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Exogenous GR24 Inhibits Strawberry Tillering by Affecting the Phytohormone Signaling and Sugar Metabolism Pathways

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Abstract: Tillering is an important part in strawberry growth, and strawberries can reproduce nutritionally through stolons to generate genetically stable offspring. However, excessive tillering during the fruit-growing stage can negatively impact fruit yield and quality. In this study, different concentrations of exogenous rac-GR24 (GR24) are used to treat the strawberry plants. It was found that GR24 effectively inhibited the sprouting of strawberry stolons, while promoting the growth of the stems and leaves. Among the treatments, the most effective concentration was found to be 5 µmol/L GR24. This treatment resulted in a decrease in the glucose content in the strawberry crowns and also caused changes in the contents of two endogenous phytohormones, gibberellic acid (GA_3) and trans-zeatin riboside (tZR). Transcriptome data further suggested that exogenous GR24 may inhibit strawberry plant tillering by affecting various phytohormone signaling pathways and the sugar metabolism pathway. In 5 µmol/L GR24-treated plants, the expression level of type-B response regulator (*B-ARR*) was down-regulated and the expression level of CYTOKININ RESPONSE 1 (*CRE1*), histidine-containing phosphotransfer protein (*AHP*), and type-A response regulator (*A-ARR*) were up-regulated, suggesting the inhibition of the cytokinin (CTK) signaling pathway. The down-regulation of auxin (*AUX*) and auxin response factor (*ARF*), as well as the up-regulation of auxin/indole-3-acetic acid (*AUX/IAA*), led to the inhibition of the indole-3-acetic acid (IAA) signaling pathway. Additionally, the up-regulation of pyrabactin resistance 1/ pyrabactin resistance 1-like (*PYR/PYL*), non-fermenting 1-related protein kinase 2 (*SnRK2*), and ABRE binding factors (*ABF*) and the down-regulation of protein phosphatase 2C (*PP2C*) were observed in the up-regulated abscisic acid (ABA) signaling pathways. In the sugar metabolism pathway, the up-regulation of invertase (*INV*), hexokinase (*HK*), and fructokinase (*FRK*) and the down-regulation of trehalase (*TREH*) and beta-amylase (*BMY*) led to a decreased glucose synthesis and an increased glucose consumption. Therefore, GR24 can effectively inhibit strawberry plant tillering through these pathways, making it an effective reagent for tillering inhibition.

Keywords: strigolactone; stolon; cytokinin; auxin; abscisic acid

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1. Introduction

Strawberries (*Fragaria* × *ananassa* Duch.) are perennial herbaceous plants in the Rosaceae family, known for their high nutritional and economic value [1]. In the production of cultivated strawberries, the propagation of stolons is a commonly used breeding method to preserve the plants' excellent traits. However, in China, during mid-October every year, favorable external environmental conditions lead to a significant sprouting of stolons. This excessive growth can disrupt the balance between the reproductive and nutritional growth

processes of strawberries, resulting in yield reduction and loss of fruit quality [2]. Therefore, it is critical to effectively control the formation of stolons during the cultivation process of strawberries.

As perennial rosette-forming herbaceous plants, strawberries have dense verticillate leaves with long petioles. Axillary buds, which are located in the leaf axils, can germinate into branches under low-temperature and short-day conditions [3]. These branches can develop into long branches called stolons [4]. The occurrence of stolons is influenced by various factors, such as the development and location of axillary buds, phytohormones, as well as environmental conditions. It has been found that the duration of sunlight can affect the asexual differentiation of strawberry axillary buds [5]. Long-day conditions promote the formation of stolons [6]. Gibberellin (GA) has been found to enhance the ability of strawberry plants to sprout stolons [7]. The photoperiod also plays a role in regulating stolon formation by affecting the GA biosynthesis or balancing asexual and sexual reproduction patterns in axillary meristems [8]. The *GIBBERELLIN20-oxidase 4* gene (*FvGA20ox4*) is essential for stolon growth in woodland strawberries (*Fragaria vesca* L.) [9]. In addition, 6-Benzylaminopurine (6-BA) can break the dormancy of axillary buds and promote stolon sprouting by increasing the soluble sugar content in strawberries [10]. In other plant species, such as creeping bentgrass (*Agrostis stolonifera* L.), an elevated carbon dioxide (CO_2) concentration promotes stolon growth and increases the accumulation of glucose, sucrose, fructose, and endogenous IAA at the stolon nodes and internodes, resulting in longer stolons and larger stolon shoot biomass [11]. Kentucky bluegrass (*Poa pratensis* L.) with more ramets also exhibits a significant increase in the soluble sugar content in the rhizome [12]. In addition, studies on strawberries have revealed that auxin and CTK play antagonistic roles in controlling the development of axillary buds. Reduced auxin accumulation promotes stolon formation, while exogenous CTK promotes the formation of flower buds [13]. In wheat (*Triticum aestivum* L.), a lower red light/far-red light ratio up-regulated CTK degradation genes (*TaCKX5* and *TaCKX11*) and GA biosynthesis genes (*TaGA20ox1* and *TaGA3ox2*) in the tillering nodes, leading to a decreased CTK level and an increased GA level. This promotes CTK degradation and inhibits tillering in wheat [14]. The temperature mainly affects the dormancy of buds or the germination of buds. In experiments with roses, it has been shown that a low temperature can promote the signaling pathway of strigolactone, which in turn affects the gradient germination rate of lateral branches [15]. Additionally, phosphorus (P) and nitrogen (N) have important effects on the development of plant lateral branches, which can be influenced by the synthesis and transportation changes of IAA, CTK, and SLs, thereby affecting the development of lateral branches [16].

Strigolactone (SL) is a carotenoid-derived phytohormone that plays a crucial role in regulating various plant activities. It was initially discovered as a germinating agent [17,18] and later found to be an effective inhibitor of branching [19]. The function of SL in plants is mainly achieved through its complex interaction with other phytohormones. Research has shown that reducing the activity of IAA4/5, which is a downstream component of the auxin signaling pathway in the pea (*Pisum sativum* L.) plant, can promote auxin signaling transduction. This, in turn, leads to higher content of SL and a lower content of CTK, resulting in the inhibition of axillary bud branching [20]. Furthermore, SL and CTK have antagonistic effects on shoot growth regulation [21]. SL also affects root development through the transduction of GA signaling [22].

GR3, GR7, GR5, and GR24 are common artificially synthesized SL analogues; among them, GR24 is the most effective [23]. GR24 ($C_{17}H_{14}O_5$) is a triterpene lactone analogue of SL, which has been found to promote seed germination, inhibit plant branching, and promote anthocyanin accumulation in various plant species [24,25]. In addition, it has been found that GR24 can affect the cell size of cherry rootstocks and inhibit their stem growth [26]. GR24 treatment can also reduce the susceptibility of tobacco and grapevine plantlets to *Botrytis cinerea* infection [27]. However, studies on SL in strawberries are limited to its effects on strawberry fruit quality and endogenous SL affecting branching. It was found that exogenous SL alleviated the oxidative damage of strawberries by improving the

defense capability of the antioxidant system [28]. And it was reported that the expression of most SL biosynthetic genes was high in developing carpel, anther, and style, while that of SL signaling genes was high in carpel and style, but low in anther. This indicated that SL plays a role in the early stages of woodland strawberry fruit development [29]. Meanwhile, research has also shown that branching features or the number of branches are dependent on the content of SL in strawberry plants [30]. However, the role of exogenous SL in regulating strawberry stolons has not been reported. Therefore, this study intends to analyze the physiological and transcriptome changes in strawberry plants treated with different concentrations of GR24. We also examine the interaction between endogenous phytohormones and sugar in response to exogenous SL. By studying the effects of exogenous SL on strawberry plants, we hope to gain insights into the methods of controlling plant tillering in strawberry cultivation.

2. Materials and Methods

2.1. Plant Materials and Treatments

Cultivated strawberries (*Fragaria* × *ananassa* cv. 'Benihoppe') were used as the experimental materials at the strawberry picking base in Jinggang Village, Pidu District, Chengdu City, Sichuan Province. Strawberry seedlings were planted in September 2022. One month after planting, uniform and robust strawberry plants were selected and sprayed with 50 mL of the GR24 (Coolabar, Beijing, China) solution at concentrations of 0 μmol/L, 5 μmol/L, 10 μmol/L, and 20 μmol/L on their crowns. Each treatment consisted of 15 plants, and the spraying was conducted every 9 days for a total of 3 times, with 3 replicates. One functional leaf (15 days old) with a uniform growth state from each plant was marked for the subsequent measurement of leaf physiological indicators. Stem diameter, plant height, petiole length, leaf thickness, and the number of stolons were measured 9 days after each spraying. The crowns, 9 days after the 3rd spraying of 5 μmol/L GR24, were quickly frozen in liquid nitrogen, ground into a uniform powder, and stored in an ultra-low temperature refrigerator at −80 °C for the subsequent determination of plant endogenous phytohormones and transcriptome analysis.

2.2. Measurement of Plant Growth Indexes

The stem diameter, plant height, petiole length, and the diameter of the flowering branches were measured with vernier calipers and straightedges. The leaf thickness was measured using a thickness gauge (Qifeng Digital High Precision Thickness Gauge MIMZI, Hangzhou, China). The number of stolons and flowering branches were counted through visual observation.

