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Effects of Exogenous ALA on Leaf Photosynthesis, Photosynthate Transport, and Sugar Accumulation in *Prunus persica* L.

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Abstract: Peaches/nectarines (*Prunus persica* L.) are widely cultivated worldwide. As with other species, the sugar content is the most important trait for fruit quality, especially for precocious cultivars. Most fruits need to improve their sugar content in order to be more profitable under fierce market competition. 5-Aminolevulinic acid (ALA), a naturally occurring δ-amino acid, has been shown to improve leaf photosynthesis and fruit quality, especially sugar content. However, the mechanisms are not clear. The objective of this study is to determine the effects of exogenous ALA on leaf photosynthesis, assimilate transport, and sugar accumulation during fruit development. We used the field-cultivated precocious nectarine 'Zhongyoutao 4' and potted cultivated peach 'Zhongai 33' as materials, whereas in the second experiment, we used ^{14}C radiolabeling to trace ^{14}C fixation in leaves, transport in branches, and distribution in different organs. The results showed that ALA significantly enhanced the photosynthetic gas exchange capacity, and the effects were maintained for at least one month. The results of the ^{14}C radiolabel experiment showed that ALA enhanced ^{14}C fixation in leaves, promoted the transport to fruits, and reduced the allocation rate of young leaves. This suggests that ALA enlarges "source" volume and strengthens "sink" competition; therefore, assimilate translocation to fruits is promoted. It was observed that sucrose contributed the main saccharide for peach fruit quality at maturity, which might not be converted from glucose or fructose but from starch degradation. ALA improved starch accumulation in the young fruits as well as degradation during maturity. The RT-qPCR showed that the expression of most genes involved in sugar metabolism did not correlate or even negatively correlate with fruit sucrose content. However, the expressions of *SWEET1/6/7/8/15/16/17* were highly correlated with the sucrose content, and exogenous ALA treatment up-regulated the gene expression at fruit maturity, suggesting they might play an important role in fruit sugar accumulation. These results provide important theoretical support for ALA application in fruit quality improvement, as well as a regulatory mechanism study on sugar accumulation in fruits.

Keywords: 5-Aminolevulinic acid (ALA); ^{14}C-assimilate translocation; peach/nectarine; photosynthesis; soluble sugar content; source sink

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1. Introduction

Peach/nectarine (*Prunus persica* L.) is a deciduous stone fruit tree belonging to the Rosaceae family. It originated from the northwest of China and has been cultivated for more than 3000 years [1]. Among all the countries in the world, China has the largest cultivated area and the highest production of peaches [2]. Growing and selling peaches with different mature periods not only enriches the fruit market but also brings huge income to growers. It is often the case that the sugar content in precocious varieties is generally not very high, so many consumers buy early ripening fruits for the fresh taste. Unless they are good enough, consumers may buy them often and consume them as a necessity. Methods

of enhancing their sugar content and improving fruit quality has an important practical significance and economic value [3]. In fruit trees, assimilates are synthesized through photosynthesis in the leaves, the "source" organ, and transported in the form of soluble sugars over long distances through the phloem into fruits, the "sink" organ [4]. The sugars are then unloaded here and undergo biochemical reactions to converse or store in fruit cells [5,6]. In addition, sugars are not only the most important component of fruit quality but also the energy source for metabolic activities, which are involved in regulating cellular osmotic pressure, enhancing the resistance to environmental stresses, and participating in signal transduction [7]. During recent years, many key genes and proteins involved in fruit sugar accumulation have been identified by transcriptomic and proteomic analysis [8–10]. On the other hand, not-targeted metabolomics using ^{1}H-NMR, UPLC, LC-MS, and GC-MS combined with multivariate statistics to determine the differences and regularity of various chemical components in sugar metabolism has been established [11–13]. All these theoretics and technological advances are helpful in elucidating the sugar accumulation and mechanisms underlying fruits.

5-Aminolevulinic acid (ALA) is a natural δ-amino acid commonly occurring in plants, animals, and microorganisms. It is a key precursor for the biosynthesis of all porphyrin compounds, such as chlorophylls and heme. Therefore, it is closely related to plant photosynthesis and respiration [14,15]. However, experiments have demonstrated that exogenous ALA treatments significantly increase the sugar content of fruits and, sometimes, decrease the titratable acids, with a consequently higher ratio of sugars/acids and better tastes [16,17]. ALA also significantly increases the content of vitamin C and soluble proteins in fruits [18,19]. Additionally, ALA promotes the accumulation of anthocyanin and flavonoids, resulting in improved fruit coloration [19–21]. In tomatoes, exogenous ALA promotes postharvest fruit ripening [22]. In peaches, ALA treatment promotes fruit quality and anthocyanin accumulation in the skins, which has been ascribed to it inducing the expression of structural genes and transcription factors related to anthocyanin biosynthesis [23]. These results suggest that ALA has important application prospects in producing high-quality fruits. However, most studies until now have only ascribed the promotive effect of ALA on fruit quality to its leaf photosynthesis improvement. The deeper mechanisms need to be clarified. There are few reports regarding the regulation of ALA on the "sink-source" relationship and coordination during fruit development, which is important for high-quality fruit production.

In this study, the effects of exogenous ALA treatment on photosynthesis, assimilate transport, sugar accumulation, and related gene expressions during peach fruit development were investigated by using radioisotope ^{14}C labelling, HPLC, and RT-qPCR. The aim of the study was to investigate the effects of ALA on leaf photosynthesis, photosynthate transport, and sugar accumulation in fruits in order to provide a scientific basis to elucidate the mechanism of ALA in improving fruit quality and promoting ALA application in fruit production.

2. Materials and Methods

2.1. Experimental Materials and Treatment

Two experiments were conducted from March 2021 to June 2022; the first was carried out at Doukou Agricultural Scientific and Technological Orchard in Hushu Community. It is located in Nanjing, Jiangsu Province, China, with a subtropical monsoon climate, abundant rainfall, and four distinct seasons. Four-year-old nectarine (*Prunus persica* L. cv. Zhongyoutao 4) trees were used, which were arranged in east–west rows with a plant spacing of 1 m × 4 m and Y-shaped pruning. Two treatments were set up, including the control (spraying with clean water) and the ALA treatment (spraying with 10 mg L^{-1} ALA). Eight trees with similar growth potential were selected for each treatment. The ALA solution was foliar sprayed on 10 April, 20 days after full blooming, when the young fruits were at the cell division stage. After that, the leaf photosynthetic gas exchange

was measured weekly, and fruit samples were collected and stored in the laboratory for subsequent analysis.

The second experiment was conducted in Nanjing Agricultural University radioisotopic laboratory, where 4-year-old potted peach (*Prunus persica* L. cv. Zhongai 33) trees were used. ALA solution of 10 mg L^{-1} was sprayed at the second rapid expansion period of fruit development (23rd May, 60 d after full blooming). After 12 days, the mature middle leaves of new shoots of the treated and the control trees were fed with ^{14}C, and the abundance of radiolabel distribution in leaves and fruits was analyzed after 24 h. Three trees each were used for the control and treatment.

2.2. Analysis Methods

2.2.1. Determination of Photosynthetic Gas Exchange

To determine the gas exchange parameters of the nectarine leaves, a portable photosynthetic system analyzer (Li-6800, Lincoln, NE, USA) was used. The measurements were taken every week after ALA treatment on a sunny or less cloudy day between 9:00 AM and 12:00 AM, which continued for five weeks. The measurement conditions were: air flow rate 500 μmol s^{-1}, air chamber pressure 0.1 kPa, air humidity 60%, CO_2 concentration 400 μmol mol^{-1}, fan speed 10,000 rpm, leaf temperature 27.0 °C, and light intensity of leaf chamber 1500 μmol m^{-2} s^{-1}. The measurement was repeated with twenty leaves, and then the means and standard error were calculated.

