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Grapevine Berry Inner Necrosis Virus (GINV) and Grapevine Yellow Speckle Viroid 1 (GYSVd1) Exhibit Different Regulatory Effects on Soluble Sugars and Acids in 'Welschriesling' Grape Berries and Wine

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Abstract

This study investigates the roles of grapevine berry inner necrosis virus (GINV) and grapevine yellow speckle viroid 1 (GYSVd1) in regulating the soluble sugar and organic acid metabolism of grape berries and wine. The contents of soluble sugar and organic acid components and the activity and expression levels of critical enzymes of the soluble sugar acid metabolism pathway were measured in 'Welschriesling' grape berries and wine carrying the virus GINV, the viroid GYSVd1, and a mixed infection of both GINV and GYSVd1 (GINV + GYSVd1), respectively. The results show that the virus GINV and the viroid GYSVd1 decreased the soluble sugar and increased the organic acid in berries and wine. GINV decreased glucose content and increased malic acid content by regulating AI, NADP-IDH, PEPC, and NAD-MDH activity, as well as *VvHT4*, *VvSWEET10*, *VvPEPC*, and *VvMDH* expression levels. GYSVd1 decreased glucose content and increased malic acid content by regulating AI and CS activity and *VvHT4*, *VvSWEET15*, and *VvPEPC* expression. The results suggest that the viroid GYSVd1 negatively impacts berries and wine more than the virus GINV. Moreover, in the mixed infection with GINV + GYSVd1, the negative effects of GINV and GYSVd1 on soluble sugars do not seem to be observed.

Keywords: virus; viroid; sugar–acid metabolism; quality; *Vitis vinifera* L.

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1. Introduction

Grape (*Vitis vinifera* L.) berries are widely appreciated worldwide for their delicious flesh, distinctive flavor, and high economic value. The wine made from grape berries is attractive, sweet, and sour and has high nutritional value and health benefits. In recent years, the proliferation of grapevine virus disease, facilitated by the continuous expansion of cultivation areas and widespread circulation of planting material, has emerged as a significant constraint on the sustainable development of the grape industry. Grapevines are particularly susceptible to a range of viruses and viroids, including grapevine berry inner necrosis virus (GINV) and grapevine yellow speckle viroid (GYSVd) [1]. Currently, 86 grapevine viruses and eight species of viroids have been reported worldwide [2], and

22 viruses and seven species of viroids have been reported in China [3]. Infection of grapes with viruses seriously affects their yield, physiological functions, and quality [4]. After infection with viruses, the flowers and berries of grapes fall significantly, the berries are dull in color and flavor, the contents of anthocyanins and total sugars are significantly decreased, and the ripening is delayed [5]. The contents of phenolic substances and free amino acids are also significantly decreased, negatively effecting the quality of the grape berries and wine [6]. Grape viruses also affect primary metabolites throughout the berries by decreasing total sugars and increasing total acids, thereby directly affecting the flavor of the wine [7].

Grapevines are asexually propagated plants; once infected with a virus, they will carry the virus for life. The primary mode of virus and viroid transmission in grapevines is through the use of infected clonal propagation material [8–10]. GINV, as an RNA virus, is part of the genus *Trichovirus* of the *Betaflexiviridae* family [11]. GINV infection causes growth rate reduction in grapevines, eliciting symptoms such as branch necrosis, internode shortening, chlorotic mottling on leaves, and berry necrotic discoloration. GINV can interact with protein to destroy and interfere with chloroplast function and the photosynthetic rate of leaves, resulting in the reduction of sucrose, glucose, and fructose contents, which significantly impacts the quality of the grape berries and wine [12]. Grapevine yellow speckle viroid (GYSVd1) belongs to the family *Potyviridae* and the genus *Apscaviroid*. It spreads rapidly through seeds and insects in the hot summer, causing serious yellow spots on leaves [13]. However, as far as we know, the implications of GYSVd1 for grape berries and wine quality have not been reported.

The content and proportion of the soluble sugars and organic acids determine the taste of the berries and the wine and their quality reflects this [14]. Sugar accumulation during fruit growth and development is mainly determined by the balance of biosynthesis, transportation, and storage [15]. This process is catalyzed by several essential enzymes, such as sucrose synthase (SS), sucrose phosphate synthase (SPS), acid invertase (AI), and neutral invertase (NI) [16]. Glucose and fructose are the main soluble sugars in grape berries, with the fruit of most varieties containing very little sucrose [17]. In addition, sugar transporter proteins (SWEETs) play a vital role in the transmembrane transport of sugars in grape berries [18]. The hexose transporter protein (HT) is mainly responsible for transporting glucose, fructose, and other monosaccharides in the body of the fruit [19]. Grape berries also contain organic acids, mainly tartaric and malic acids, with a small amount of citric acid and other acids [20–22]. The balance of organic acids in fruit is mainly regulated directly or indirectly by phosphoenolpyruvate carboxylase (PEPC), malic enzyme (NADP-ME), malate dehydrogenase (NAD-MDH), citrate synthase (CS), and isocitrate dehydrogenase (NADP-IDH) [23].

This study investigated the effects of the virus GINV, the viroid GYSVd1, and a mixed infection of the two on the sugar–acid metabolism in 'Welschriesling' grape berries and wine, aiming to elucidate the impacts of GINV and GYSVd1 infections on grape berries and wine quality.