2.3. Determination of the Endogenous Phytohormone Content

We accurately weighed 0.5 g of the sample in a test tube and added 10 mL acetonitrile solution and 8 μL phytohormone standard solution. The sample was extracted overnight at 4 °C and then centrifuged (−80 °C, 12,000× *g*) for 5 min. After extracting the supernatant, 5 mL acetonitrile solution was added to the precipitate to be extracted twice. The supernatant was combined and the impurities were purified by adding the appropriate amount of C18 and graphitized carbon black (GCB). Then, the liquid was centrifuged (4 °C, 12,000× *g*) for 5 min, and retain supernatant was retained. The sample was blown dry under nitrogen, re-dissolved with 400 μL of methanol, passed through a 0.22 μm organic filter membrane, and placed in in a −20 °C refrigerator for further analysis. The endogenous phytohormones IAA, ABA, tZR, and GA_3 were determined using an Agilent 1290 HPLC system in series with an AB's SCIEX-6500Qtrap mass spectrometer. The samples were detected using a linear gradient eluent program: A total of 0.1% formic acid in methanol as eluent A and 0.1% formic acid in water as eluent B. A 2 μL sample was injected, and the flow rate remained at 0.3 mL/min. From 0 to 1 min, the eluent A was at 20%, and it increased from 20% to 50% and it kept on increasing until reaching 80% from 3 to 9 min. After staying at 80% for 1.5 min, the eluent A decreased to 20% in 1 min and remained at 20% for

2.9 min. The concentration of endogenous phytohormones was quantified by comparing them with the corresponding external standards, all of which were HPLC-grade standards purchased from Sigma (St. Louis, MI, USA). The experiments were independently repeated three times.

2.4. Determination of Sucrose, Fructose, and Glucose Contents

The contents of sucrose, glucose, and fructose were determined by high-performance liquid chromatography (Agilent HPLC 1260 Infinity II, Santa Clara, CA, USA) [31]. After weighing 0.3 g of the sample, it was extracted with 2 mL ultra-purified water and subsequently passed through an Agilent Athena NH2-RP column using an Agilent HPLC system with a differential refractive index detector. The elution program was: 75% acetonitrile for 10 min with a 10 µL injection and a flow rate of 1 mL/min.

2.5. Transcriptome Analysis

The total RNA was isolated using the cetyltrimethylammonium bromide (CTAB) method [32]. Libraries were then constructed and sequenced by Genepre in Chengdu, China. The quality of the libraries was assessed with an Agilent 2100 bioanalyzer. A total of 6 libraries were clustered and sequenced (150 bp, pair-end) on a Hiseq-2500 platform, with three replicates for both the control (CK) and the samples treated with 5 µmol/L of the GR24 treatment. To ensure data quality, low-quality reads with a quality score (Q) below 20 were screened using the FASTQ software (v0.11.9). The adaptors were trimmed using the Trim Galore software (v0.6.6). The resulting cleaned reads were then mapped onto the reference strawberry genome (v1.0.a2) and quantified using the Hisat 2 and stringtie (v) pipeline with the default parameters. The DESeq 2 (v3.34.1) R package was used to detect the differentially expressed genes (DEGs). The genes with $\log_2$-fold changes (FCs) >1 or <−1 and an adjusted $p \leq 0.05$ were considered as significant DEGs.

2.6. Real-Time Fluorescence Quantification (RT-qPCR)

A real-time quantitative PCR (RT-qPCR) assay was performed to verify the expression levels of the key genes (*FaAHP1-like*, *FaHK2-like*, *FaARR1-like*, *FaLAX2*, *FaMETTL5*, *FaAUX28-like*, *FaIAA8-like*, *FaIAA9*, *FaIAA27*, *FaARF5*, *FaPP2C10*, *FaPP2C12*, *FaPP2C13*, *FaPP2C27*, *FaABF1*, and *FaABF5*) involved in the CTK, IAA, and ABA signaling pathway. A 10 µL reaction mixture was prepared, consisting of 1 µL of cDNA template, 1 µL of gene specific primer pairs (Table S1), and 5 µL of TB Green Premix Ex Taq II (TaKaRa, Dalian, China). The qPCR assay involved a three-step PCR reaction, with a denaturation step at 94 °C for 30 s, an annealing step at 58 °C for 10 s, and an extension at 72 °C for 10 s. The total reaction circle was set to 40. The *FaActin2* gene (LOC101313255) was selected as an internal control. The relative expression levels of the detected genes were calculated by the $2^{-\Delta\Delta Ct}$ method. Three wells of each sample were conducted as three technological replicates, and three independent biological replicates were conducted for all the qPCR reactions.

2.7. Statistical Analysis

The software SPSS 22.0 was used for the statistical analysis of the data obtained from the experiments. A one-way ANOVA analysis using Duncan's test was performed to identify significant differences between the means ($p < 0.05$). The results are expressed as the mean ± standard deviation (SD). Three different biological replicates were obtained from each measurement.

3. Results

3.1. Effects of Different Concentrations of GR24 on Stem Diameter and Its Growth

The stem diameter of strawberry plants is a key index of their strength and resilience. In this study, the stem diameter of the plants treated with different concentrations of GR24 gradually increased over time. Starting from the day of treatment, the diameter was recorded as DAT 0, which represents day 0 after treatment. At DAT 18, the stem

diameters of the plants treated with 5 µmol/L, 10 µmol/L, and 20 µmol/L GR24 were significantly increased by 1.43 mm, 1.47 mm, and 1.17 mm, respectively, compared to the control. However, there was no significant difference among the different GR24 treatments. At DAT 27, the stem diameters of the plants treated with 5 µmol/L and 10 µmol/L GR24 were significantly larger than that of CK by 1.67 mm and 0.94 mm, respectively. But there was no significant difference between the strawberry stem diameters treated with 20 µmol/L GR24 and the control (Figure 1A). To further understand the growth of the strawberry stem diameter after the treatments, the increase in the stem diameter was counted between each treatment. It was found that the increase in stem diameter under the 5 µmol/L GR24 treatment at DAT 9 was 1.11 mm, which was significantly smaller than that of the other three groups. Between DAT 9 and DAT 18, the increase in the stem diameter under the 5 µmol/L and 20 µmol/L GR24 treatments was 2.17 mm and 1.92 mm, respectively, which was significantly greater than that of CK at 1.07 mm. From DAT 18 to DAT 27, the increase in the plant stem diameter for all four treatments was 0.81 mm, 1.04 mm, 1.10 mm, and 0.93 mm, respectively, which were significantly smaller than the previous two increases, and there was no significant difference between each treatment and the control (Figure 1B). Based on these findings, it can be concluded that GR24 may have a promotion effect on the growth of strawberry stem diameter, especially at concentrations of 5 µmol/L and 10 µmol/L. The 20 µmol/L treatment also had a positive effect, although it was not significantly different from the control.

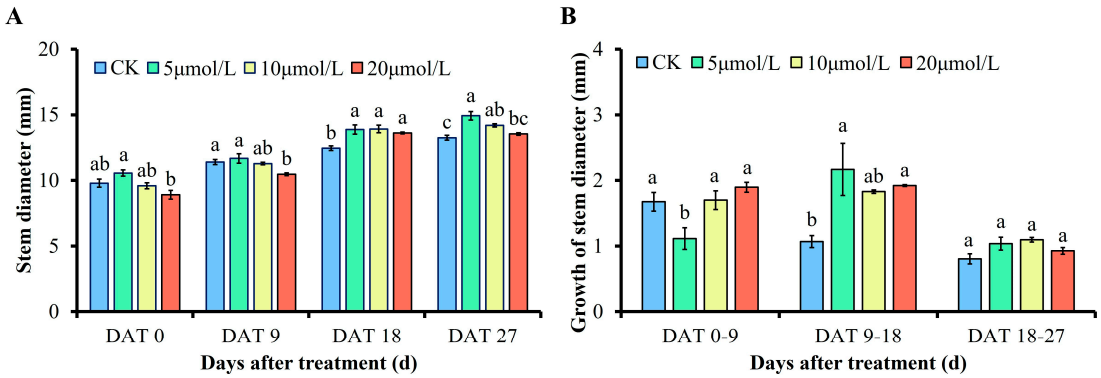

Figure 1. Effect of different concentrations of GR24 on the strawberry stem diameter (**A**) and its growth (**B**). DAT 0: the day when the 1st treatment was administered; DAT 9: the day when the 2nd treatment was administered and also the 9th day after the 1st treatment; DAT 18: the day when the 3rd treatment was administered and also the 9th day after the 2nd treatment; DAT 27: the 9th day after the 3rd treatment. The bars in the graph indicate the standard error (SE) (n = 3). Means followed by different letters are significantly different at $p \leq 0.05$ according to Tukey's multiple test (n = 3). Different letters indicate significant differences ($p < 0.05$). This legend also applies to the table below.

3.2. Effects of Different Concentrations of GR24 on Plant Height, Petiole Length, and Their Growth

Plant height and petiole length are two important indexes reflecting the nutritional growth of strawberry. The plant height and petiole length of strawberry under different concentrations of GR24 increased as the experiment progressed. It was found that the height of strawberry plants under the 5 µmol/L and 10 µmol/L GR24 treatments was significantly higher than that of CK at DAT 9, DAT 18, and DAT 27 (Figure 2A). The increase in the plant height showed that the overall growth rate of the strawberry plant height was the greatest in the first 9 days, with a maximum increase of 2.67 cm and 2.58 cm under the 5 µmol/L and 10 µmol/L GR24 treatments, respectively. However, from DAT 9 to DAT 18, there was no significant difference in the increase in the plant height between the strawberry plants under the three concentrations of GR24 treatments and the control. The obvious increase in the plant height was observed from DAT 18 to DAT 27 under the 10 µmol/L GR24 treatment, reaching 1.9 cm (Figure 2B).