2.2.2. Radiolabeling and Measurement of ^{14}C

The $^{14}CO_2$ labelling and measurement were conducted following the method described by Wu [24]. In the morning of 4 June 2022, a sunny day, the bases of the fruit branches of the peach trees selected for labelling were girdled to prevent ^{14}C transport outward to other branches. Then, the above leaves close to the fruit were singly sealed by a transparent polyethylene bag. After confirming the air-tightness of the bags, $NaH^{14}CO_3$ (NEC086H, PerkinElmer, Waltham, MA, USA) and the excess amount of 1 mol L^{-1} HCl solution were injected sequentially to quantitatively produce $^{14}CO_2$. The injection pinhole was then sealed immediately to prevent ^{14}C labelling escape to the atmosphere and ensure that the feeding quantity in a single leaf was about 1.85 × $l0^5$ Bq. After 2 h, NaOH solution was injected to recover the unassimilated $^{14}CO_2$ in the polyethylene bag in order to avoid the radioactive substance contaminating the environment.

Different tissues, including the labeled mature leaves, unlabeled young leaves, branch sections from the labeled leaves to the fruit, and fruits, were collected every 24 h after feeding, which were then washed by tap water, dried to constant mass, crushed, and screened through 100 mesh. Samples of 0.2 g were accurately weighed and mixed with 3 mL of $HClO_4$ and 2 mL of 30% H_2O_2. The mixture was thoroughly mixed in a hot water bath at 70 °C for 1 h. After filtration, the radioactivity was determined by liquid scintillation counter (LS-6500, Beckman coulter, Brea, CA, USA). Three biological replicates were conducted, and the average was calculated.

Total amount of ^{14}C radioactivity in an organ (cpm) = radioactive activity of the samples/dry weight of sample (g) × dry weight of organ (g)

^{14}C distribution rate of organs (%) = (total ^{14}C in an organ/total ^{14}C in all organs) × 100%

2.2.3. Radiographic Autoradiography of Fruit Slices

The radiographic autoradiography of fruit slices was performed according to the method of Li [25]. The peach fruits collected at different times after ^{14}C labelling were cut from the middle to remove the stone and cut into fruit slices of 2 mm thickness, which were then flattened and dried in a 60 °C oven for 72 h. Then, the dried flesh discs were wrapped with transparent plastic films, ensuring the surface was smooth and wrinkle-free. The samples were placed flatwise on the white exposure surface of a phosphor screen to expose them to radiation autofluorescence in a dark room for 72 h. The phosphor screen was scanned with a multifunctional laser molecular imager (Typhoon Trio, GE

Healthcare, Fairfield, CT, USA) under a storage phosphor imaging mode with a pixel size of 1000 microns.

2.2.4. Extraction and Determination of Soluble Sugars and Starch

The nectarine fruits of the field experiment were collected after ALA treatment. The method of extraction and determination of soluble sugars in fruit samples was based on Guo et al. [26]. Firstly, 1 g fresh fruit sample was weighed, ground, and extracted in total 6 mL of 80% ethanol solution three times. Then, the homogenate was transferred to a centrifuge tube with a plug, which was incubated in an 80 °C water bath for 10 min and centrifuged at 4000 rpm for 10 min. The supernatant was evaporated in a boiling water bath. Next, 3 mL of ultrapure water was added to dissolve the residue, and the redissolved solution was filtered with a 0.45 μm pore size filter for soluble sugar determination. On the other hand, 30% (v/v) of $HClO_4$ solution was added to the fruit extraction residue overnight at room temperature. Then, the extraction solution was placed in an 80 °C water bath for 10 min, centrifuged for 10 min, and the supernatant volume was fixed to 25 mL. The total soluble sugars and starch content were determined using the colorimetric method of anthraquinone H_2SO_4 [27]. Three biological replicates were taken and averaged.

2.2.5. Determination of Sorbitol, Sucrose, Glucose, and Fructose

The soluble sugars of nectarine fruits were determined using high-performance liquid chromatography (HPLC). The chromatographic conditions were as follows: liquid chromatography (Acquity UPLC H-class, Waters, Milford, MA, USA), Prevail carbohydrate ES 5u column (100 mm × 4.6 mm, 5 μm), ELSD detector, column temperature 50 °C, mobile phase: acetonitrile/water = 80/20 (v/v), injection volume 2 μL, and flow rate 1.0 mL min^{-1}. The ELSD2000 evaporative light scattering detector parameters were as follows: nitrogen as the carrier gas, gas flow 1.5 mL min^{-1}, and drift tube temperature 80 °C. The content of sorbitol, sucrose, glucose, and fructose in the fruits was calculated according to the sample peak areas and standard curves of each sugar. Three biological replicates were taken and averaged.

2.2.6. Extraction of Total RNA from Fruit and RT-qPCR Assay

Nectarine fruit samples in the field experiment were snap-frozen in liquid nitrogen, and total RNA was extracted using the Biofit RNA kit (RN35040, Chengdu, China). The primers were designed using Primer 5.0 (Premier, Vancouver, BC, Canada) and synthesized by Generay Biotechnology (Shanghai, China) (Table A1). Genomic DNA removal and cDNA synthesis were according to TransScript (AT-311-03, TransGen Biotech, Beijing, China) instructions. Taking cDNA as the template, RT-qPCR reactions were performed on a real-time quantitative PCR instrument (ABI, Los Angeles, CA, USA) according to the instructions of ChamQ SYBR qPCR master mix (Vazyme, Nanjing, China). The $2^{-\Delta\Delta CT}$ method was used for the calculation of gene expression. *PpTEF* (translation elongation factor) was used as s the internal control. Three biological replicates were conducted.

2.3. Data and Statistical Analysis

All data were calculated using Microsoft Excel. Graphs were drawn using GraphPad Prism 9.0.1, TBtools 1.108, and Photoshop CC 2015. SPSS 17.0 was used for statistical analysis. *T*-test (Student's *t*-test) analysis was used to compare the significance of differences between the two groups (* $p < 0.05$, ** $p < 0.01$).

3. Results

3.1. Effect of ALA Treatment on Leaf Gas Exchange of Nectarine in the Orchard

Figure 1 shows the effect of exogenous ALA sprayed 20 d after full blooming on the gas exchange parameters of 'Zhongyoutao 4' nectarine leaves. The net photosynthetic rate (P_n) in the treatment was 19% higher than the control at 7 d after ALA spraying, significant at $p = 0.01$. Additionally, the transpiration rate (E), stomatal conductance to water vapor (G_{sw}),

intercellular CO_2 concentration (C_i), and instantaneous carboxylation efficiency (P_n/C_i) were also significantly higher than the control, indicating that ALA promoted nectarine leaf gas exchange with photosynthetic ability. These effects of ALA could be detected 35 d after treatment, indicating that the promotion of ALA was maintained for at least more than one month. The total increase of net photosynthetic rate after ALA treatment was 16% within 35 d. On the other hand, ALA treatment significantly reduced the leaf water use efficiency (WUE). After 7 days of ALA treatment, the WUE of the treated leaves was only 82% of the control ($p < 0.01$). This effect was present at all time points except for at 21 d, which was not significant ($p > 0.05$). This implies that ALA may be more advantageous in areas with adequate moisture than in arid regions.