2. Materials and Methods

2.1. Plant Material and Virus Detection

The grapes (Welschriesling) were all picked from the grape planting orchard of Zhangyu Winery in Shihezi Reclamation Area, China (44°15′–44°19′ N latitude, 429 m above sea level). The grapevines, all ten years old, were grown under identical environmental conditions and cultivation practices. During May to June, grape leaves were collected in the field. (The five-point sampling method was used: the grape planting orchard was divided into 4 equal quadrants. Sampling points were set at the intersection of diagonals and 10 m

inward from each corner. At each point, 5 grapevines were sampled.) A systematic detection was performed for 12 viruses and 7 viroids, all of which are common viruses and viroids in the Xinjiang region. The primers are listed in Table S1 in the Supporting Information. The grapevines without viruses and viroids are regarded as the control. The grapevines infected with the virus GINV (forward primer: 5′-CTCCGGATCTTCTTGCTTGTGGTTC -3′; reverse primer: 5′-CCTCTTAGCGGGGGTTCCGGGGATT -3′) or the viroid GYSVd1 (forward primer: 5′-ATGTCGATCAGACAGGAATTGAGGGA -3′; reverse primer: 5′-TTACATAGTAAAAGCACCCTCGCTCG -3′) are referred to as GINV or GYSVd1. The grapevines infected with both GINV and GYSVd1 are referred to as GINV + GYSVd1. After ripening (15–30 September), healthy and whole berries were collected from each grapevine at different orientations (top, middle, and bottom). In each treatment, 10 kg of berries were collected for quality determination, and another 10 kg were used to make wine. The winemaking method is described in the Supporting Information.

2.2. Determination of Soluble Sugar Fractions

The soluble sugars fraction measure method was taken from Rajendar et al. [24]. Frozen grape berry powder (3 g) was mixed with 75% ethanol and centrifuged to collect the supernatant. The reaction solution contained 100 μL of the supernatant, 500 μL of 75% ethanol, and 1.5 mL of anthrone sulfurate. For fructose content, the solution was incubated at 50 °C for 30 min, and the absorbance at 640 nm was measured. For the measurement of glucose content, the solution was boiled for 5 min, and the absorbance at 640 nm was measured. The reaction solution for determining sucrose content contained 100 μL of the supernatant, 90 μL of anhydrous ethanol, 200 μL of 2 mol L^{-1} potassium hydroxide (KOH), and 3 mL of anthrone sulfate, and the absorbance at 640 nm was recorded. The sugar contents of the berries and wine were expressed as mg g^{-1}, based on fresh weight, and mg mL^{-1}, based on the volume of wine, respectively.

2.3. Determination of Organic Acid Fractions

The organic acids were measured with HPLC (Varian, Palo Alto, CA, USA) [25]. The column was a Phenomenex Luna 5u NH_2 100A column (250 mm × 4.60 mm, 5 micron) (Agilent, Santa Clara, CA, USA); the column temperature was 30 °C; the mobile phase was 0.02 mol·L^{-1} potassium dihydrogen phosphate (KH_2PO_4). Grape berries (10 g) were homogenized with 50 mL of ultrapure water and centrifuged at 3000× *g* for 15 min, and the supernatant was collected and filtered through a 0.22 μm membrane (Varian, USA). Tartaric acid, malic acid, and citric acid in the supernatant were qualified with the authentic standards, and the contents of these acids were calculated and expressed as μmol·g^{-1} or μmol·mL^{-1}, respectively.

2.4. Measurement of Sugar Metabolism Essential Enzyme Activity

The activity of enzymes essential for sugar metabolism was determined according to Samkumar et al. [26]. Grape berries (1 g) or wine (1 mL) were homogenized with Hepes sodium salt (Hepes-NaOH) (pH 7.5). The homogenization was centrifuged, and then the supernatant was collected.

For SPS activity, the supernatant (20 μL) was mixed with 70 μL of 50 mmol·L^{-1} Hepes-NaOH (containing 7.5 mmol·L^{-1} uridine diphosphate glucose (UDPG), 7.5 mmol·L^{-1} D-fructose 6-phosphate (F6P), and 5 mmol·L^{-1} magnesium chloride ($MgCl_2$)), incubated at 37 °C for 40 min. After 70 μL of 1 mmol·L^{-1} NaOH was added, the mixture was boiled for 10 min. Then, 750 μL of 30% hydrochloric acid (HCl) and 250 μL of 0.1% resorcinol were added, and the mixture was incubated at 80 °C for 8 min. The absorbance was measured at 520 nm.

For SS activity, the reaction solution contained 20 μL of the supernatant and 70 μL of 50 mmol·L^{-1} Hepes-NaOH (pH 7.5, including 5 mmol·L^{-1} $MgCl_2$, 7.5 mmol·L^{-1} UDPG, and 7.5 mmol·L^{-1} fructose), and was incubated at 37 °C in a water bath for 40 min. Then, 70 μL of 1 mmol·L^{-1} NaOH was added, and the mixture was boiled for 10 min. After being cooled to room temperature, the solution was supplemented with 250 μL of 0.1% resorcinol and 750 μL of 30% HCl, and incubated in an 80 °C water bath for 8 min. The absorbance was measured at 520 nm.

For AI activity, the reaction system contained 0.1 mL of the supernatant, 0.7 mL of 80 mmol·L^{-1} acetate buffer (HAc-NaAc) (pH 4.5), and 0.2 mL of 100 mmol·L^{-1} sucrose, incubated at 37 °C for 30 min. The reaction was terminated with the addition of 1.5 mL of 3,5-dinitrosalicylic acid (DNS) reagent and was boiled for 5 min. The absorbance values at 540 nm were determined.

The determination protocol of NI activity was similar to that of AI, except that the buffer used for the determination of NI activity in grape berries was phosphate buffer (pH 7.5).

A change of 0.01 in light absorption value per gram of grape berries or milliliter of grape wine per hour was defined as one enzyme activity unit (U).

2.5. Measurement of Acid Metabolism Essential Enzyme Activity

The activity of essential enzymes for acid metabolism was determined using kits from Suzhou Grace Biotechnology Co., Ltd. (Suzhou, China). Grape berries (1 g) were homogenized with enzyme extraction. The homogenization was centrifuged. The supernatants from the grape berries and the wine were used to measure enzyme activity, respectively.