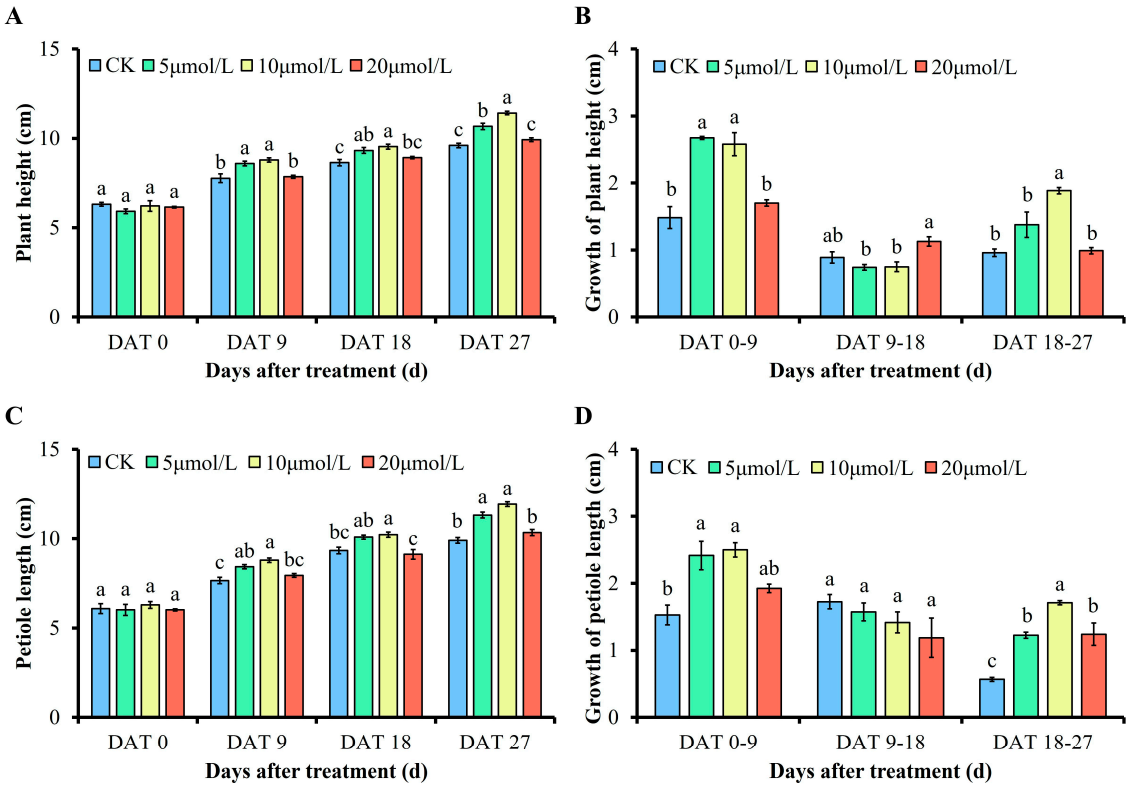

Figure 2. Effect of different concentrations of GR24 on the strawberry plant height (**A**) and its growth (**B**) and the petiole length (**C**) and its growth (**D**). Different letters indicate significant differences ($p < 0.05$).

The results of the petiole length measurement showed that a concentration of 10 μmol/L GR24 was the most effective in promoting the growth of the petiole length in strawberry, and the petiole length in this treatment was significantly higher than that of CK on DAT 9, DAT 18, and DAT 27, with increases of 1.13 cm, 0.88 cm, and 2.03 cm, respectively (Figure 2C). In addition, during the first 9 days, the maximum growth of the petiole length was found under the 5 μmol/L and 10 μmol/L GR24 treatments, with increases of 2.42 cm and 2.50 cm, respectively, which were significantly higher than the 1.53 cm increase in CK. From DAT 9 to DAT18, there was no significant difference in the growth rate of the petiole length between the three treated groups and the CK. However, from DAT 18 to DAT27, the increase in the strawberry petiole length was the highest (1.71 cm) under the treatment with 10 μmol/L GR24, which was significantly higher than that of CK (Figure 2D). Thus, GR24 may have a promotion effect on the growth of the strawberry plant height and petiole, and adding 10 μmol/L GR24 at the early growth stage promoted the increase in the plant height and petiole length more effectively.

3.3. Effect of Different Concentrations of GR24 on Leaf Thickness and Its Growth

We found no significant differences in the leaf thickness of plants in these four treatment groups at any of the four periods, and we subsequently measured the amount of growth in leaf thickness. But there was no significant difference between the treatments at any time (Figure 3). When studying the growth of leaf thickness, it was discovered that the increase in leaf thickness under the 10 μmol/L GR24 treatment was 0.09 mm from DAT 9 to DAT18, which was significantly higher than the 0.06 mm increase in CK (Figure 3). Therefore, the overall effect of GR24 in promoting leaf thickening was not significant, but 10 μmol/L GR24 could significantly promote strawberry leaf thickening in the middle term of the treatment.

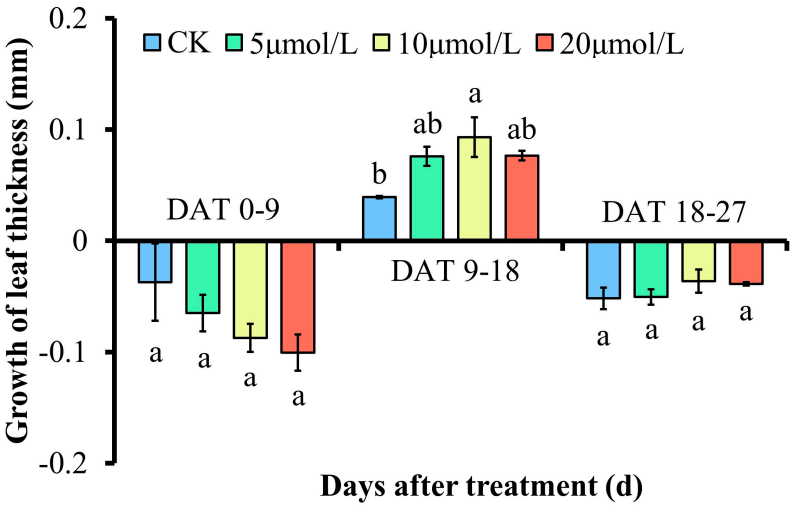

Figure 3. Effect of different concentrations of GR24 on the growth of strawberry leaf thickness. Different letters indicate significant differences ($p < 0.05$).

3.4. Effect of Different Concentrations of GR24 on Strawberry Stolons and Flowering Branches

The stolon is an important index of the vigorous growth of strawberry plants and is closely related to yield. This study found that the number of strawberry stolons gradually increased as the strawberry plants grew. However, the number of strawberry stolons in all three treatment groups was significantly lower than in CK at DAT 9 and DAT 18. By DAT 27, only the number of stolons under the 10 μmol/L GR24 treatment (1.91) was significantly lower than that in CK at 2.38 (Figure 4A). Based on the diameter and number of flowering branches of the plants after the last treatment, it was found that the plants treated with 5 μmol/L GR24 had the best promotion effect on these two indicators (Figure 4B,D). Since the purpose of reducing stolon growth is to increase fruit yield, based on the above results, we decided to use the strawberry plants under the 5 μmol/L GR24 treatment for the subsequent determination of the sugar and phytohormone contents as well as for the transcriptome analysis. Considering the above results, GR24 had a significant inhibitory effect on the stolon sprouting of the strawberry plants.

3.5. Effect of GR24 on the Sugar Content of the Strawberry Crowns

Since soluble sugars have been found to promote strawberry runner production in previous studies [10] 2020, this study also measured the sugar content of the crowns in strawberry plants under the different treatments. Sucrose was not detected in the strawberry crowns, but fructose and glucose were detected (Figure 4C). There was no significant difference in the fructose content between the CK and GR24-treated plants, but the glucose content in the 5 μmol/L GR24-treated plants was significantly lower than that in the plants of CK, accounting for 70% of the glucose content in CK.

3.6. Effect of GR24 on the Content of Endogenous Phytohormones

To systematically study the effect of GR24 on endogenous hormones in strawberry plants, we measured the content of four endogenous phytohormones in the crown. The results show that GR24 significantly promoted the production of tZR and GA_3 (Figure 5). Among them, GR24 most obviously promoted tZR, which was 4.5 times higher than that of CK after the treatment, while GA_3 levels increased by 39.1%. However, the contents of IAA and ABA were not significantly different compared to those of CK. In summary, the exogenous spraying of GR24 can influence the content of endogenous phytohormones in strawberry plants and thus regulate the growth of the plants.

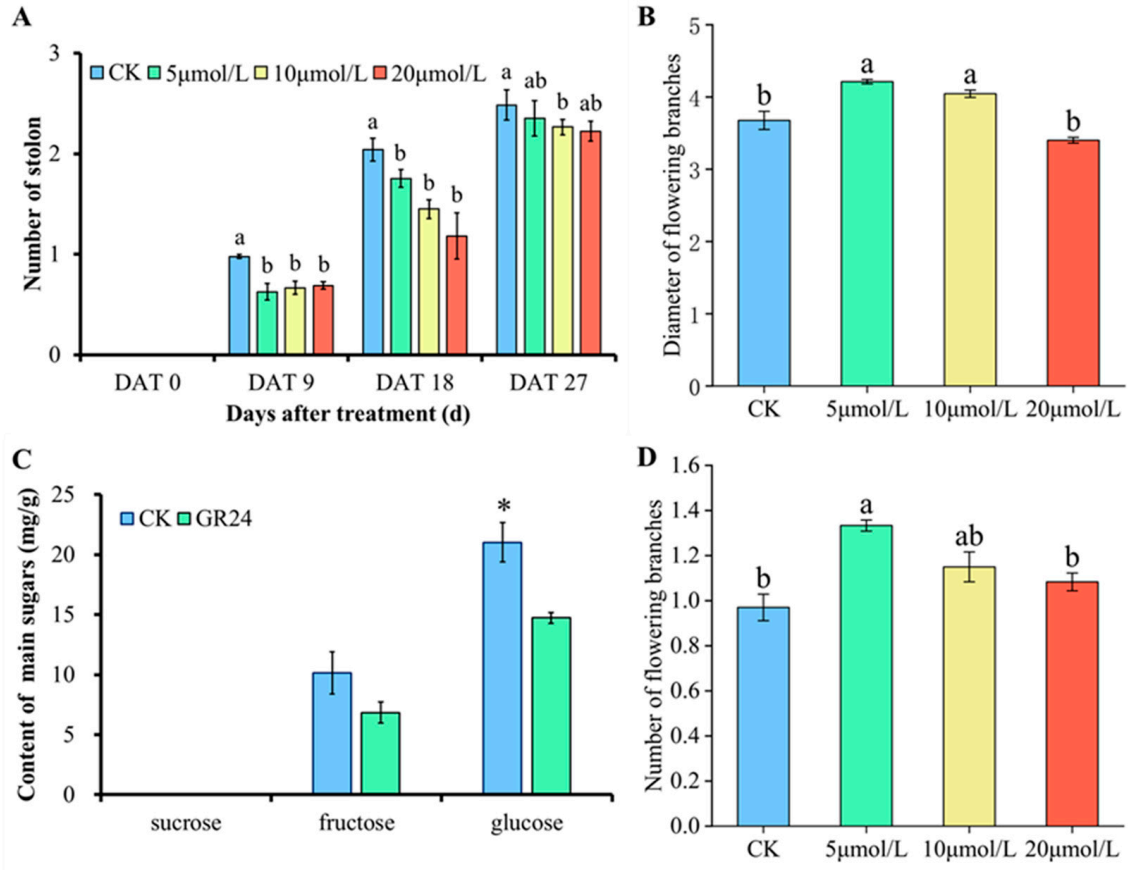

Figure 4. Effect of different concentrations of GR24 on the number of stolons (**A**), the diameter of the flowering branches (**B**), the content of the main sugars (**C**), and the number of the flowering branches (**D**). Different letters and * indicate significant differences ($p < 0.05$).