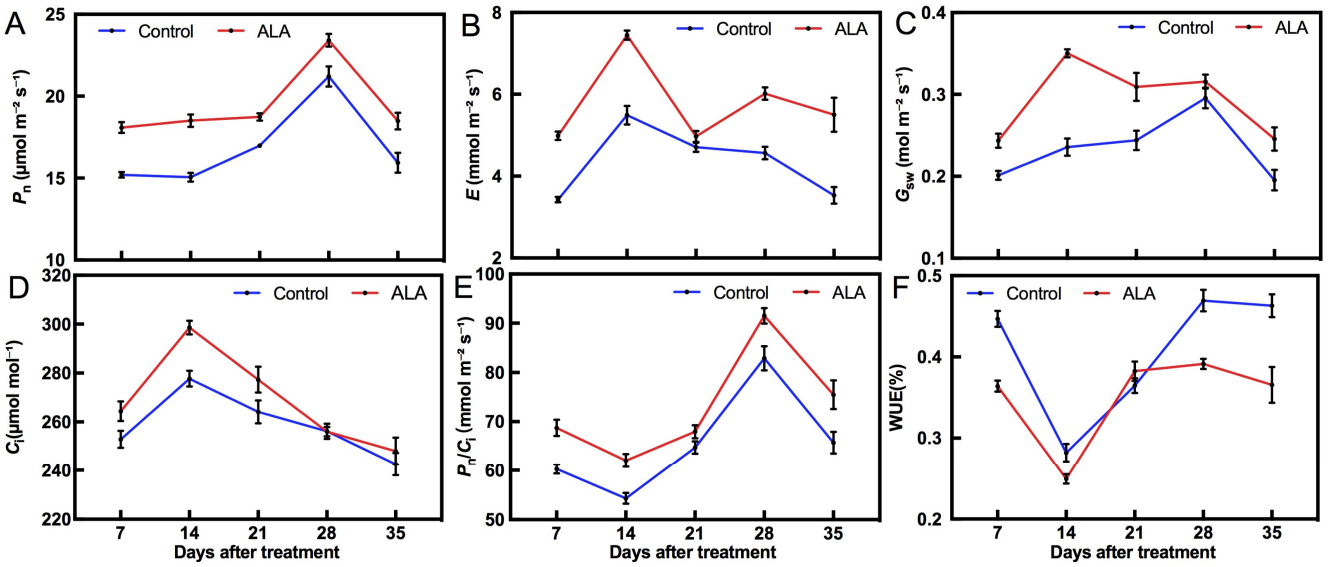

Figure 1. Effect of ALA treatment on photosynthetic gas exchange parameters of nectarine leaves. (**A**) P_n, net photosynthetic rate. (**B**) E, transpiration rate. (**C**) G_{sw}, stomatal conductance to water vapor. (**D**) C_i, intercellular CO_2 concentration. (**E**) P_n/C_i, instantaneous carboxylation efficiency. (**F**) WUE, water use efficiency. The data are the means ± standard error of twenty biological replicates.

3.2. Effect of ALA Treatment on ^{14}C-Assimilate Transport from Leaves to Peach Fruits

Figure 2A shows the results of radioactive autoradiography of 'Zhongai 33' peach fruits, whose leaves were fed with ^{14}C label. Among them, the blue part of Figure 2A is the natural background, and the green part is the ^{14}C radiolabel. The stronger the radioactivity, the greener the color is. It can be seen that the intensity of fruit radioactivity gradually increased as the time of leaf ^{14}C labelling was prolonged (Figure 2A). At 24–48 h, a small amount of radiolabeling could be seen in the control; at 72 h, the radiolabeling increased substantially, indicating that the photosynthates were transported from the labelled leaves to fruits within 1–2 d, which was increased substantially by the third day. Exogenous ALA treatment significantly promoted the transport of ^{14}C from leaves to fruits. At 24 h, the autofluorescence image of the fruit was vaguely visible, whose basic outline could be observed at 48 h, and the complete outline could be clearly seen at 72 h. These indicate that ALA treatment promoted the transport of assimilates from leaves to fruits.

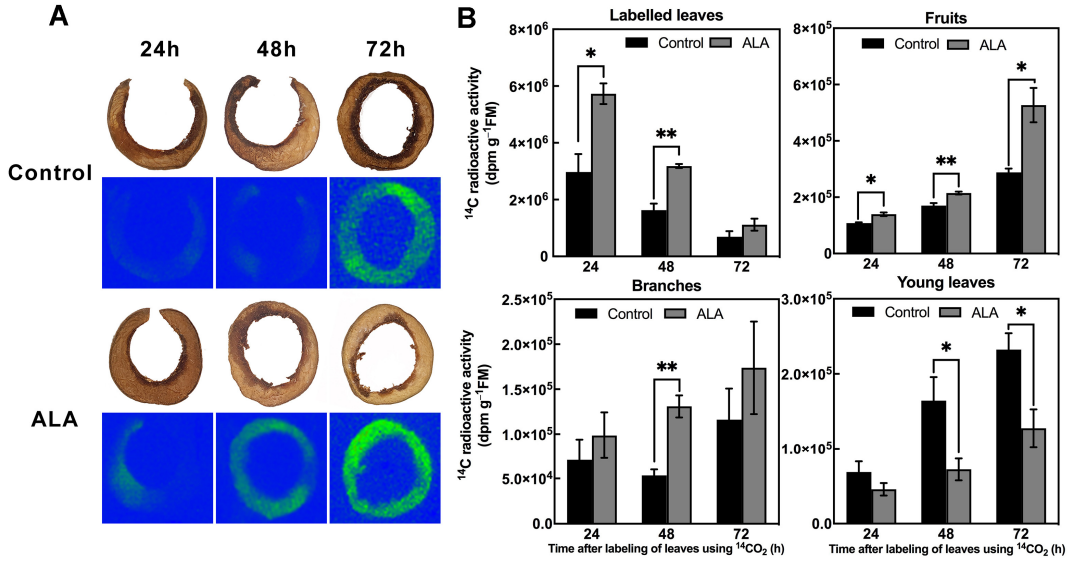

Figure 2. (**A**) The images of autoradiography of flesh discs after leaf ^{14}C labelling. (**B**) Comparison of ^{14}C radio activity of different organs of peach after leaf labelling. The data are the means ± SE from three biological replicates. Bars with * indicate the differences significant according to *t*-test. (* $p < 0.05$, ** $p < 0.01$).

Figure 2B shows the comparison of radioactivity in labelled leaves, fruits, branches, and young leaves above the branches after leaf ^{14}C labelling for different times. It can be seen that the radioactivity of ^{14}C in the labelled leaves tended to decrease with time, while that in the fruits, branches, and young leaves tended to increase, indicating that the assimilated products synthesized in the mature leaves were gradually transported to the fruits and young leaves throughout the branches. At 24–48 h of labelling, the radioactivity of ALA-treated leaves was 93%–96% higher than that of the control ($p < 0.01$), indicating that the carbon fixation capacity in ALA-treated peach leaves was almost doubled; after 72 h, this promotion trend still remained, although the difference was not significant at $p = 0.05$ because of the large experimental error. In the fruits, the radioactivity of ALA-treated samples collected at different times was significantly higher than that of the control, with an increase of 82.87% at 72 h. This corroborates with the radioactive autoradiographic images in Figure 2A. It once again indicates that ALA treatment promotes the transport of assimilated products from leaves to fruits. Similar results were obtained from measurements of branch sections, where the radioactivity of the ALA treatment was generally higher than that of the control, and the difference at 48 h after ^{14}C labelling was significant at $p = 0.01$, indicating that ALA promoted the assimilate transport through branches. Conversely, ALA treatment depressed the radioactivity of young leaves in the upper part of the labelled leaves ($p < 0.05$), suggesting that ALA decreased the ability of young leaves to compete for photosynthetic products from mature leaves.