The absorption value of CS at 412 nm was recorded. The production of 1 nmol acetyl-CoA per gram of grape berries or per milliliter of grape wine per minute was defined as one unit of enzyme activity (U).

The changes in the absorption value at 340 nm were used to calculate PEPC activity. The consumption of 1 nmol nicotinamide adenine dinucleotide (NADH) per gram of grape berries or milliliter of grape wine per minute was defined as one unit of enzyme activity (U).

The absorption value of NADP-IDH at 450 nm was read. A reading of 1 nmol of nicotinamide adenine dinucleotide phosphate (NADPH) generated per gram of grape berries or milliliter of grape wine per minute was defined as one unit of enzyme activity (U).

The absorption value of NADP-ME was read at 340 nm. The production of 1 nmol NADPH per gram of grape berries or milliliter of grape wine per minute was defined as a unit of enzyme activity (U).

The absorption value of NAD-MDH at 340 nm was recorded. The consumption of 1 nmol of NADPH per gram of grape berries or milliliter of grape wine per minute was defined as a unit of enzyme activity (U).

2.6. Analysis of the Relative Gene Expression

The total RNA of grape berries was extracted using a Quick RNA isolation kit from Bejing HuaYueYang Biotechnology Co., Ltd. (Beijing, China) and reverse transcribed using an EasyScript First-Strand cDNA Synthesis SuperMix kit from Beijing TransGen Biotech Co., Ltd. (Beijing, China). The qRT-PCR protocol includes the following: 94 °C for 30 s (initial denaturation), then 40 cycles of 94 °C for 5 s, and 60 °C for 30 s. The qRT-PCR primers for the target genes and the internal reference gene β-Actin are shown in Table S2. All qRT-PCR reactions were set up with three biological replicates, and the relative expression levels were calculated using formula $2^{-\Delta\Delta CT}$.

2.7. Statistical Analysis

There were 5 biological replicates per treatment. Data were analyzed using a one-way analysis of variance (ANOVA). Different letters indicate significant differences when the p-value < 0.05.

3. Results

3.1. GINV, GYSVd1, and Mixed Infection Regulated Soluble Sugar and Organic Acid Content

The soluble sugar content of grape berries infected with GINV and GYSVd1 showed a significant decrease ($p < 0.05$), reaching only 86.51% and 78.94% of the control, respectively (Figure 1A). In contrast, the organic acid content of berries carrying GYSVd1 and GINV + GYSVd1 was significantly higher than that of the control ($p < 0.05$), being 1.39 and 1.47 times that of the control (Figure 1B). The sugar–acid ratio of berries with GINV, GYSVd1, and GINV + GYSVd1 was significantly decreased ($p < 0.05$), to only 63.97%, 56.75%, and 65.08% of the control, respectively (Figure 1C).

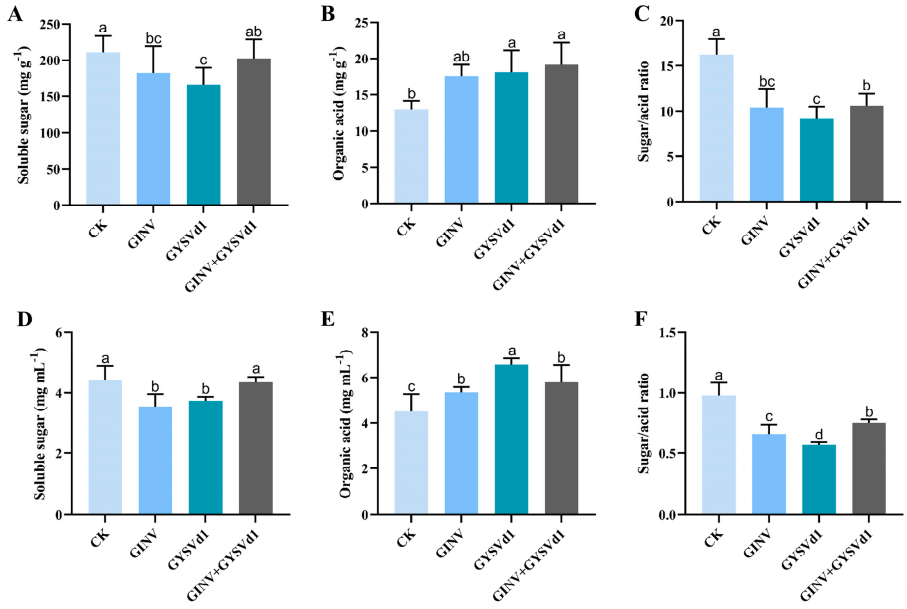

Figure 1. Effects of different viruses on the soluble sugar and organic acid content of berries (**A**–**C**) and wine (**D**–**F**) of 'Welschriesling' grapes. Soluble sugar (**A**,**D**), organic acid (**B**,**E**), and sugar/acid ratio (**C**,**F**). CK: virus-free berries or wine made from virus-free berries. Vertical bars indicate standard error (n = 5), and different letters indicate significant differences between samples ($p < 0.05$).

The soluble sugar content of wine made from grape berries infected with GINV and GYSVd1 was significantly lower ($p < 0.05$) than that of wine made from the control, being 80.29% and 84.58% of the control, respectively. However, no significant difference was found between the soluble sugar content of wine made from the berries infected with GINV + GYSVd1 and that of the control (Figure 1D). The organic acid content of wine made from berries carrying GINV, GYSVd1, and GINV + GYSVd1 was significantly higher than that of the control ($p < 0.05$), being 1.19, 1.46, and 1.29 times that of the control, respectively (Figure 1E). The sugar–acid ratio of wine made from grape berries infested with GINV, GYSVd1, and GINV + GYSVd1 was significantly lower ($p < 0.05$) than that of wine made from the control, being 67.37%, 57.81%, and 76.51% of the control, respectively (Figure 1F).