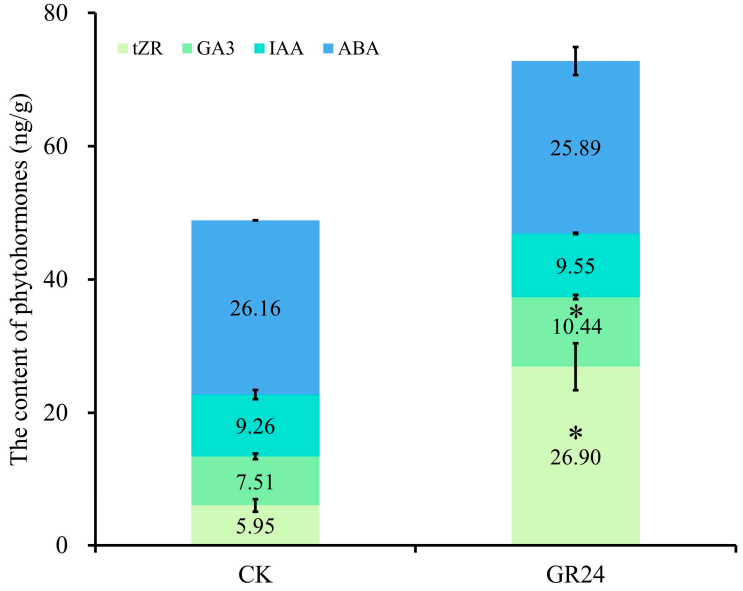

Figure 5. Effect of GR24 on the content of the endogenous phytohormones of strawberry plants. Comparison of the tZR content, GA_3 content, IAA content, and ABA content in CK and the strawberry plants treated with 5 μmol/L GR24 on the 9th day after the 3rd treatment. The * indicates statistically significant differences ($p \leq 0.05$, Student's *t*-test).

3.7. Transcriptome Analysis of the Cytokinin Anabolic and Signaling Pathways

Since higher levels of tZR and GA_3 were detected in the plants treated with 5 µmol/L GR24 (Figure 5), we sequenced and analyzed the transcriptome of crowns from both groups of materials to investigate the underlying molecular mechanisms. The results of the differential gene expression analysis of the CTK anabolic pathway as well as its signaling pathway (Figure 6) showed that *CKX* (FxaC_4g32960, FxaC_2g04210, FxaC_2g12570, FxaC_3g07760, FxaC_7g41590, FxaC_8g06620, and FxaC_6g42650), *ZOG* (FxaC_23g16160 and FxaC_24g30610), and *UGT76C2* (FxaC_26g11300), the key enzymes involved in CTK degradation, were significantly down-regulated in the GR24-treated plants. At the same time, the rate-limiting enzyme for cytokinin synthesis, *IPT* (FxaC_5g12240), and the key enzyme for CTK synthesis, *CYP735A* (FxaC_21g11720, FxaC_22g10010, FxaC_23g54500, and FxaC_24g54290), were significantly up-regulated. It is thus speculated that the significant increase in the tZR content in the GR24-treated plants may be attributed to the inhibition of the degradation pathway and the enhancement of CTK synthesis, leading to the accumulation of tZR in plants.

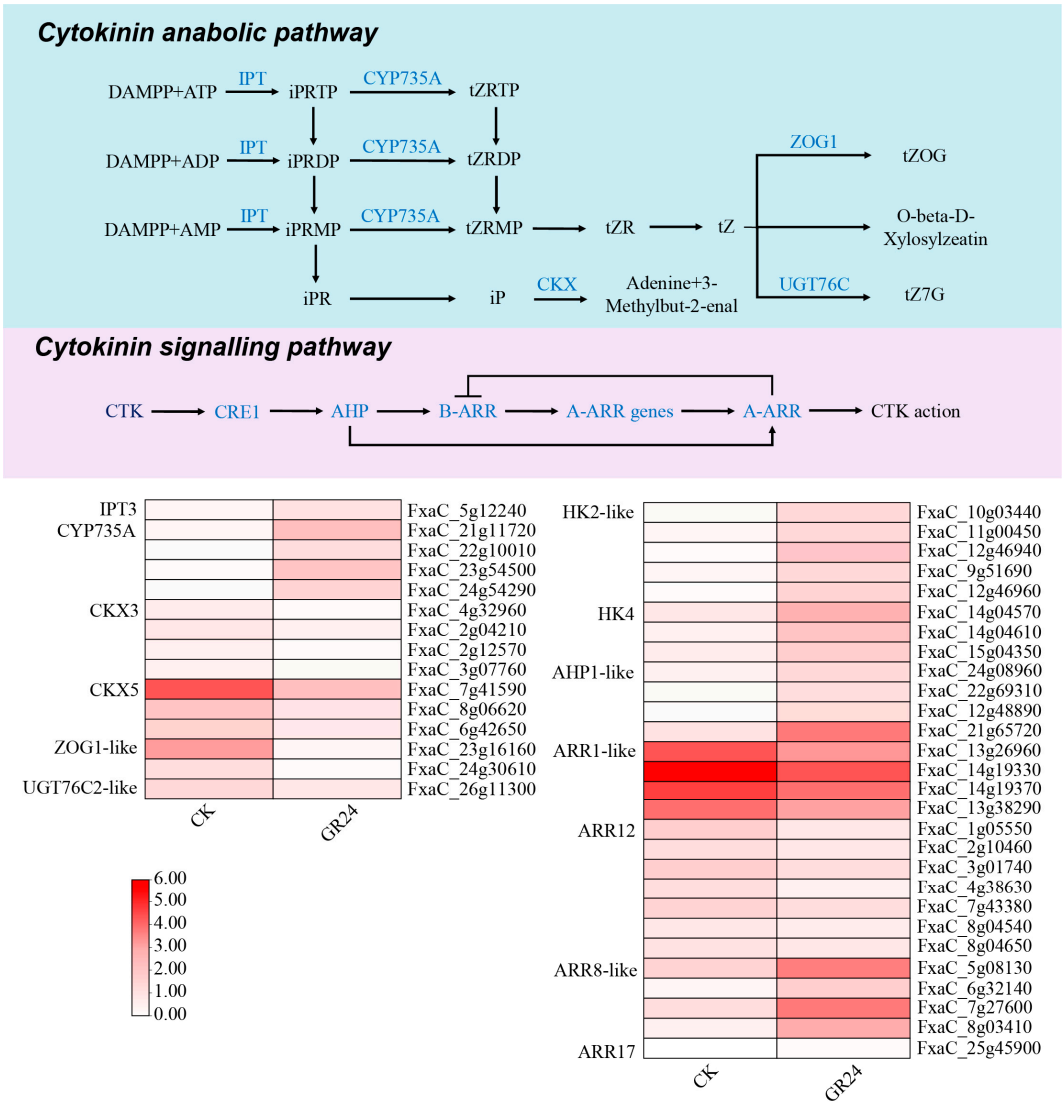

Figure 6. Transcriptome analysis of the cytokinin anabolic and signaling pathways. IPT, isopentenyl transferase; CYP735A, cytochrome P450 monooxygenase 735A; CKX, cytokinin oxidase; ZOG1,

zeatin O-glucosyltransferase; UGT76C, UDP-glycosyltransferase 76C2; CRE1, histidine kinase; AHP, histidine-containing phosphotransfer protein; B-ARR, B-type Arabidopsis response regulator; A-ARR, A-type Arabidopsis response regulator. For the gene expression comparison, the log_2-transformed CPM (counts per million) values were presented as a heatmap, with CK on the left and the material treated with 5 μmol/L GR24 on the right. Substances labeled in blue indicate that they were differentially expressed in CK and 5 μmol/L GR24-treated plants. The expression level gradually increases from white to red. The legend also applied to the figure below.

The CTK signaling pathway is a dual signaling system with the following core steps: the transmembrane protein CRE1 serves as a receptor for cytokinin from the extracellular environment, which are then transferred to AHP. The AHP proteins then transfer phosphate groups to response regulator (ARR) proteins and functions. B-ARR in the system is a v-myb avian myeloblastosis viral oncogene homolog (MYB) transcription factor that is activated upon phosphorylation and promotes the expression of downstream genes to regulate the growth and development of the plant. In addition, B-ARR can activate the transcription of A-ARR, while A-ARR can inhibit the activity of B-ARR, forming a negative regulatory network. In this study, the transcriptional results show that the expression of *CRE1* (FxaC_10g03440, FxaC_11g00450, FxaC_12g46940, FxaC_9g51690, FxaC_12g46960, FxaC_14g04570, FxaC_14g04610, and FxaC_15g04350) and *AHP* (FxaC_24g08960, FxaC_22g69310, FxaC_12g48890, and FxaC_21g65720) was significantly up-regulated in the GR24-treated plants. Conversely, the transcription factor *B-ARR* (FxaC_13g26960, FxaC_14g19330, FxaC_14g19370, FxaC_13g38290, FxaC_1g05550, FxaC_2g10460, FxaC_3g01740, 4g38630, FxaC_7g43380, FxaC_8g04540, and FxaC_8g04650) was significantly down-regulated and its repressor *A-ARR* (FxaC_5g08130, FxaC_6g32140, FxaC_7g27600, FxaC_8g03410, and FxaC_25g45900) was significantly up-regulated. This suggests that the signal transduction of CTK may be weakened in response to the suppression of B-ARR.