The results in Figure 3 show that at 24 h after leaf ^{14}C labeling, 82.59% of radioactive substances were present in leaves, while 15.07% were transported to the fruits, and 0.81% and 1.53% were distributed in young leaves and branches, respectively. Subsequently, the percentage of radiolabeled compound distribution in the leaves decreased rapidly but increased rapidly in other parts. By 72 h, only 35.30% were present in leaves, while 49.26% were in fruits. The percentages of branches and young leaves continued to increase; however, they were significantly lower than the "source" leaves. The distribution of ^{14}C indicates that only about 1/3 of the assimilates were retained in the mature leaves; about 1/2 were transported to the fruit, and a smaller portion to the upper young leaves. A similar situation was found in ALA-treated plants. At 24 h after ^{14}C labelling, most of the radiolabeled compounds were present in the leaves; as the time increased, the percentage of ^{14}C assimilates in the labelled leaves gradually decreased, but increased in other parts.

ALA treatment significantly promoted the distribution of ^{14}C assimilates to the fruits, about 15 percentage points higher than the control at 72 h. At the same time, the percentage of self-retaining in labelled leaves, branches, and young leaves decreased by 6.52, 2.11, and 6.26 percentage points, respectively.

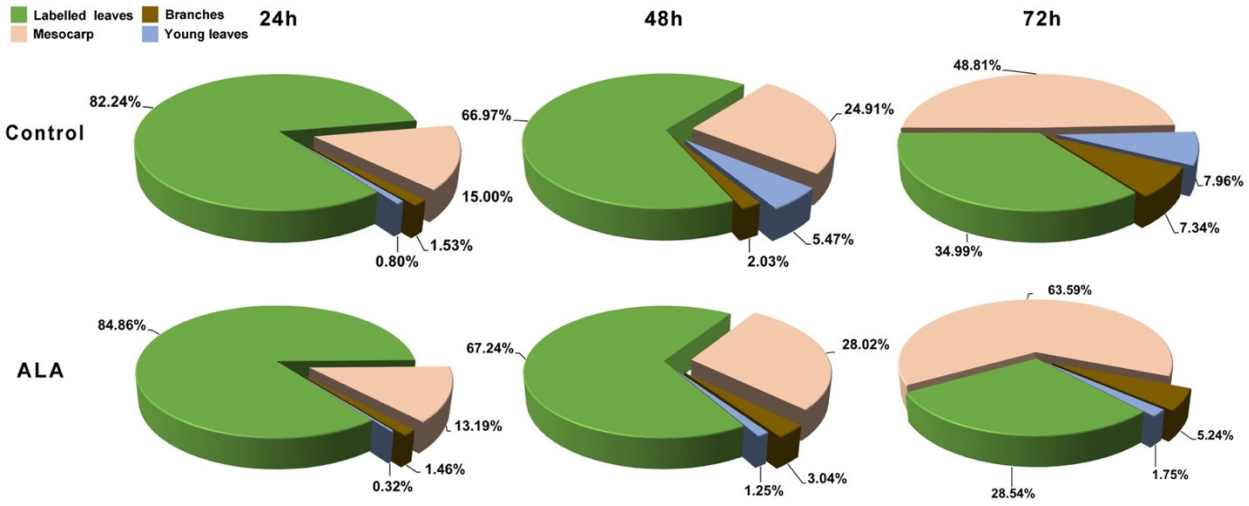

Figure 3. Distribution ratio of ^{14}C radioactivity in different organs after leaf labelling. The data are the means from three biological replicates.

3.3. Effect of ALA Treatment on the Sugar Content in the Fruits during Nectarine Development

Figure 4 shows the effect of ALA treatment on the sugar content during fruit development of 'Zhongyoutao 4' nectarine cultivated in the orchard. In order to better understand, it is necessary to show the relationship among the ALA treatment time, days after full blooming, and fruit development stages. The 14–28 d after ALA treatment in the X-axis of Figure 4 was 34–48 d after full blooming, and fruits were at the stone hardening stage. Here, we call this "the early stage" of fruit development. The 40 d after ALA treatment was 60 days after full blooming, when fruits began secondary expansion. We call this "the middle stage". Then, the 50 d and 60 d were the stage close to maturity and the maturity stage, respectively.

As can be seen, there was a clear peak of fructose, glucose, and sorbitol content in the early stage at 28 d after ALA treatment. After this, the content of the three soluble sugars fell back and remained stable until the fruit matured. It is worth noting that the fructose and glucose content in the ALA-treated fruits was significantly higher than that of the control at the early stage, although no significant differences can be found after then. These results indicate that the effect of ALA on nectarine fruit quality at maturity did not depend on the content of the three monosaccharides. Contrarily, the sucrose content increased almost linearly during fruit development while the starch content decreased gradually, especially towards maturity (50–60 d after treatment), and the starch content decreased sharply. These indicate that the final sugar content of nectarines was closely related to sucrose accumulation and starch hydrolysis. The sucrose content in the ALA-treated fruits was generally higher than that of the control, where the differences at 28, 50, and 60 d after ALA treatment was significant at $p = 0.05$, which caused a significant increase in the total fruit sugar content at maturity. Furthermore, ALA treatment caused a significant increase in the starch content at the early stage and, conversely, a significant decrease in fruit starch content at maturity. This seems to show that ALA treatment promoted the early accumulation of starch as well as degradation into sucrose at maturity, which may contribute to the increase in sugar content at fruit maturity.

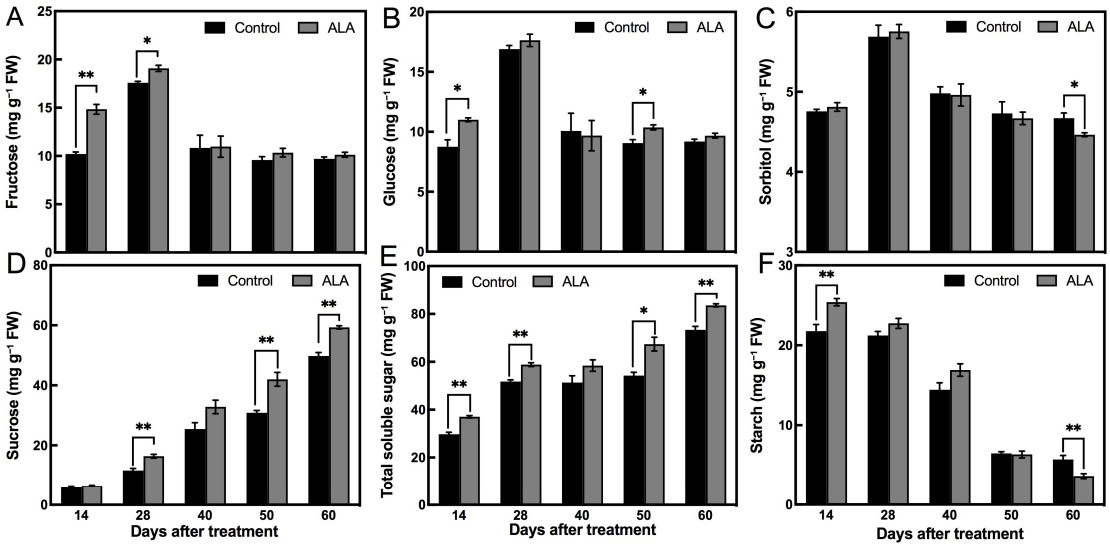

Figure 4. Effect of ALA treatment on the sugar content during nectarine development. (**A**) Fructose content. (**B**) Glucose content. (**C**) Sorbitol content. (**D**) Sucrose content. (**E**) Total soluble sugar content. (**F**) Starch content. The data are the means ± SE from three biological replicates. Bars with * indicate the differences significant according to *t*-test. (* $p < 0.05$, ** $p < 0.01$).