3.2. GINV, GYSVd1, and Mixed Infection Regulated Soluble Sugar Fraction Content

The glucose content of berries carrying GINV and GYSVd1 was significantly lower ($p < 0.05$) compared to the control, being 92.19% and 88.06% of the control, respectively. However, there was no significant difference in soluble sugar and glucose content between berries carrying GINV + GYSVd1 and the control (Figure 2A). There were no significant differences in fructose and sucrose content between the control and grape berries infected with GINV, GYSVd1, or GINV + GYSVd1 (Figure 2B,C).

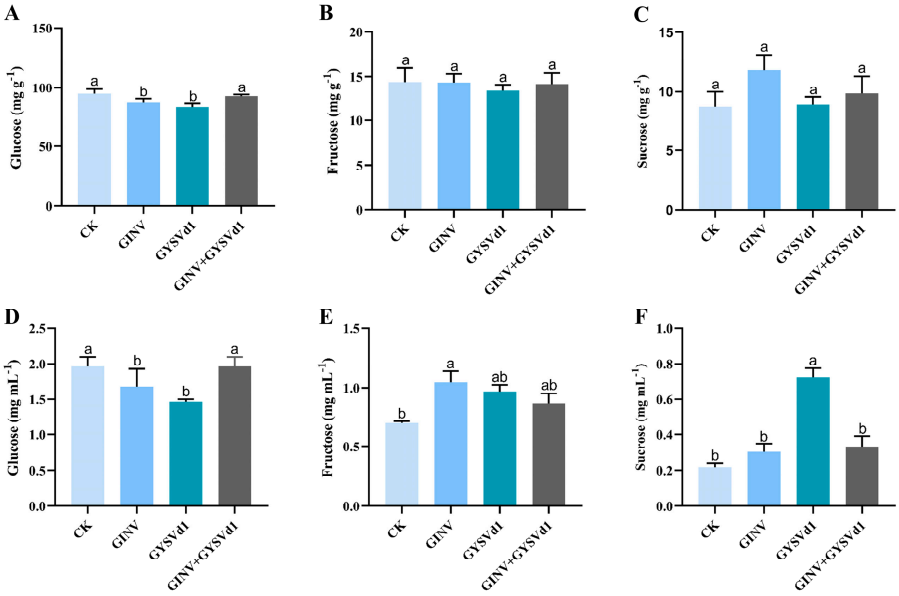

Figure 2. Effect of different viruses on the soluble sugar fraction content of the berries (**A**–**C**) and wine (**D**–**F**) of 'Welschriesling' grapes. Glucose (**A**,**D**), fructose (**B**,**E**), and sucrose (**C**,**F**). CK: virus-free berries or wine made from virus-free berries. Vertical bars indicate standard error (n = 5), and different letters indicate significant differences between samples ($p < 0.05$).

The glucose content of wines made from grape berries infected with GINV and GYSVd1 was significantly lower ($p < 0.05$) than that made from the control, being 84.24% and 73.29% of the control. However, there was no significant difference between the glucose content of wines made from berries with GINV + GYSVd1 and that made from the control (Figure 2D). The fructose content of wine made from berries infected with GINV was significantly higher ($p < 0.05$) and was 1.50 times that of wine made from the control (Figure 2E). The sucrose content of wine made from berries infected with GYSVd1 was also significantly higher ($p < 0.05$) and was 3.34 times that of wine made from the control (Figure 2F).

3.3. GINV, GYSVd1, and Mixed Infection Regulated Activity of Enzymes for Sugar Metabolism

No significant differences were observed in the activity of SPS, SS, and NI between the control and grape berries infected with GINV, GYSVd1, or GINV + GYSVd1 (Figure 3). The AI activity in the berries infected with GINV and GYSVd1 significantly decreased, to 81.23% and 83.31% of the control. However, GINV + GYSVd1 had no significant impact on the AI activity in grape berries (Figure 3C).

GINV, GYSVd1, and GINV + GYSVd1 had no significant effects on the activity of SPS, SS, AI, or NI in wines made from berries (Figure 3E–H).

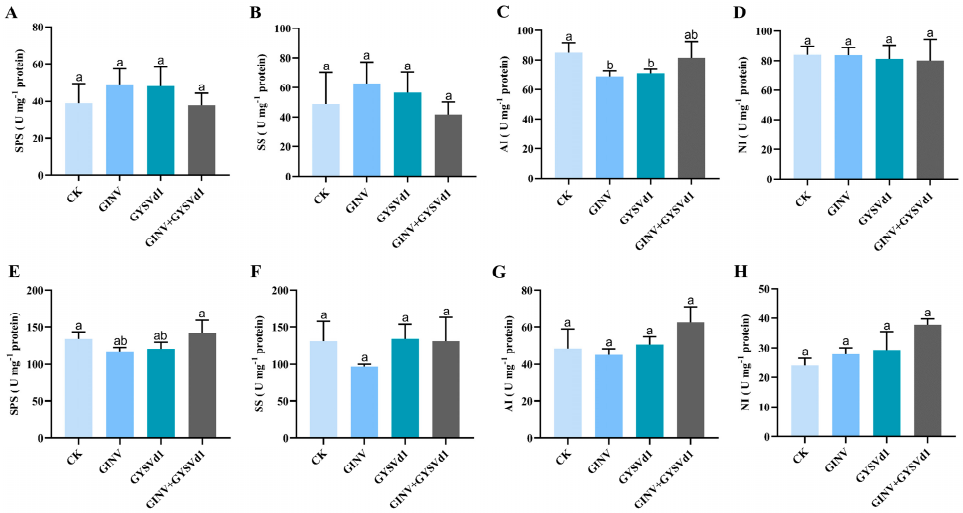

Figure 3. Effect of different viruses on sugar metabolism enzyme activity in grape berries (**A**–**D**) and wine (**E**–**H**) of 'Welschriesling'. SPS (**A**,**E**), SS (**B**,**F**), AI (**C**,**G**), and NI (**D**,**H**). CK: virus-free berries or wine made from virus-free berries. Vertical bars indicate standard error (n = 5), and different letters indicate significant differences between samples ($p < 0.05$).

