present study, i.e., that the electron transport pathway rate was stable under the CK treatment, and CP is a dominant pathway [77,78]. The suboptimal low temperature significantly reduced V_{CP}, while V_{AP} significantly increased (days 2 to 4) in this experiment. Some researchers believe that the significantly enhanced respiratory rate of the AP under stress can enable the EMP and the TCAC to operate continuously, while simultaneously releasing enough heat to maintain the internal plant temperature and inhibit the production of ROS [75,79,80]. Nevertheless, the increase in V_{CP} and the decrease in V_{AP} contributed to the application of GABA in this experiment. Wang et al. [81] reported that succinic dehydrogenase as a key enzyme in the TCAC is closely related to energy metabolism located in mitochondria. The addition of exogenous substances can effectively increase succinic dehydrogenase activity, which further promotes the CP activity, to reduce ROS production [82]. There is some basis between the TCAC activity and CP activity. Thus, it is speculated that exogenous GABA can alleviate the inhibition effect of suboptimal low-temperature on V_{CP} by enhancing the TCAC activity, providing more ATP for low-temperature adaptability in roots. However, the specific mechanism by which exogenous GABA enhances V_{CP} requires further study. Under the LV treatment, V_{CP} decreased, V_{AP} increased (Figure 5e,f), and MDA levels increased. Thus, the activity of the GABA shunt was inhibited by exogenous VGB, which could aggravate stress injury. Finally, the PCA showed that GABA application mainly affected V_{Total}, V_{EMP}, V_{TCAC}, V_{CP}, and root vitality in response to suboptimal low root-zone temperatures (Figure 6a). In addition to this, the comprehensive score (Figure 6b) showed that the rank was LG > CK > L > LV, indicating that GABA was a positive factor promoting root respiratory activity to advance the suboptimal low root-zone temperature tolerance of *M. baccata* Borkh.

5. Conclusions

Suboptimal low root-zone temperature has a negative effect on roots. In contrast, exogenous GABA application has a significant effect on the roots of *Malus baccata* (L.) Borkh., including enhancing the root vitality, reducing the MDA content in roots, enhancing the TCAC activity through increasing the GABA shunt activity, and finally improving total respiration and energy synthesis. Based on the changes, exogenous GABA has the potential to improve the tolerance of apple rootstocks to suboptimal low temperatures in early spring.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9020268/s1, Table S1: The eigenvalues of principal component analysis (PCA); Table S2: The scores of principal component analysis (PCA); Table S3: The loadings of principal component analysis (PCA).

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