3.4. Effect of ALA Treatment on the Relative Expressions of the Sugar-Related Genes during Nectarine Development

To investigate the regulatory role of exogenous ALA on sugar accumulation during nectarine development, we determined the relative expressions of the genes involved in sucrose synthesis/degradation (including sucrose synthase, *SUSs*, and sucrose phosphate synthase, *SPSs*), sucrose conversion (including neutral convertase, *Nis*, cell wall convertase, *CWINV*, and acidic convertase, *AI*), sucrose transport (including sucrose transporters, *SUTs*, and sugars will eventually be exported transporters, *SWEETs*), and starch synthesis (soluble starch synthase, *SS*, and granule-bound starch synthase, *GBSS*) and degradation (α-1,4-glucan starch phosphorylase, *α-GP*, and β-starch hydrolase, *BAMs*).

From Figure 5, it can be seen that (1) the genes involved in sucrose synthesis/degradation, such as *SUS2/3/4*, were expressed at high levels in the early stage of fruit development, and *SUS5* and *SPS1* were expressed at high levels in the early-to-middle stages, while *SUS1* and *SPS2* were expressed at high levels in the mid-to-late stage. *SPS3* was expressed at high levels at fruit maturity. These indicate that the sucrose synthesis/degradation genes are involved in the whole process of fruit development, and different genes may act at different stages. (2) Most of the genes involved in sucrose conversion (including *NIs*, *CWINV*, and *AI*) and sucrose transporter genes (including *SUT2/4*) were expressed at high levels in the early stage of fruit development and then gradually decreased, while *SUT1* was expressed at high levels in the early and middle stages and increased somewhat near maturity. (3) *SS* involved in starch synthesis was expressed at high levels in young fruits and at moderate levels in other stages. *GBSS* was expressed at low levels in the early stages of fruit development and at moderate levels in the middle and late stages. (4) The genes involved in starch degradation, such as *α-GP*, *BAM1*, and *BAM2*, were expressed at high levels in the early stage, then gradually decreased to low levels in the middle and late stages. (5) *SWEETs* are a family of energy-independent sugar transporter genes, and their different members have different expression characteristics at different stages of nectarine fruit development. *SWEET1/6/7/8/15/16/17* had similar expression patterns, with low expression at early stages but high expression near and at maturity, which suggests that they may be related to sugar storage accumulation. The relative expression of *SWEET2/3/10/11* showed a peak in the early and middle stages, followed by a decrease, and then increased again when maturity was close, forming two expression peaks. *SWEET5/9/13/14* showed a similar expression pattern, with high expression in the early stages of fruit development, followed by a

gradual decrease, but maintaining a certain level. The expression pattern of *SWEET4/12/13* was similar, with high expression only at the early stage of fruit development and low expression at other stages.

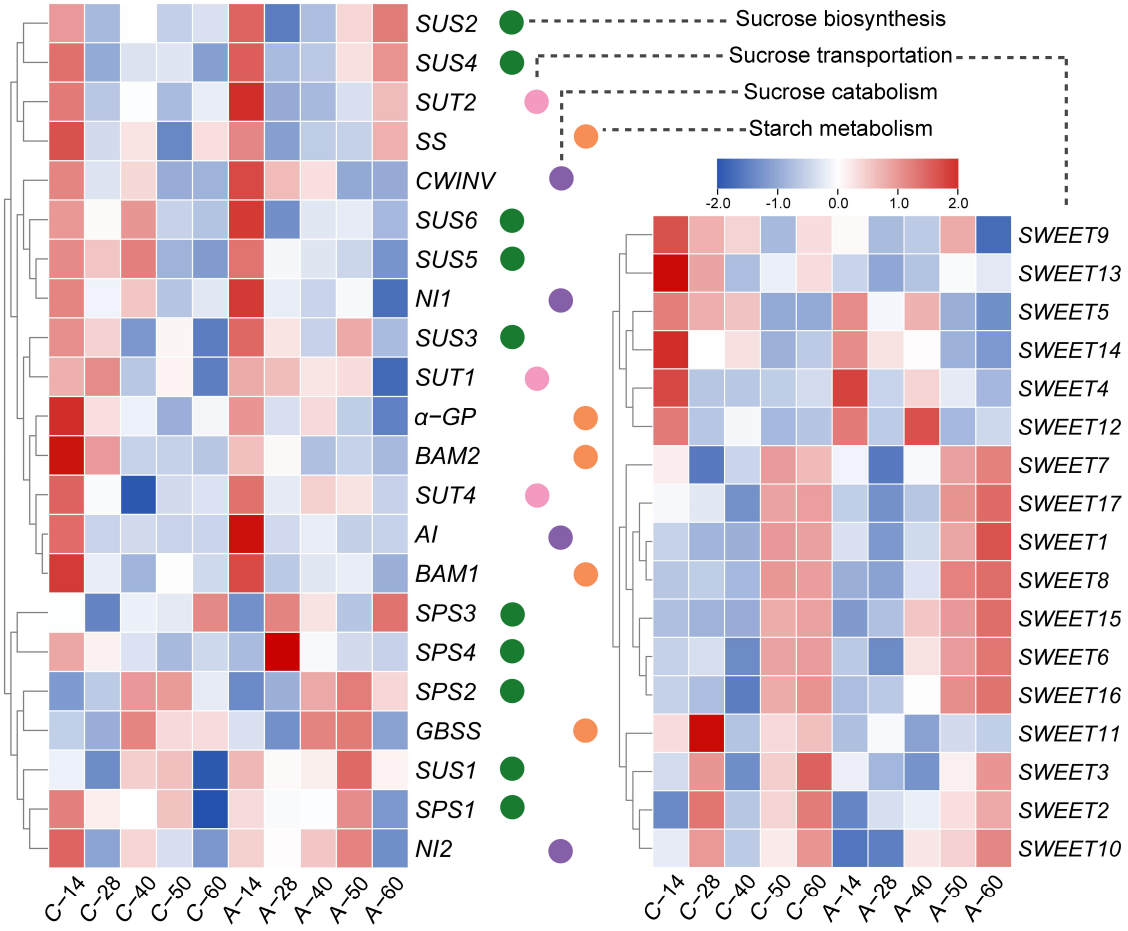

Figure 5. Effect of ALA treatment on the relative expressions of the sugar-related genes during nectarine development. C-14 to C-60 and A-14 to A-60 represent the fruit samples collected 14 to 60 days after spraying clear water and ALA, respectively. The color gradient from blue to red corresponds to fold changes from low to high.

Exogenous ALA treatment played an important role in regulating the expression of genes related to sugar accumulation in nectarine fruits. As shown in Figure 5, the expression of sucrose synthesis, catabolism, and conversion-related genes, such as *SUS2/3/4/5/6*, *SUT2*, *NI1*, *CWINV*, and *AI*, were significantly up-regulated by ALA at 14 d after treatment, indicating that ALA promoted sucrose accumulation and monosaccharide increase in young fruits. Before fruit maturity (e.g., A-50 in Figure 5), the expressions of *SUT1*, *SUS1/2/3/4*, *SPS1/2*, and *GBSS* were up-regulated by ALA; at fruit maturity (A-60), *SS*, *SUS2/4*, and *SPS2/3* were up-regulated by ALA treatment. For *SWEETs*, the expression of ALA-treated fruits was overall lower than that of the control at early developmental stages, but the expression of several genes, including *SWEET1/6/7/8/15/16/17*, was significantly higher than that of the control at pre-mature and mature stages, suggesting that they may be closely related to the ALA-induced rise in sugar content of nectarine fruits. A phenomenon worthy of attention was the starch-degrading genes *α-GP*, whose relative expression was down-regulated by ALA treatment at the young fruit stage but up-regulated at the middle stage (A-40) and before maturity (A-50). This expression pattern is likely to be associated with starch accumulation at early stages, as well as starch degradation at later stages.