3.8. Transcriptome Analysis of the GA Anabolic and Signaling Pathways

A total of 25 differentially expressed transcripts directly related to the GA anabolic pathway were detected from the transcriptome data (Figure 7). *CPS* (FxaC_6g28150), *KS* (FxaC_9g40940, FxaC_11g13021, FxaC_9g41050, and FxaC_11g13020), and *KAO* (FxaC_28g30380 and FxaC_26g31590) are genes involved in the synthesis of GA_{12}-aldehyde from geranylgeranylpyrophosphate (GGPP), whose expression levels were significantly up-regulated in the GR24-treated plants. The conversion of GA_{12}-aldehyde to differentially structured GAs is a complex oxidation process and the expression of the key gene *GA3ox* (FxaC_2g07540 and FxaC_22g32160) involved in this process was significantly up-regulated in the GR24-treated plants compared to the control. In contrast, GA2ox, which is involved in the inactivation of GA, was significantly down-regulated in the GR24-treated plants. Among the 16 differentially expressed transcripts, the expression of seven differentially coding genes in the GR24-treated plants was significantly down-regulated by more than 10 times compared to the control. To sum up, these finding suggest that the GR24 treatment enhanced the expression of genes encoding key enzymes in the GA biosynthesis pathway, while significantly inhibiting the expression of genes encoding key enzymes in the GA metabolism pathway, leading to an increase in the final GA_3 content in strawberry plants.

In addition, we analyzed the transcriptome of the GA signaling pathway. This pathway is mainly regulated by the GA receptor *GID1*, the GA transduction inhibitor DELLA, and the DELLA degradation complex $SCF^{GID2/SLY1}$. GA first binds to *GID1* and DELLA to form a GA-GID1-DELLA trimer, followed by the ubiquitination modification of the DELLA protein by $SCF^{GID2/SLY1}$, which initiates the degradation of DELLA by the 26s proteasome under the regulation of the transcription factor PIF3/4. This process relieves the repressive effect of DELLA on the downstream genes of the GA signaling pathway and exerts a bioregulatory effect on GA (Figure 7). In this study, the transcriptome data showed that the expression level of the GA receptor *GID1* (FxaC_10g12560, FxaC_11g09750, FxaC_22g63620, FxaC_22g69290, and FxaC_23g38030) was significantly up-regulated in the GR24-treated

plants, and the inhibitory factor DELLA protein (FxaC_14g13060, FxaC_13g20790, and FxaC_15g13990) was significantly down-regulated. The expression levels of four DEGs encoding transcription factors *PIF3* and *PIF4* (FxaC_23g34990, FxaC_21g60640, FxaC_24g47710, and FxaC_22g16130) were all more than 3-fold up-regulated in the GR24-treated plants compared to the control. In summary, the analysis revealed an up-regulation in the expression of differential genes encoding both *GID1* and the transcription factors *PIF3/4*, indicating an increase in the binding of GA to GID1 and the subsequent ubiquitination and degradation of DELLA protein. This further suggests that the inhibitory effect of the DELLA protein on the GA signaling pathway was diminished, leading to an enhanced GA signal.

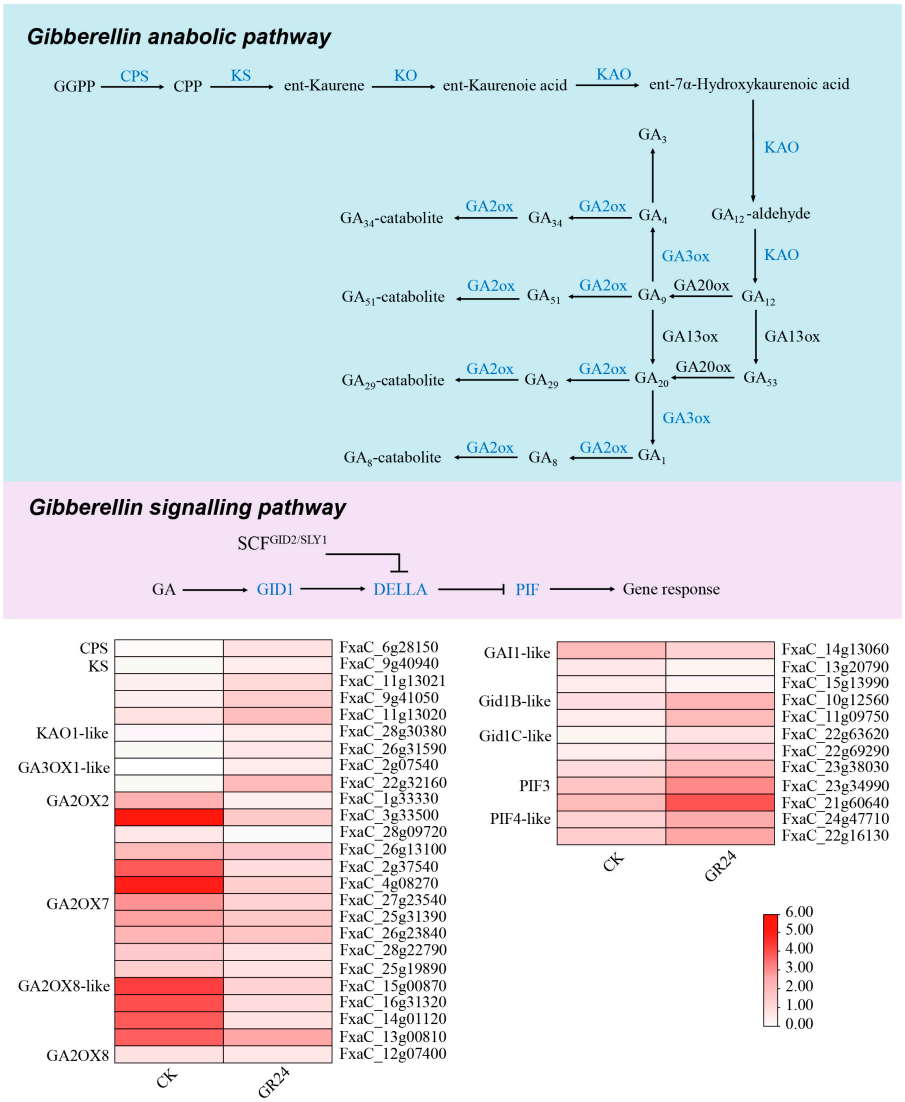

Figure 7. Transcriptome analysis of the GA anabolic and signaling pathways. The anabolic pathway of GA is divided into 3 steps: the synthesis of ent-kaurene by GGPP, the synthesis of GA_{12}-aldehyde from ent-kaurene, and the oxidation of GA_{12}-aldehyde to gibberellins with different structures. CPS, ent-copalyl diphosphate synthase; KS, ent-kaurene synthase; KAO, ent-kaurenoic acid oxidase; GA20ox, gibberellin 20 oxidase; GA3ox, gibberellin 3-beta-dioxygenase; GA2ox, gibberellin 2-beta-dioxygenase; GID1, gibberellin insensitive dwarf1; SCF, skp1-cullin-F-box protein; PIF, phytochrome-interacting factor.

3.9. Transcriptome Analysis of the Auxin Signaling Pathway

Since the contents of IAA and ABA were not significantly different between the GR24-treated plants and the control, and IAA and ABA were closely related to strawberry stolon sprouting [33,34], this experiment focused on the transcriptome analysis of the IAA and ABA signaling pathways. In the auxin signaling pathway (Figure 8), IAA binds to the auxin input carrier AUX1 and then the complex SCF^{TIR1}. This complex then modifies the inhibitory factor AUX/IAA through ubiquitination, leading to the release of the transcription factor ARF that it inhibits, and subsequently activating downstream response elements. The transcriptome results show that, after the GR24 treatment, the expression levels of auxin 1 (*AUX1*) (FxaC_21g55080, FxaC_21g56310, 22g56080, and FxaC_23g31230) and *ARF* (FxaC_7g41100, FxaC_17g12650, FxaC_17g16080, FxaC_19g11690, FxaC_27g35540, FxaC_27g31570, FxaC_28g16990, and FxaC_26g18090) were up-regulated. Additionally, the inhibitor *AUX/IAA* was also significantly up-regulated, thereby inhibiting the transmission of the auxin signal.