3.4. GINV, GYSVd1, and Mixed Infection Regulated Expression Levels of Genes for Sugar Metabolism

Both GINV and GYSVd1 exhibited no significant effect on the expression level of *VvHT2* (Figure 4A). The expression level of *VvHT4* in berries carrying GINV was 1.93 times that of the control, while the expression level of gene *VvSWEET10* was only 48.33% of that of the control (Figure 4C,E). The expression levels of genes *VvHT3*, *VvHT4*, and *VvSWEET15* in berries carrying GYSVd1 were significantly ($p < 0.05$) higher compared to the control, being 3.40, 1.46, and 2.88 times the expression levels of the control, respectively (Figure 4B,C,F). However, GINV + GYSVd1 exhibited no significant effects on the expression levels of these genes (Figure 4).

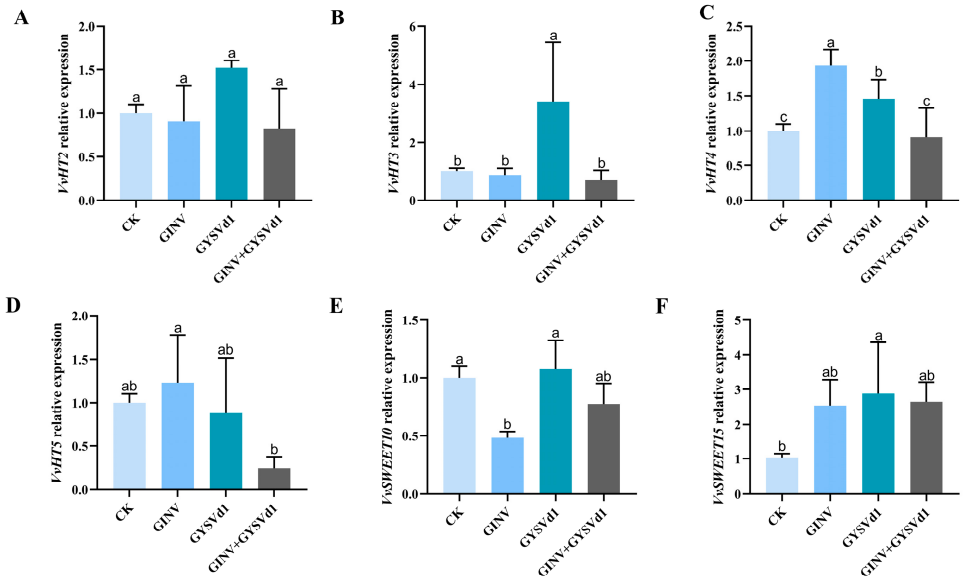

Figure 4. Effects of different viruses on the expression levels of essential genes for sugar metabolism in 'Welschriesling' berries. *VvHT2* (**A**), *VvHT3* (**B**), *VvHT4* (**C**), *VvHT5* (**D**), *VvSWEET10* (**E**), and *VvSWEET15* (**F**). CK: virus-free berries or wine made from virus-free berries. Vertical bars indicate standard error (n = 5), and different letters indicate significant differences between samples ($p < 0.05$).

3.5. GINV, GYSVd1, and Mixed Infection Regulated Organic Acid Fraction Content

The malic acid content in berries infected with GINV, GYSVd1, or GINV + GYSVd1 significantly ($p < 0.05$) increased to 1.54, 2.13, and 1.94 times that of the control, respectively (Figure 5B). The virus and viroid had no significant effects on the content of tartaric acid and citric acid in grape berries (Figure 5A,C).

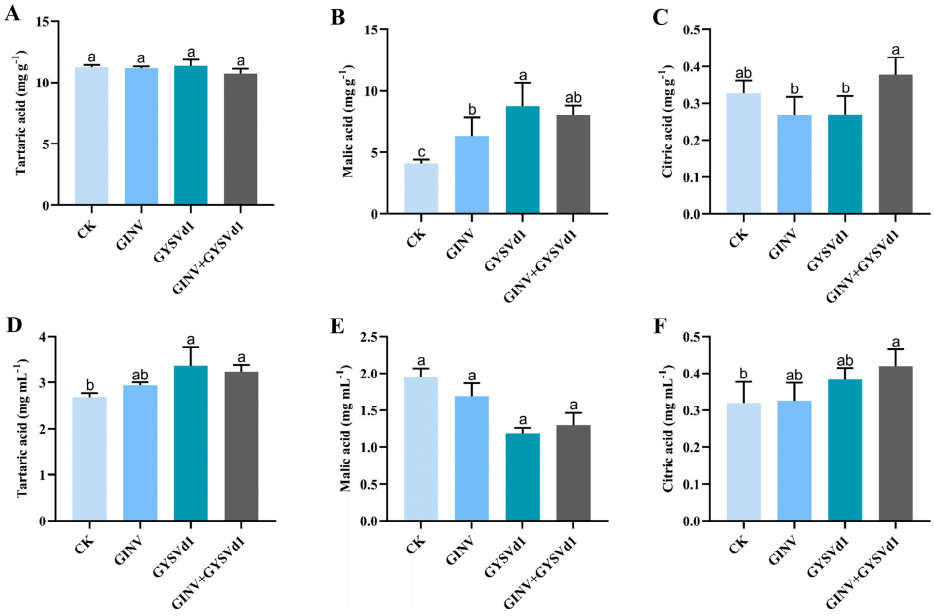

Figure 5. Effect of different viruses on the content of organic acid fractions in the berries (**A**–**C**) and wine (**D**–**F**) of 'Welschriesling' grapes. Tartaric acid (**A**,**D**), malic acid (**B**,**E**), and citric acid (**C**,**F**). CK: virus-free berries or wine made from virus-free berries. Vertical bars indicate standard error (n = 5), and different letters indicate significant differences between samples ($p < 0.05$).