3.5. Correlation Analysis between Expressions of the Sugar-Accumulation-Related Genes and the Sugar Content in Nectarine

The results of correlation analysis between different sugars during nectarine fruit development (Figure 6) showed that the sucrose content was highly significantly positively correlated with total soluble sugar content (r = 0.924 **) and negatively correlated with starch content (r = −0.918 **); fructose, glucose, and sorbitol were highly correlated with each other ($p < 0.01$), fructose and sorbitol also positively correlated with starch content ($p < 0.05$), but low-correlated with sucrose or total soluble sugar content of fruits. This suggests that the three monosaccharides may be related to fruit starch accumulation, but not to sucrose accumulation. Sucrose accumulation may be closely related to starch hydrolysis.

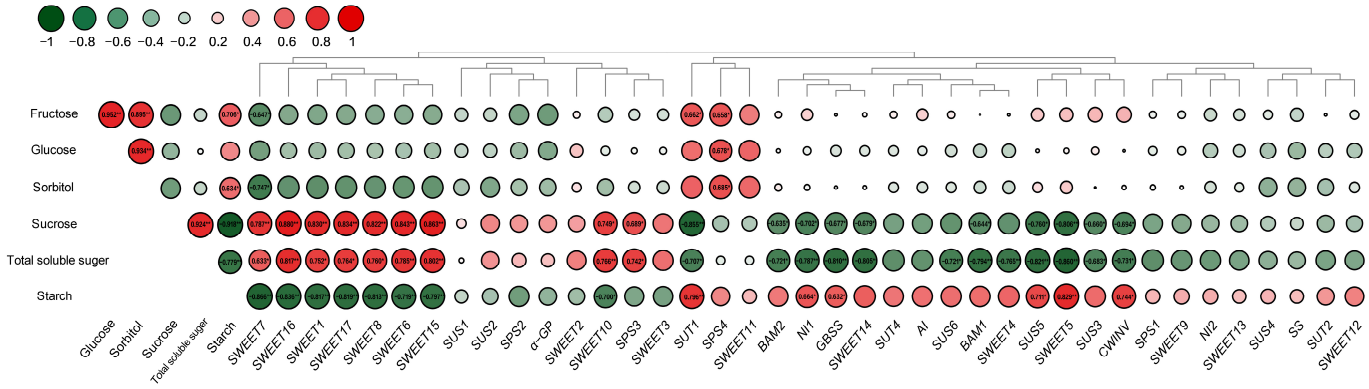

Figure 6. Correlation analysis between expressions of the sugar-related genes and the sugar content in nectarine. The circular bubble sizes represent the correlation coefficient from low to high. The color gradient from green to red corresponds to correlation coefficient changes from negative to positive. The value in the bubble is the correlation coefficient. * indicate the differences significant. (* $p < 0.05$, ** $p < 0.01$).

Sucrose or total sugar content was significantly positively correlated ($p < 0.05$ or 0.01) with the relative expression of nine genes, including *SWEET1/6/7/8/10/15/16/17* and *SPS3*. It was significantly negatively correlated with the relative expression of seven genes, including *SUT1*, *SUS3/5*, *CWINV*, *SWEET5/14*, *BAM1/2*, *NI1*, and *α-GP*. Starch content was significantly positively correlated with the relative expression of six genes, including *SUT1*, *SUS5*, *SWEET5*, *CWINV*, *NI*, and *α-GP*, while it was significantly negatively correlated with the relative expression of eight genes, including *SWEET1/6/7/8/10/15/16/17*. The glucose content was positively correlated with *SPS4* only; fructose content was positively correlated with the relative expression of *SUT1* and *SPS4* and also negatively correlated with *SWEET7* expression. Sorbitol was similarly positively correlated with *SPS4* and negatively correlated with *SWEET7*.

4. Discussion

Improving fruit quality is the main way to increase the production benefits of fruit trees and enhance market competitiveness. Soluble sugars are the core element that affects the flavor quality of fruits. It not only directly determines fruit sweetness, but is also closely related to other factors, such as fruit taste, texture, aroma, and flavor, and is essential for fruit quality formation [28,29]. It has been shown that ALA can improve fruit quality. The earliest report was on apple fruit coloration. Wang et al. [30] found that 100–300 mg L^{-1} ALA significantly promoted anthocyanin accumulation in apple fruit peel, thus improving fruit appearance quality. Later, this effect was successively confirmed in apple fruit discs [31] and callus [19,32]. In peach, Guo et al. [33] suggested that 300 mg L^{-1} ALA promoted anthocyanin accumulation in the pericarp before coloring, but if 10 mg L^{-1} ALA was sprayed, it increased specific leaf weight, promoting fruit softening and single fruit quality but without affecting fruit color, soluble sugar, and organic acid content [34].

Ye et al. [23] demonstrated that ALA enhanced the content of vitamin C, soluble sugars, the activity of antioxidant enzymes, and the ratio of soluble sugars to titratable acids in the peach fruit flesh. In recent years, Wang et al. [22] found that ALA could not only improve the nutritional and flavor quality of fruit but also promote postharvest fruit ripening. In the present experiment, the total soluble sugar content of mature nectarines after ALA treatment was 14% higher than the control (Figure 4). In addition, we also examined the soluble solids content of ALA-treated nectarines in the field at different times, and found that the highest was 14 °Brix on 29 May, 21 °Brix on 4 June, and 27.6 °Brix on 12 June 2022, respectively. Such high sugar content in precocious nectarines has rarely been reported before. These results indicate that the effect of ALA on improving fruit sugars is astonishing.

Research on the mechanism of ALA to increase fruit brix is still scarce. Most scholars ascribe the effect to its improving leaf photosynthetic performance [16,35,36]. This is definitely correct, because ALA is a key precursor of tetrapyrrole biosynthesis in all organisms [14,15], and exogenous application promotes leaf chlorophyll synthesis and increases photosynthetic accumulation, which naturally improves fruit quality. In addition, ALA can improve plant stress tolerance [37]. Higher photosynthetic performance of leaves can be maintained under stresses such as low light [38], salinity [39], drought [40], low temperature [41], suboptimal temperature, and light [42]. This is important because plants often encounter various adverse stresses during growth and development. ALA enhances the ability of plants to accumulate carbohydrates under stressful conditions, which is valuable for improving fruit quality. In this study, we did not specifically set up stressful conditions; however, in the orchard, the spraying of low-concentration ALA solution on trees 20 d after full blooming significantly increased the net photosynthetic rate of leaves, and this effect was maintained at least one month with an average increase of 16% in P_n (Figure 1). Meanwhile, transpiration rate, stomatal conductance to water vapor, intercellular CO_2 concentration, and transient carboxylation efficiency were significantly higher than those of the control. This indicates that ALA improves peach/nectarine fruit quality in close relationship with photosynthetic gas exchange characteristics. In addition, we observed that ALA induced a decrease in WUE (Figure 1F). This was related to an ALA-induced increase in stomatal opening [43]. This is not a problem in the rainy regions in southern China, although it may be a concern in northern arid regions.