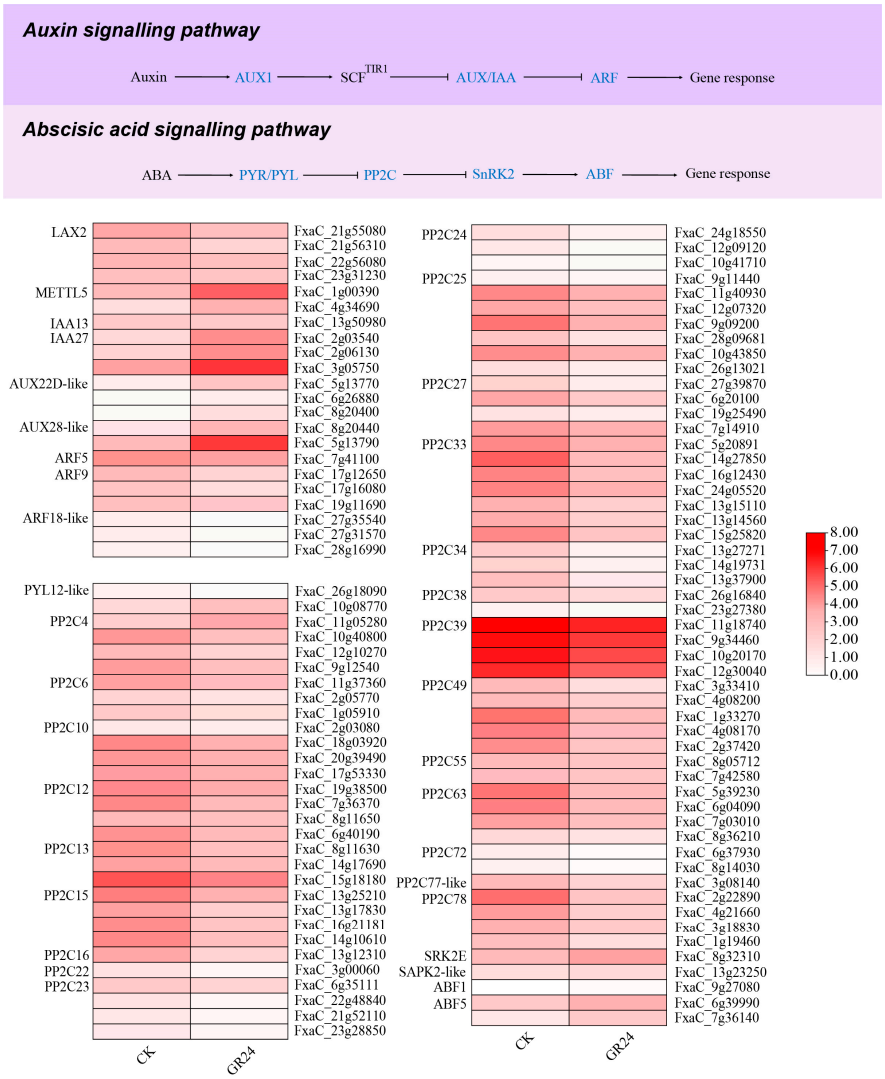

Figure 8. Transcriptome analysis of the auxin signaling pathway and the ABA signaling pathway. AUX/IAA, auxin/indole-3-acetic acid; ARF, auxin response factor; PYR/PYL, pyrabactin resistance 1/pyrabactin resistance 1-like; PP2C, protein phosphatase 2C; SnRK2, sucrose non-fermenting 1-related protein kinase 2; ABF, ABRE-binding factors.

3.10. Transcriptome Analysis of the ABA Signaling Pathway

In the ABA signaling pathway (Figure 8), PYL acts as an ABA receptor and regulates a series of downstream reactions. PP2C is also a key protein in the ABA signaling pathway, and PYR/PYL controls the ABA signal by inhibiting PP2C. The results of this study show that the GR24 treatment significantly up-regulated *PYL* (FxaC_10g08770 and FxaC_11g05280) expression while down-regulating *PP2C* expression. Additionally, the expression levels of *SnRK2* (FxaC_13g23250 and FxaC_8g32310) and the transcription factor *ABF* (FxaC_9g27080, FxaC_6g39990, and FxaC_7g36140) were also significantly up-regulated in the GR24-treated plants. In summary, the GR24 treatment may suppress ABA signal transduction by up-regulating the inhibitory factor *PYL*, which in turn inhibits *PP2C*.

3.11. Transcriptome Analysis of the Sugar Metabolism Pathway

The sugar metabolism pathway is divided into two parts: soluble sugar transformation metabolism and starch synthesis and metabolism in plastids. The metabolism of soluble sugars mainly involves the interconversion of sucrose, fructose, glucose, and trehalose. These monosaccharides are then further metabolized through glycolysis. Glucose-6-phosphate (D-glucose-6P) is converted into glucose 1-phosphate (D-glucose-1P) under the catalysis of glucose phosphate mutase (PGM), and then is polymerized to form starch under the catalysis of a series of enzymes in plastids. Subsequently, starch is broken down to maltose catalyzed by BMY or to glucose catalyzed by amylase (AMY). Maltose can also be further broken down to glucose by α-glucosidase (GAA) (Figure 9). The transcriptome data showed that INV (FxaC_24g24690, FxaC_25g57790, FxaC_25g48110, FxaC_28g06710, FxaC_25g48920, and FxaC_27g43570), involved in the decomposition of sucrose into glucose and fructose, was significantly up-regulated in the GR24-treated plants. On the other hand, TREH (FxaC_19g11430, FxaC_17g12410, and FxaC_20g12180), involved in the conversion of trehalose to glucose, was significantly reduced in the GR24-treated plants. Additionally, HK (FxaC_3g06340, FxaC_22g23850, FxaC_8g07590, FxaC_6g43410, and FxaC_7g40800) and FRK (FxaC_7g21700, FxaC_24g17000, FxaC_15g07820, FxaC_14g07810, FxaC_13g09120, FxaC_16g24240, FxaC_15g28090, FxaC_1g38700, and FxaC_26g01820) were also significantly up-regulated in the GR24-treated plants. And in the plastid, BMY (FxaC_13g49830, FxaC_15g35160, FxaC_16g05110, FxaC_17g12490, FxaC_17g15860, FxaC_18g34720, FxaC_19g11530, FxaC_25g19860, FxaC_25g31350, FxaC_26g23910, FxaC_27g23620, and FxaC_28g22720) participated in the reaction of starch decomposition to maltose, which was significantly down-regulated in the GR24-treated plants. This suggests that the decrease in the glucose content in the crowns of the GR24-treated plants may be due to the up-regulation of HK and the down-regulation of TREH and BMY.

3.12. Real-Time Quantitative PCR of the Key Genes

To verify the accuracy of the transcriptome data, we selected 16 key DEGs (FaAHP1-like, FaHK2-like, FaARR1-like, FaLAX2, FaMETTL5, FaAUX28-like, FaIAA8-like, FaIAA9, FaIAA27, FaARF5, FaPP2C10, FaPP2C12, FaPP2C13, FaPP2C27, FaABF1, and FaABF5) involved in the CTK, IAA, and ABA signaling pathways to perform a real-time quantitative PCR (Figure 10). The results show that the expression levels of FaAHP1-like and FaHK2-like in the CTK signaling pathway were significantly increased in the 5 μmol/L GR24-treated plants, while the expression of FaARR1-like was significantly down-regulated (Figure 10A–C). Additionally, the expression of FaMETTL5, FaAUX28-like, FaIAA8-like, FaIAA9, and FaIAA27 in the IAA signaling pathway was significantly up-regulated compared to the control, whereas the expression levels of FaLAX2 and FaARF5 were significantly down-regulated (Figure 10D–J). Among the key genes involved in the ABA signaling pathway, the expression levels of FaABF1 and FaABF5 were significantly higher in the 5 μmol/L GR24-treated plants than that in the control, while the expression levels of FaPP2C10, FaPP2C12, FaPP2C13, and FaPP2C27 were significantly lower in the 5 μmol/L GR24-treated plants than in the control (Figure 10K–P). In summary, the real-time quantita-

tive PCR results of these 16 key genes are consistent with transcriptome data, indicating the reliability of the transcriptome data.

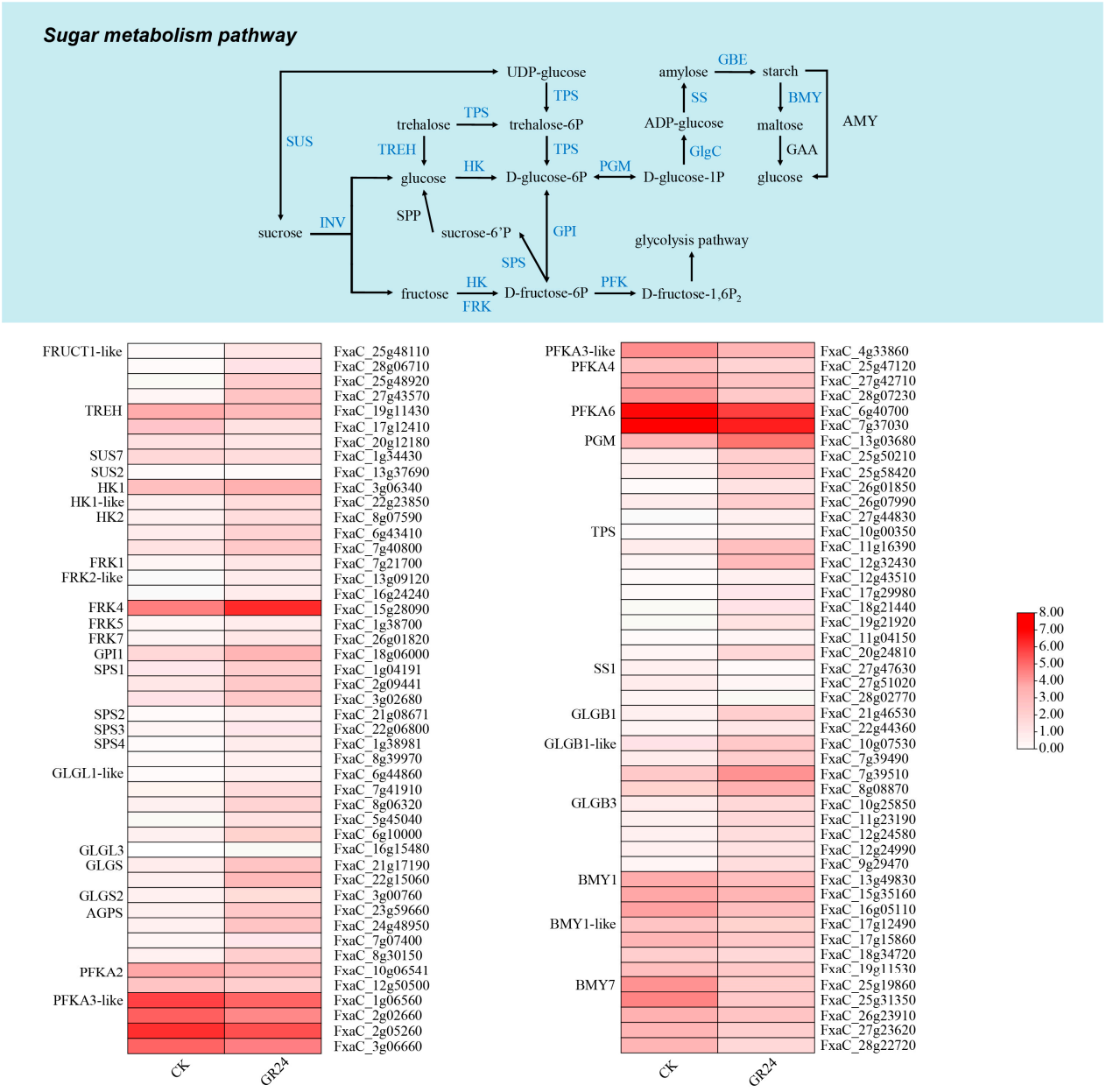

Figure 9. Transcriptome analysis of the sugar metabolism pathway. SUS, sucrose synthase; INV, invertase; TREH, trehalase; TPS, terpene synthase; HK, hexokinase; FRK, fructokinase; SPS, sucrose phosphate synthase; GPI, glucose phosphate isomerase; PGM, phosphoglucomutase; PFK, phosphofructokinase; SS, starch synthase; GlgC, ADP-glucose pyrophosphorylase; GBE, glycogen-branching enzyme; BMY, beta-amylase.