The content of tartaric acid in wines produced from grape berries infected with GYSVd1 and GINV + GYSVd1 was 1.26, 1.21 times that of the control ($p < 0.05$) (Figure 5D). However, GINV and GYSVd1 did not affect malic acid content in wine (Figure 5E). The citric acid content of wine made from grape berries carrying GINV + GYSVd1 increased to 1.31 times that of the control (Figure 5F).

3.6. GINV, GYSVd1, and Mixed Infection Regulated Activity of Enzymes for Acid Metabolism

The activity of CS in berries with GYSVd1 was significantly decreased to only 74.70% of the control (Figure 6A). GINV and GYSVd1 had no significant effects on the NADP-ME activity in berries (Figure 6C). The activity of NADP-IDH and PEPC in grape berries with GINV was 3.48 and 1.82 times that of the control, respectively (Figure 6B,D). The activity of NAD-MDH in berries infected with GINV and GINV + GYSVd1 was 1.84 and 2.06 times that of the control ($p < 0.05$) (Figure 6E).

There were no significant differences in the activity of NADP-IDH, NADP-ME, and NAD-MDH between wines made from berries infected with GINV, GYSVd1, and GINV + GYSVd1 and the control (Figure 6G,H,J). The activity of CS in wines made from berries infected with GINV, GYSVd1, and GINV + GYSVd1 was higher than that in wines made from the control ($p < 0.05$), and was 2.48, 1.76, and 1.71 times higher, respectively (Figure 6F). The activity of PEPC in wines made from berries infected with GINV + GYSVd1 was 3.11 times that of wine made from the control ($p < 0.05$) (Figure 6I).

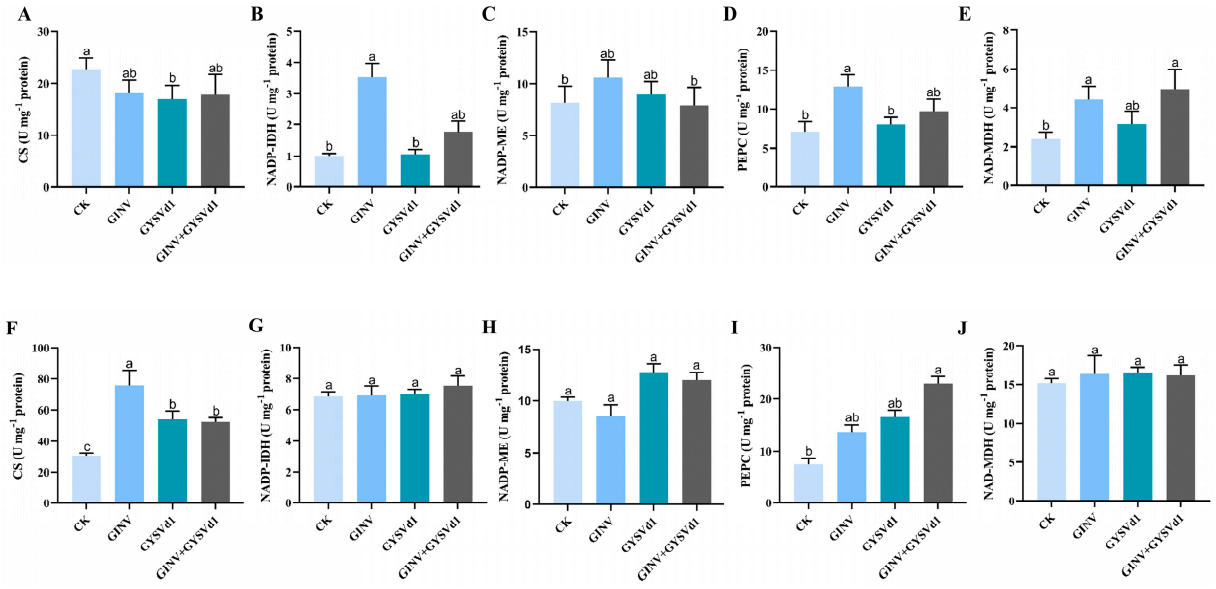

Figure 6. Effects of different viruses on the activity of enzymes for acid metabolism in grape berries (**A**–**E**) and wine (**F**–**J**) of 'Welschriesling'. CS (**A**,**F**), NADP-IDH (**B**,**G**), NADP-ME (**C**,**H**), PEPC (**D**,**I**), and NAD-MDH (**E**,**J**). CK: virus-free berries or wine made from virus-free berries. Vertical bars indicate standard error (n = 5), and different letters indicate significant differences between samples ($p < 0.05$).

3.7. GINV, GYSVd1, and Mixed Infection Regulated Expression Levels of Genes for Acid Metabolism

The expression level of the gene *VvPEPC* in berries carrying GINV and GYSVd1 was 1.72 and 1.57 times that of the control ($p < 0.05$), respectively (Figure 7A). No significant difference was found in *VvPEPC* gene expression level between berries carrying GINV + GYSVd1 and the control. The expression level of *VvMDH* in berries infected with GINV was 1.66 times ($p < 0.05$) that of the control. However, there was no significant difference in the expression level of *VvMDH* in berries carrying GYSVd1 and GINV + GYSVd1 compared with the control (Figure 7B). No significant difference in the expression level of gene *VvME* was found between the control and berries carrying GINV and GYSVd1 (Figure 7C).