Yao et al. [44] found that ALA promoted phosphorus uptake and distribution in rice grains using ^{32}P, suggesting that ALA improved plant nutrition and crop yield. In this experiment, the effect of ALA on photosynthetic product transport and distribution in nectarine leaves was investigated using ^{14}C labelling (Figure 2). The results showed that: (1) ALA significantly promoted $^{14}CO_2$ fixation in leaves. The comparison of ^{14}C radioactivity of all samples at three time points showed that ALA treatment resulted in a 71% increase in total ^{14}C radioactivity in nectarines. The data are much higher than the P_n increase (Figure 1A). This may be due to the super-sensitivity of radioisotopes, but it suggests that ALA significantly promoted CO_2 fixation in nectarine leaves. (2) ALA promoted the transport of leaf-fixed ^{14}C to fruits. There are at least four pieces of evidence. One is the ^{14}C activity of branches which increased overall after ALA treatment, where the difference was statistically significant at 48 h. The second is the fruit. The radioactivity in ALA-treated fruits collected at 24 h and 48 h after ^{14}C labelling increased by 26%–29% compared to the control; at 72 h, it increased by 83%. These indicate that ALA not only accelerates assimilate transport, but also increases the amount of transportation. The third is that ALA treatment depressed the ^{14}C transport from mature leaves to young ones. The mean value of ^{14}C in young leaves at three times was only 58% of the control. This may be due to the fact that ALA treatment enhances fruit "sink strength" while reducing the ability of young leaves to compete for carbohydrates. This dual effect is important for fruit nutrient accumulation and quality formation. The fourth is that ALA treatment reduced the retention of ^{14}C in leaves. At 72 h after labelling, 35.30% of ^{14}C was retained in control leaves, but only 28.78% in the ALA treatment. This indicates that ALA enhances fruit

"sink strength"; then more of the ^{14}C from leaf "source" was allocated to the fruit. This process is similar to the reports of Fan et al. [45,46], who suggested that a large amount of carbohydrate is required during the secondary expansion stage and fruit maturity in peach fruits. They found that the ^{14}C retention in the labelled leaves was 28.21%, while 67.67% ^{14}C was allocated to fruits when it was detected 7 d after labelling. According to Shu [4], the amount of assimilates transported to the fruit depends on the photosynthetic capacity of the leaves, the "sink strength" of the fruit, and the loading and unloading of photosynthetic products. In this experiment, we observed that ALA treatment enhanced the photosynthetic carbon fixation of nectarine leaves, increased fruit "sink strength," promoted assimilate transport and distribution, and reduced the competition of young leaves. Therefore, regulating the "source-sink" relationship of fruit trees with ALA deserves further study.

In this experiment, the regulation of ALA on sugar accumulation during fruit development of 'Zhongyoutao 4' nectarine was analyzed. 'Zhongyoutao 4' is ecologically adaptable and has become one of the main precocious cultivars in many facilities [47,48]. In our experiment, it was observed that in Nanjing, it bloomed in early-to-middle March, and fruits matured in the middle of June, with a fruit development period of about 80 days. The glucose and fructose content in the fruit were close to each other (Figure 4A,B), belonging to the fructose/glucose $\approx$ 1 type variety [49]. Except for early accumulation, the two hexoses were not too high throughout development. Furthermore, they were not closely related to sucrose content. However, they were positively correlated with starch content (Figure 5). This suggests that they may be converted to starch in young fruit to store in leucoplast. Therefore, glucose and fructose are not the direct sources of sucrose in mature fruits. ALA treatment promoted the increase of two hexoses in the early stages of fruit development. This effect is beneficial to increasing the osmotic potential of flesh cells [50] and promoting cell division and fruit size at maturity [51]. In addition, sorbitol is the main form of long-distance transport and storage of sugars in peach trees [29]. 'Zhongyoutao 4' fruits had a sorbitol accumulation peak at 48 d after full blooming, and remained stable in other time periods. ALA treatment significantly decreased the sorbitol content in the mature fruits (Figure 4C). Accordingly, there is no direct relationship between ALA in improving fruit quality and sorbitol content. On the contrary, starch was accumulated in the early stage of fruit development and gradually decreased with fruit development (Figure 4F). At the same time, sucrose increased linearly (Figure 4D), implying that starch was first accumulated during fruit development, then converted into sucrose that finally became the main sugar affecting nectarine fruit quality (Figure 4E). This is consistent with the current theory of sugar accumulation in peach fruit [29] and is exactly similar to that reported by Guo et al. [26]. In this experiment, we sprayed exogenous ALA at the fruit cell division stage (20 d after full blooming), and the starch content of young fruit was significantly higher than that of the control 14 d later (Figure 4F). This effect was prevalent in both early and middle fruit development. However, at maturity, the starch content of ALA-treated fruits was significantly lower than that of the control, while the sucrose and total soluble sugar content were significantly increased. This implies that ALA promotes starch accumulation in early young fruits and also promotes starch degradation to sucrose when maturity is imminent. This may be a biochemical mechanism by which ALA promotes sugar conversion and accumulation in nectarine fruits.

We further analyzed the regulatory effects of ALA on the relative expressions of the sugar-related genes during fruit development. Sucrose phosphate synthase (SPS), sucrose synthase (SUS), acid invertase (AI), neutral invertase (NI), and cell wall invertase (CWINV) have been reported to be the major enzymes of sucrose metabolism in peach fruits [52]. In this experiment, we observed that *SPS3/4* expression in fruits was significantly higher than the control at A-28; *SPS1/2* expression was significantly higher than the control at A-50; and *SPS2/3* expression was higher than the control at A-60 (Figure 5). However, only *SPS4* expression was positively correlated with sorbitol, glucose, and fructose content, and the relative expression of other *SPSs* did not correlate with sugar content (Figure 6). This

may indicate that (1) different *SPSs* genes are involved in sugar metabolism in fruits at different developmental stages; and (2) *SPSs* are mainly associated with monosaccharide metabolism, which has little relationship with sucrose accumulation, especially fruit quality at maturity. This is similar to the results of Muhammad et al. [53], who showed that SPS*4* expression was positively correlated with glucose and fructose, while *SPS2* had a negative correlation with glucose and sorbitol. However, our results differ from the reports of Moriguchi et al. [54] or Zhang et al. [52]. The former suggested that SPS activity was undetectable during peach fruit development, whereas the latter proposed that *SPS* expression during peach fruit development was negatively correlated with glucose and fructose content and positively correlated with sucrose content. More bizarrely, Zhang et al. [52] reported a lack of correlation between *SPS* gene expression and enzyme activity. These suggest that the relationship between *SPS* and sugar metabolism in peach fruits needs further study.

In our data, *NI1* and *CWINV* were significantly positively correlated with starch content and significantly negatively correlated with sucrose and total soluble sugar content (Figure 6). ALA treatment up-regulated *AI*, *NI1*, and *CWINV* expression at the young fruit stage (Figure 5), suggesting that these genes may play a role in early starch accumulation. The correlation between soluble starch synthase *SS* expression and fruit sugar content was not significant, but the expression of granule-bound starch synthase *GBSS* was significantly correlated with starch content, while sucrose and total soluble sugar content were significantly negatively correlated. The effect of ALA on the gene expressions was not significant, indicating that ALA may not regulate *GBSS* expression to regulate fruit sucrose content. On the other hand, the expressions of starch amylase genes *BAM1* and *BAM2* were significantly and negatively correlated with sucrose and total soluble sugar content (Figure 6). ALA had no significant effect on *BAM1* expression, but significantly down-regulated starch degradation gene α-GP and *BAM2* expressions at the young fruit stage. It may favor starch accumulation in young fruits.

The sucrose synthase SUS reversibly catalyzes the generation of UDP-glucose and fructose from sucrose and UDP (uridine diphosphate) [55]. Zhang et al. [56] identified *SUS* family members in peaches and suggested high expression levels of *SUS3/5* during fruit development, while *SUS1* is expressed only in mature fruits. However, in this experiment, the expression of *SUS3/5* in nectarine was significantly negatively correlated with sucrose content, and *SUS5* expression was positively correlated with starch content, implying that *SUSs* are closely related to starch accumulation (Figure 6). In addition, multiple *SUSs* expressions were higher in early and middle fruit development, whereas only *SUS1* expression was higher near maturity. This is similar to the results of Zhang et al. [56]. It seems that *SUS3/5* may be involved in early young fruit starch accumulation, while high *SUS1* expression at later stages may be related to sucrose accumulation. Notably, ALA up-regulated *SUS1/2/3/4/5/6* expression at different stages, suggesting that ALA promotes sucrose accumulation in nectarine fruits through the regulation of *SUSs*, but this needs to be studied in further detail.