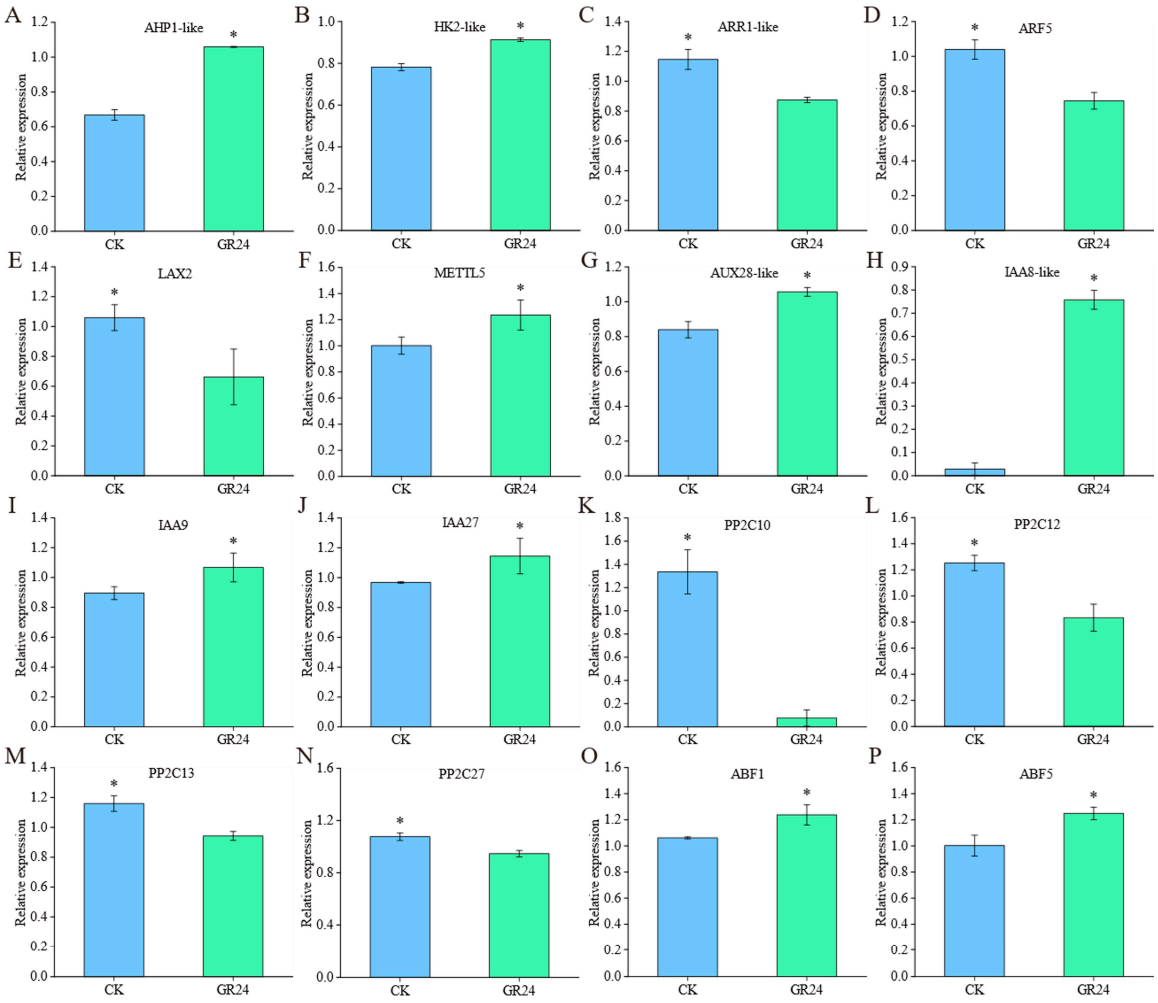

Figure 10. Real-time quantitative PCR analysis of the DEGs of CK and the 5 μmol/L GR24-treated plants. The relative expression levels of *FaAHP1-like* (**A**), *FaHK2-like* (**B**), *FaARR1-like* (**C**), *FaLAX2* (**D**), *FaMETTL5* (**E**), *FaAUX28-like* (**F**), *FaIAA8-like* (**G**), *FaIAA9* (**H**), *FaIAA27* (**I**), *FaARF5* (**J**), *FaPP2C10* (**K**), *FaPP2C12* (**L**), *FaPP2C13* (**M**), *FaPP2C27* (**N**), *FaABF1* (**O**), and *FaABF5* (**P**). The * indicates statistically significant differences ($p \leq 0.05$, Student's *t*-test).

4. Discussion

The occurrence of stolons in strawberries before fruit formation can hinder the allocation of nutrients for reproductive growth, resulting in a decline in both the yield and quality of strawberry fruits. In this study, we investigated the effects of spraying different concentrations of the SL analogue GR24 on the strawberry crown. We found that GR24 not only promoted the growth of strawberry plants but also effectively inhibited the sprouting of stolons. A further study revealed that GR24 inhibited the growth of the stolons by affecting the accumulation of soluble sugars and the signaling pathways of phytohormones in the strawberry crown.

SL participates in plant morphogenesis. Previous studies have shown that SL signaling in the vascular cortex can stimulate cortex growth and promote stem thickening in *Arabidopsis* [35]. SL can also promote the thickening of stems during maize (*Zea mays* L.) development [36]. In the present study, we found that the exogenous GR24 treatment at different concentrations (5 μmol/L, 10 μmol/L, and 20 μmol/L) promoted stem thickening in strawberry plants (Figure 1), which was consistent with previous research. In addition, after knocking out the SL receptor gene *dwarf14* in *Arabidopsis*, the plants showed dwarfing and shortened petioles [37]. In our experiment, both the 5 μmol/L and 10 μmol/L GR24 treatments promoted plant height (Figure 2A,B) and petiole length in strawberry plants

(Figure 2C,D). However, GR24 had no significant effect on the strawberry leaf thickness. In summary, exogenous GR24 promotes stem thickening, plant height, and petiole growth in strawberry plants, leading to an overall growth promotion.

The branches of plants develop from axillary buds (AXBs) in the leaf axils, which in turn are formed by axillary meristems (AMs) under suitable conditions [38,39]. The most prominent effect of SL in plants is to regulate the growth of the above-ground parts and inhibit plant branching. The levels of SL were found to be significantly reduced in the branching mutants of certain plants, but the application of SL inhibited branching in these mutants [40]. The mutants *d14* and *d10* in *Arabidopsis* exhibited increased branching and reduced plant height, while exogenous SL effectively inhibited tillering in the *d10* mutant [41]. Exogenous GR24 has been found to effectively inhibit the occurrence of secondary branches in rapeseed (*Brassica napus* L.) and improve the quality and yield of siliques [42]. In this study, exogenous GR24 effectively reduced the number of stolons in the strawberry plants (Figure 4), which is consistent with the results of previous studies.

Many studies have shown that SL can interact with various endogenous phytohormones in plants to jointly regulate plant growth and development. These phytohormones include CTK, GA, auxin, jasmonic acid, and ABA [43–46]. SL and CTK have opposite effects on axillary bud growth. For example, one study reported that exogenous GR24 inhibited the expression of CTK-synthesis-related genes in non-heading Chinese cabbage (*Brassica campestris ssp.* chinensis Makino). It also inhibited the promotion of axillary shoot growth by the CTK content in peas and increased the expression of the CTK-synthesis-related gene *PsIPT1* in the SL mutants [21,47]. Other studies have also shown that, after 12 h of the exogenous GR24 treatment, the tZR content in rice tillering buds significantly decreased, while the tZR content in the tillering nodes significantly increased [48]. SL can also inhibit the expression of *A-ARR* in rice buds [49]. In addition, the activation of *CRE1* and *ARR5* in the CTK signaling pathway was detected in plants with increased strawberry stolons [38], while the mutation of the B-type *ARR1* in *Arabidopsis* led to increased branching [50]. In this study, we found that the exogenous GR24 treatment significantly increased the tZR content and the expression of *IPT* and *CYP735A* in the CTK synthesis pathway in strawberry crowns (Figure 5). However, it significantly inhibited the expression of *CKX*, *ZOG*, and *UGT73C1* in the CTK metabolic pathways, resulting in an enhanced CTK synthesis and lowered metabolism (Figure 6). Additionally, the exogenous GR24 treatment activated the expression of *CRE1* and *AHP*, leading to the activation of *A-ARR* and the suppression of *B-ARR* in the CTK signaling pathway, thereby inhibiting this pathway (Figure 6). Therefore, we believe that, although exogenous GR24 promoted the accumulation of CTK in the strawberry crowns, it significantly inhibited the CTK signaling pathway. As a result, the development of strawberry axillary buds was hindered, and the sprouting of stolons was reduced.