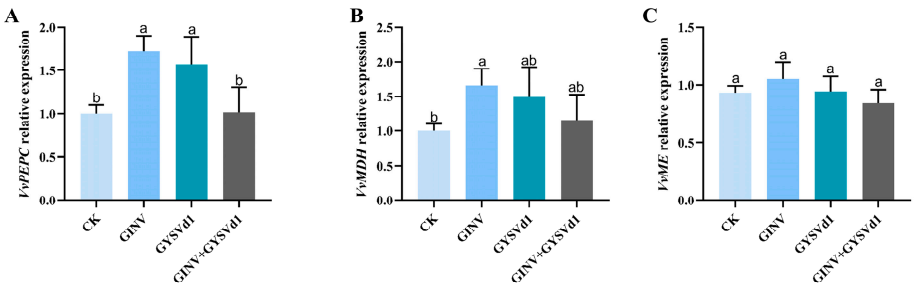

Figure 7. Effects of different viruses on the expression levels of essential genes for acid metabolism in "Welschriesling" grape berries. *VvME* (**A**), *VvMDH* (**B**), and *VvPEPC* (**C**). CK: virus-free berries or wine made from virus-free berries. Vertical bars indicate standard error (n = 5), and different letters indicate significant differences between samples ($p < 0.05$).

4. Discussion

Soluble sugars and organic acids are essential influencers of berry and wine quality. Grapevine berry inner necrosis virus (GINV) and grapevine yellow speckle viroid 1 (GYSVd1) reduce soluble sugar content, increase organic acid content, and reduce the sugar–acid ratio of grape berries and wine. GINV decreases glucose content and increases

malic acid content by regulating acid invertase (AI), isocitrate dehydrogenase (NADP-IDH), phosphoenolpyruvate carboxylase (PEPC), and malate dehydrogenase (NAD-MDH) activity, as well as *VvHT4*, *VvSWEET10*, *VvPEPC*, and *VvMDH* expression levels. GYSVd1 decreases glucose content and increases malic acid content by regulating AI and citrate synthase (CS) activity and *VvHT4*, *VvSWEET15*, and *VvPEPC* expression (Figure 8A).

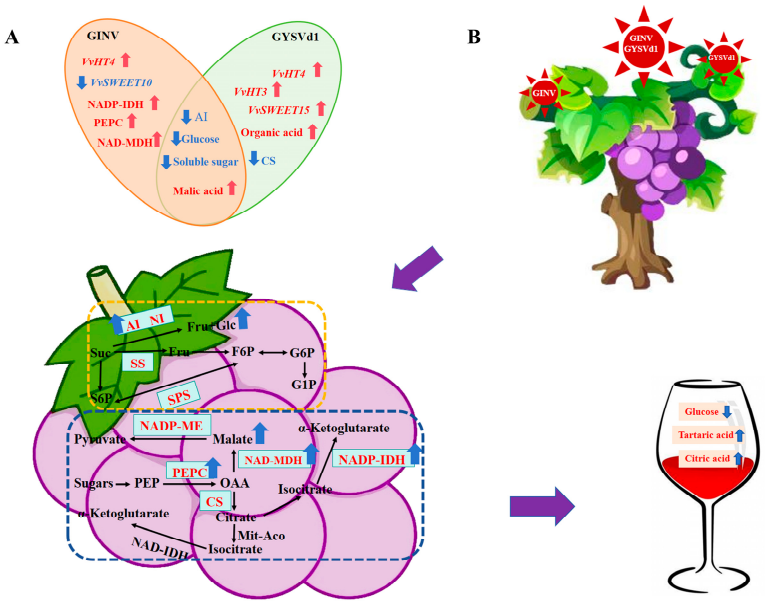

Figure 8. Virus regulatory effects on the metabolic processes of soluble saccharic acid in the berry and wine of the 'Welschriesling' grapevine. (**A**): Venn diagram showing the shared and specific effects of GINV and GYSVd1 on quality components of both grape berries and wine. (**B**): Mechanism by which viruses affect grape berries and wine quality through regulation of sugar-acid metabolism.

Sugar is not only an energy substance for fruit growth and development but also a substrate for the alcoholic fermentation of wine [27]. Soluble sugar contents and composition impact the quality of fruit. This study shows that GINV and GYSVd1 decrease the total soluble sugar content of grape berries, mainly because they promote a decrease in glucose content but not in fructose and sucrose. The effects of GINV and GYSVd1 on the soluble sugars in berries are similar. The sugar content of wine grapes significantly reduces after infection with GRBV and GRLV [5,28], which are pathogens similar to those used in this study, suggesting that viruses and viroids decrease the quality of grape berries. SS catalyzes the conversion of sucrose to UDPG (uridine diphosphate-glucose) and fructose. SPS catalyzes the synthesis of UDPG and fructose 6-phosphate (F6P) into sucrose phosphate (S6P) and then produces sucrose under the action of phosphodiesterase [4]. Invertase decomposes sucrose to produce glucose and fructose, with AI localized in the vacuole or cell wall and NI localized in the cytoplasm [17]. In this study, GINV and GYSVd1 decreased AI activity in berries, which delayed the conversion from sucrose to glucose and fructose. A study by Shimizu-Yumoto et al. showed results similar to those in this study [29], revealing that AI activity is positively correlated with glucose content in berries. Sugar transport proteins transport sugars from the exosomes into the pulp cells, and the level of gene expression is closely related to the accumulation of sugars in the reservoir tissues [30]. *VvHT1* is involved in the recycling of monosaccharides during early grapevine development, and *VvHT4* and *VvHT5* are functionally similar to *VvHT1* [31]. SWEETs have also been identified in plants, animals, and bacteria, and constitute a new family of sugar transport proteins [32]. In this study, GINV and GYSVd1 up-regulated the expression levels of *VvHT* and *VvSWEET*, which promoted the transportation of glucose and fructose to be metabolized. It should be noted that GYSVd1 up-regulated the expression levels of