SUT is a sucrose transmembrane transporter [57]. In peach, *SUT1* is localized in the cytoplasmic membrane, involved in assimilate loading or unloading. *SUT4* is localized in the vesicular membrane of fruit cells and may be involved in intra-vesicular sucrose transmembrane transport [58]. Peach *SUT2* exhibits a functional compensatory effect in sucrose-uptake-deficient yeast, demonstrating its involvement in sucrose transportation [59]. Thus, *SUTs* are the main genes involved in the long-distance transport of "source-sink" sugars [60,61]. In our study, *SUT1* expression in nectarine fruits was positively correlated with fructose and starch content and negatively correlated with sucrose and total soluble sugar content, suggesting that it may not be related to fruit sucrose accumulation but, instead, may be involved in the unloading of sugars from leaf transport in the fruit vascular bundles and conversion to starch. In addition, the expression of *SUT1/2/4* in ALA-treated fruits was up-regulated at the mid-to-late stage, implying that ALA may

promote leaf sugar transport to fruits by regulating the expression of *SUTs* genes. This coincides with our results of ^{14}C radioactivity in Figure 2.

SWEETs are a family of low-affinity sugar transporters whose work does not depend on primary drive forces (including energy gradients and pH gradients). SWEETs can bind to sugars as long as a concentration gradient exists and achieve bidirectional transmembrane transport of sugars with the assistance of amino acids at the corresponding sites [62,63]. There have been reports that *SWEET* genes were found in apple [64,65], sweet orange [66], pear [67], grape [68], and peach [69,70] fruit trees. Predictions show that most peach SWEETs are localized in the cytoplasmic membrane, while SWEET4/8/16 (Prupe.1G133300/Prupe.3G034900/Prupe.2G245600) are localized in the vesicular membrane and SWEET12 in the chloroplast membrane [69]. In addition, *SWEET5* (Prupe.5G146500) was specifically expressed in fruits. In our experiment, we examined the relative expressions of 17 *SWEET* genes in fruit development. Broadly, they can be divided into two categories: one of them was expressed mainly in the early stages of young fruit development, including *SWEET4/5/9/12/13/14* (Figure 5). This group had a low or negative correlation with fruit sucrose content (Figure 6). The other group was mainly expressed at high levels before fruit maturity, including *SWEET1/6/7/8/10/15/16/17*. They were significantly positively correlated with the fruit sucrose content and up-regulated by ALA, suggesting that they may play an important role in the promotion of fruit sugar accumulation by ALA. The subsequent molecular regulatory mechanism deserves profound study.

5. Conclusions

In summary, this study explored the possible mechanisms of exogenous ALA on peach/nectarine fruit quality enhancement from the perspectives of leaf photosynthetic efficiency, assimilate transport, fruit sugar accumulation, conversion, and gene expression regulation. Our study firstly demonstrated that ALA increased source volume and improved "sink" competition for assimilation products by ^{14}C radiolabeled technology. We propose the possibility that fructose and glucose in young fruits are first converted to starch and the latter gradually degraded to sucrose, which provides a theoretical basis for the screening of key genes for ALA to regulate peach fruit quality improvement.

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Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

Appendix A

Table A1. Primer sequences designed for RT-qPCR of genes.

Primer	Gene ID	Forward Primer (5′–3′)	Reverse Primer (5′–3′)
PpTEF	Prupe.4G138700	gcacggatgggcttttac	aatagggcactgctggctgg
PpSUS1	Prupe.7G192300	agaggctgacatcattccac	gcttcgcattcttaccatacc
PpSUS2	Prupe.1G483200	agtggcacagaacacacaca	ccctgcaactcagcagctat
PpSUS3	Prupe.8G264300	gccatttatttcccttactctg	tgtaaccagcaaccacgac
PpSUS4	Prupe.1G131700	ttggagaaaacaaagcaccc	cccaggaagagtgaaagctgtgtgg
PpSUS5	Prupe.3G014100	cgttttgacatctatccctacc	caatagttgcctgagtaatccc
PpSUS6	Prupe.5G241700	taaggcggaaaacgatga	ttggatttggatgggtca
PpSUT1	Prupe.8G052700	ctgcttcttcctttccatca	cccatccaatcagtgtcg
PpSUT2	Prupe.1G271500	tcaacccggacacaaacaca	caatgtctgcagaaaacccga
PpSUT4	Prupe.1G542000	ctctgtttatggcggttgg	tcctgagctgctgatatgct
PpSPS1	Prupe.7G249900	tactggagcgtaaactgcg	tcttcatttgcttctgtctcac
PpSPS2	Prupe.1G483200	catggttgtaatacctcccg	gcaagtatcataggcttccgt
PpSPS3	Prupe.1G159700	gtctgcgttgccatcca	ttgccaccacccctttc
PpSPS4	Prupe.8G003700	atggcgggaaacgactg	agcacgtattctcaagcctatt
PpNI1	Prupe.1G111800	ttgaatgacaccaaggatgag	agaaagcgagtgccgatg
PpNI2	Prupe.1G556900	acattgatagcacctactcccc	ggctgcgaaagaagacca
PpCWINV	Prupe.3G009500	cccaaaatcgccaagttca	gcttctcctgtttggttcgt
PpAI	Prupe.5G075600	cttctatgttgccaaaggacc	ttgccttggttgggtaaat
PpSWEET1	Prupe.6G355900	gtgatggcggtcacactctt	acacagcacacatccatcca
PpSWEET2	Prupe.1G220700	tttccttacattgagtgccg	tcatcatcttctatgggtttgc
PpSWEET3	Prupe.8G253500	actatgccttcctcaagacca	caacactaatccacccaacaa
PpSWEET4	Prupe.1G133300	aatgttccttgctcctgtcg	tttgccctcatctttgctg
PpSWEET5	Prupe.5G146500	tctatgcccctaagaaagcc	gacaagacaaatccatcccac
PpSWEET6	Prupe.3G283400	ggtcggcattgtctgtgat	agcagcaaccatttaggaagt
PpSWEET7	Prupe.8G076100	taacgcaaagcgaaggatt	tcaagggtgaggcatacataa
PpSWEET8	Prupe.3G034900	gtctggaccatctatgcgct	caggatgatttgcaccgcac
PpSWEET9	Prupe.5G125100	caagaagcagaggctcaaag	caaacgatgcccacaataag
PpSWEET10	Prupe.8G017400	aggtcttctttggagttgttgg	aggaggcagttgagcattg
PpSWEET11	Prupe.5G146400	gttatccgaaccaagagcg	ccgaaggtgaatccaagtatg
PpSWEET12	Prupe.4G155700	ttctcagggctgccataca	atcccagcatcctcacctt
PpSWEET13	Prupe.4G072300	gtcaccactgccaacattca	acaacagcaactgccccta
PpSWEET14	Prupe.2G118600	ctcttaatgtagcctttccagc	ttagtgccaccacagttttca
PpSWEET15	Prupe.5G175500	atggagttgtctgggtggtc	ggtggcgtacaggatgagt
PpSWEET16	Prupe.2G245600	gttagcaccgttggatgga	tggcacaaattgtcaggga
PpSWEET17	Prupe.2G307800	gctagtcagcggaaatgcga	taccaagccgctgaaaggag
PpSS	Prupe.2G309400	tgcacatcagggagtggaac	cacccattccagtgctccat
Ppα-GP	Prupe.3G257600	atcctgaacacagcaggctc	gctgcttatttggggcaacc
PpGBSS	Prupe.5G132800	tatggaacggtgcctattgt	catctgctgggtcaacttca
PpBAM1	Prupe.3G221800	tcccccagtgggtacttgat	ccactaaagagacgccggtt
PpBAM2	Prupe.5G051000	ctccgggcaaagagttcgat	gcaccttggaaccattgctg

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