GA has different effects on branching in different species. It can negatively regulate branching in pea and *Arabidopsis*, but can positively regulate the growth of axillary buds in perennial strawberry and jatropha (*Jatropha curcas* L.) [43,51–53]. GA20ox4 is responsible for the production of GA. The mutation of GA20ox4 results in fewer stolons in *F.vesca*, but this GA20ox4 can be rescued by the application of exogenous GA_3 [54]. In the present experiment, exogenous GR24 significantly increased the content of GA_3 in the strawberry crown (Figure 5), but the numbers of stolons were significantly inhibited (Figure 4). The further analysis of the transcriptome data revealed that exogenous GR24 induced the expression of *CPS*, *KS*, and *GA3ox* in the gibberellin synthesis pathway, while inhibiting the expression of *GA2ox* in the gibberellin metabolic pathway (Figure 7). In addition, the decreased expression of the repressor DELLA protein led to an enhanced GA signaling pathway (Figure 7). However, contrary to previous studies, the enhanced metabolism and signaling pathways did not promote strawberry tillering in this experiment. Thus, we suggest that GA_3 may not be the main factor that caused a decrease in the number of stolons in the GR24-treated plants.

IAA signal transduction is an important segment of IAA affecting plant growth and development. Studies have shown that it is the main signaling pathway for auxin in plants, that the signal passes through $SCF^{TIR1/AFB}$ receptors, and the ubiquitination degradation of AUX/IAA proteins; ultimately, it regulates the downstream gene expression through the ARF family of transcription factors [55]. Multiple studies have shown the significance of AUX/IAA in the lateral branches' growth in plants. For instance, the down-regulation of *SlIAA2*, *SlIAA4*, *SlIAA7*, and *SlIAA9* in transgenic tomatoes (*Solanum lycopersicum*) with multi-branching has been observed, while the overexpression of *ARF* transcription factors in eggplants (*Solanum melongena* L.) has been found to promote branching [56,57]. In the present study, although exogenous GR24 did not cause a significant difference in the content of IAA in the strawberry crowns, it did lead to a down-regulation of the *LAX2* gene, which encodes the IAA receptors in the IAA signaling pathway. Additionally, it promoted the expression of the inhibitory factor AUX/IAA, while inhibiting the transcription of the transcription factor *ARF* at the same time (Figure 8). Therefore, exogenous GR24 may inhibit the IAA signaling pathway by up-regulating *AUX/IAA* and down-regulating *ARF*, inhibiting strawberry tillering as a result.

ABA is a significant phytohormone regulating axillary bud growth and development. Previous studies have shown that ABA inhibits the growth of axillary buds in plants such as *Arabidopsis* and sugarcane (*Saccharum officinarum* L.) [58,59]. The ABA receptor PYR/PYL senses ABA and undergoes a conformational change. After interacting with PP2C, it can completely cover up the active site of PP2C phosphatase, thus activating SnRK2s and transmitting signals to the downstream transcription factors ABFs [60]. The overexpression of *SlTCP26* in tomatoes led to an increase in branching and the strong inhibition of the genes *SlSAPK2*, *SlSAPK3*, *SlSnRK2C*, and *SlSnRK2I* encoding the core component SnRK2 in the ABA signaling pathway. Meanwhile, the key gene *SlABF4* in the ABA signaling response was also strongly inhibited [61]. The down-regulation of *PYR/PYL* and the up-regulation of *PP2C* were detected in the apple multi-branch mutant, resulting in the inhibition of ABA signal transduction [62]. The exogenous cyclanilide treatment promoted apple branching, and the ABA signal responsive protein PP2C was down-regulated 168 h after the treatment [63]. In this experiment, although exogenous GR24 did not affect the ABA content in the strawberry crowns, it up-regulated the expression of *PYR/PYL*, *SnRK2*, and *ABF* and down-regulated the expression of *PP2C* (Figure 8). Therefore, we suggest that exogenous GR24 may promote ABA signal transduction by promoting the expression of *SnRK2* and *ABF*, which are key factors in the ABA signaling pathway, ultimately leading to the inhibition of strawberry plant branching.

In plants, sugar can serve as both a source of energy and a signal factor for regulating plant branching. Increasing the supply of sugar to the entire plant or explant buds can stimulate bud growth. However, dormant buds exhibit transcriptional characteristics similar to those observed during carbon starvation [64,65]. In rice, sucrose is hydrolyzed into glucose and fructose, promoting the elongation of rhizomes and the germination of basal axillary buds [66]. In this study, after being treated with exogenous GR24, a decrease in the fructose and glucose contents was detected in the strawberry crowns (Figure 4). The transcriptomic data showed that the expression of *INV*, which regulates the conversion of sucrose into fructose and glucose, was up-regulated, while the expression levels of *HK* and *FRK*, which regulate the degradation of these two monosaccharides, were also significantly up-regulated. In addition, the expression of *TREH*, which is involved in the conversion of trehalose into glucose, was significantly down-regulated. Moreover, the expression of *BMY*, which regulates the breakdown of starch into maltose in plastids, was also significantly down-regulated (Figure 9). And it is universally acknowledged that maltose is a disaccharide composed of two glucoses molecules. To sum up, we propose that exogenous GR24 may inhibit the transformation of trehalose into glucose and starch into maltose by promoting the decomposition of fructose and glucose, thus reducing the content of soluble sugar in the strawberry crowns and inhibiting strawberry tillering.

5. Conclusions

Our study suggests that exogenous GR24 can promote the robust growth of strawberry plants and inhibit tillering by affecting phytohormone signaling and the sugar metabolism pathway in the crowns. Therefore, we proposed a model in which exogenous GR24 may inhibit the germination of axillary buds and reduce tillering in strawberry plants by inhibiting the CTK and IAA signaling pathways, affecting sugar metabolism, and promoting the ABA signal transduction pathway (Figure 11). And the signaling pathways of auxin and cytokinin may play a synergistic role in affecting strawberry plant tillering. Their weakened signal led to a weakened nutritional growth and reduced tillering, while the abscisic acid signaling pathway antagonized both. These findings not only provide new technical strategies for properly controlling stolon formation during strawberry cultivation, but also provide valuable insights into the mechanism of tiller sprouting.

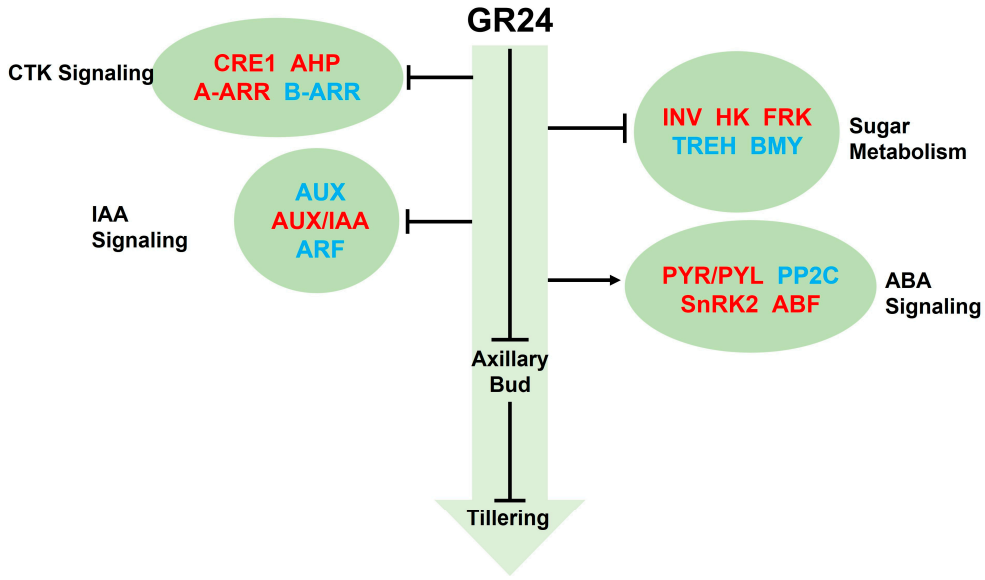

Figure 11. The model of exogenous GR24 influencing the tillering of strawberry plants. The up-regulated substances are reddened, and the down-regulated substances are marked in blue. GR24 up-regulated CRE1, AHP, and A-ARR and suppressed the expression of B-ARR, thereby inhibiting the CTK signaling transduction pathway. The IAA signaling pathway was also significantly inhibited, with AUX and ARF being repressed and AUX/IAA being up-regulated. In addition, GR24 also up-regulated INV, HK, and FRK; down-regulated TREH and BMY; and reduced the content of soluble sugars in the crown of strawberries. ABA inhibited plant tillering, and in this experiment, and its signaling transduction pathway was promoted. Among them, PYR/PYL, SnRK2, and ABF were up-regulated and PP2C was down-regulated. By acting on these four pathways, exogenous GR24 regulated the growth and development of axillary buds and reduced tillering in strawberries.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agronomy13123078/s1. Table S1: Gene quantitative primers.

Author Contributions: Conceptualization, Y.L. (Ya Luo) and Y.P.; methodology, Y.J.; software, M.S.; validation, Y.J. and C.H.; formal analysis, M.L. and Q.C.; investigation, Y.P. and Y.J.; resources, Y.Z. (Yong Zhang), Y.L. (Yuanxiu Lin), and Y.Z. (Yunting Zhang); data curation, Y.W.; writing—original draft preparation, Y.P. and Y.J.; writing—review and editing, Y.L. (Ya Luo); visualization, W.H.; supervision, H.T., X.W., and Y.L. (Ya Luo). All authors have read and agreed to the published version of the manuscript.

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Data Availability Statement: The genome sequence data are available from the GDR database (accessed on 10 September 2022). The data presented in the study have been deposited in the NCBI SRA database under the accession number CNP0004398.

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

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