VvHT3, *VvHT4*, and *VvSWEET15*, while GINV up-regulated the expression levels of *VvHT4* but down-regulated the expression levels of *VvSWEET10*, suggesting that the regulating effects of GINV and GYSVd1 on *VvHT* and *VvSWEET* are different. Meanwhile, GINV and GYSVd1 reduced glucose content, and GINV increased fructose content, while GYSVd1 increased sucrose content in grape wine, which was different from grape berries (Figure 8B). It is also suggested that GINV and GYSVd1 have different regulating effects on soluble sugars in grape wine. However, no expression of *VvHT* and *VvSWEET* was detected in grape wine. The possible reason is that *VvHT* and *VvSWEET* were demolished during the fermentation process of the wine.

Organic acids in wine are mainly released from the berries into the wine during the vinification process, and the acid balance in wine is essential for microorganisms during fermentation. Malic acid influences the final wine characteristics. GINV and GYSVd1 increased the organic acid contents of grape berries and wine with different acid components. The increase in organic acids in grape berries with GINV and GYSVd1 was mainly from malic acid while, in wine, it was from tartaric and citric acids. The difference in organic acid components between grape berries and wine might be due to is the existence or lack of a fermentation process. The fermentation process changes the composition of organic acids. The content of organic acids during fruit growth and development depends mainly on synthesis and degradation processes [33]. In the berry cytoplasm, phosphoenolpyruvate (PEP) is produced from sugar by glycolysis. Phosphoenolpyruvate carboxylase (PEPC) carboxylates phosphoenolpyruvate (PEP) to oxaloacetate (OAA). Oxaloacetate (OAA) is catalyzed by malate dehydrogenase (NAD-MDH) to form malic acid [34]. In addition, oxaloacetate (OAA) can also form citric acid under the catalysis of citrate synthase (CS) [35]. GINV increased the activity of NADP-IDH, PEPC, and NAD-MDH, which promoted the synthesis of malic acid and the degradation of citric acid in the berries. GYSVd1 decreased the activity of CS, which delayed the conversion of oxaloacetic acid to citric acid and, probably, more oxaloacetic acid to malic acid. GINV and GYSVd1 regulated the activity of different enzymes during organic acid metabolism, but they all promote malic acid production in berries. In wine, GINV and GYSVd1 increased the activity of CS to promote citric acid production. In contrast, the elevation of tartaric acid in wine may be attributed to the increase of related enzyme activity during winemaking (Figure 8B). Little is known about the tartaric acid metabolic pathway, and further studies are needed [36]. The regulatory effects of GINV and GYSVd1 on enzyme activity during the metabolism of organic acids are different, which might be an essential reason for the differences in the organic acid contents of berries and wine. GINV up-regulated the expression levels of *VvPEPC* and *VvMDH* and promoted malic acid synthesis. GYSVd1 up-regulated the expression levels of *VvPEPC* and promoted the production of oxaloacetate, a substrate for malic acid synthesis, promoting the production of malic acid. However, no expression of *VvPEPC*, *VvMDH*, and *VvME* was detected in grape wine. The possible reason was that *VvPEPC*, *VvMDH*, and *VvME* were demolished during the fermentation process of the wine.

Mixed infections with viruses and viroids of different pathogenic microorganisms that simultaneously infect a host plant often occur in nature [37]. Because mixed infections change the mutualistic relationship between the pathogen and the host plant, the occurrence of and damage caused by the disease are very different from that of a single infection. There are synergistic [38], competitive [39], and inhibitory [40] relationships between pathogens involved in mixed infections. In this study, interestingly, the mixed infection of GINV + GYSVd1 increased the organic acid content of berries and wine without inducing the soluble sugar reduction characteristic of single infections, suggesting that there might be an interaction between GINV and GYSVd1. It also hinted that, when grapevines are

infected with a virus or viroid, actively inoculating them with another viroid or virus might preserve the sugar–acid ratio of the grape berries and wine. However, further work should be carried out to prove this. Moreover, the viral titer plays a critical role, as it directly influences both the severity of grape berry damage and the resulting wine quality.

This study indicates that both GINV infection and GYSVd1 infection significantly reduce soluble sugar content while increasing organic acid content in grape berries and wine. These negative effects can be prevented by using virus-free planting materials [41]. Previous studies have confirmed that virus-free planting materials can enhance grape productivity [42] and optimize the sugar–acid balance of the berries. The use of virus-free planting materials ensures grape quality and advances industry sustainability.

5. Conclusions

The virus GINV and the viroid GYSVd1 decrease the soluble sugar content and increase the organic acid content of berries and wine. GINV decreases glucose content and increases malic acid content by regulating AI, NADP-IDH, PEPC, and NAD-MDH activity, as well as *VvHT4*, *VvSWEET10*, *VvPEPC*, and *VvMDH* expression levels. GYSVd1 decreases glucose content and increases malic acid content by regulating AI and CS activity and *VvHT4*, *VvSWEET15*, and *VvPEPC* expression. The mixed infection of GINV + GYSVd1 increases the organic acid content of berries and wine without inducing the soluble sugar reduction characteristic of single infections. The results of this study provide important insights into the changes in sugar and acid levels in grapes due to virus and viroid infections, and contribute to a further understanding of the effects of virus and viroid infections on grape berries and wine quality.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae11080879/s1, Table S1: Primer sequences for identification and detection of viruses and viroids; Table S2: Primer sequences for relative expressions of genes.

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