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Effects of Exogenous Trans-Zeatin and Lovastatin on Abortion of Small Seeds in 'Dawuxing' Loquat (*Eriobotrya Japonica* Lindl.)

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Abstract: The small seeds of loquat possess very rich and diverse genetic characteristics which can potentially serve as precious resources for plant breeding. However, they are often aborted during the seed development. Cytokinin, as an important signaling mediator, plays a pivotal regulatory role in seed development. However, the effects of exogenous cytokinin application on the development of loquat seeds are poorly understood. In this study, we analyzed the potential effects of exogenous cytokinin on the abortion of small seeds of loquat. Cytokinin (20 mg/L trans-zeatin) and cytokinin inhibitor (60 mg/L lovastatin) were sprayed on the fruits of 'Dawuxing' loquat during an early stage of fruit expansion. The clean water treatment was used as the control group. The results showed that exogenous trans-zeatin significantly increased the weight of small seeds, the levels of soluble sugar and starch, as well as peroxidase (POD) and superoxide dismutase (SOD) activities. It also promoted a substantial increase in the expression of POD- and SOD-related genes during the process of small seed abortion. Moreover, trans-zeatin treatment significantly increased the content of endogenous trans-zeatin in the small seeds, and this increase in content showed a trend opposite to that of control (CKA). Cytokinin dehydrogenase related genes were found to be down-regulated after trans-zeatin treatment. It was found that exogenous cytokinin inhibitor (lovastatin) treatment could induce the anti-stress reaction in the small seeds during the early stage of treatment by significantly increasing the activities of POD and SOD, and the weight of small seeds at the early stage of treatment was significantly lower than that of the control group, but reverted to the level of the control group during the late stage of the treatment. Therefore, a specific concentration of trans-zeatin treatment can promote the development of small loquat seeds, while cytokinin inhibitor (lovastatin) can significantly inhibit the development of small seeds during the early stage of treatment. In summary, this study reports for the first time that application of exogenous trans-zeatin could effectively promote the development of small loquat seeds by significantly increasing the metabolism of small seeds. The small seeds which contained rich and diverse genetic characteristics often aborted during seed development. Our study thus established a foundation for the rescue of new germplasm resources of loquat by promoting the development of small loquat seeds.

Keywords: loquat; exogenous trans-zeatin; lovastatin; small seeds; abortion

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1. Introduction

Loquat (*Eriobotrya japonica* Lindl.) is a subtropical fruit tree originating in China [1]. As the fruit matures in the off-season, and has rich nutritional and medicinal value, it is widely cultivated in the world. The fruit of the loquat usually has five ventricles, each of which contains two seeds [2]. Abortion often occurs during development of the seeds. A number of studies have found that these aborted small seeds possess very rich

genetic diversity [3], and can generate germplasm resources with high-quality traits such as haploids [4] and resistance to extremely cold conditions [5]. Therefore, it is of great and far-reaching significance to promote the development of the small seeds of loquat for the breeding of novel varieties and germplasm.

Cytokinin, as an important signaling mediator, can play a significant regulatory role in the process of seed development [6]. It can affect the substantial accumulation of assimilates during seed development. When developing soybean pods were treated with Kinetin (KT), Dai Yuling et al. [7] found that a specific concentration of KT could effectively promote the unloading of photoassimilate from the seed coat and cause the distribution of photoassimilate to cotyledon. It was found to be especially beneficial for the active absorption of hexose by the embryo. Cytokinins have also been reported to regulate sucrase expression and hexose transport in *Arabidopsis thaliana* [8]. A few studies have indicated that cytokinins may mobilize carbohydrate transport by inducing an increased expression of extracellular transferase, sucrose transferase and vacuolar transferase [6,9]. In addition, cytokinins can also play an important role in regulating grain storage capacity [10,11]. Cytokinins have also been reported to be involved in cell division during endosperm development. For instance, during the seed development of maize, wheat and rice, the period of the highest cytokinin content was also observed during the period of the fastest seed differentiation and endosperm development [12–15]. A number of studies have also proved that high levels of cytokinins in seeds were consistent with augmented levels of cell division [16].

Additionally, it has been reported that exogenous cytokinin treatment can significantly increase grain yield under certain conditions, especially in legumes and cereal plants it has been found that cytokinin is the limiting factor in regulating the number and size of the grain bank [10,17]. Nagel et al. [18] treated the inflorescence tissues of soybeans [*Glycine max* (L.) Merr.] cultivated in greenhouse with 6-Benzylaminopurine(6-BA), and the results showed that application of 5×10^{-7} mol of 6-BA significantly increased pod number, seed number per pod and total seed weight per plant by 58%, 62% and 79%, respectively, as compared with non-application of 6-BA. Atkins and Pigeaire (1993) [19] also found that application of exogenous cytokinins prevented the abscission of fruits and promoted rapid seed development.

However, previous studies related to the application of exogenous hormone treatment on the development of loquat seeds have mostly focused on the effect of Gibberellin A3 (GA3) and N-(2-chlor-4-pyridy1)-N′-phenylurea (CPPU) treatment on loquat seed germination [20–22]. The potential effects of exogenous cytokinin (trans-zeatin) treatment on loquat seeds have not been reported, and the underlying mechanism was also unknown until now. Trans-zeatin (tZ), an isoprenoid cytokinin, is commonly found in higher plants. It has been reported to play a central physiological role because of its abundant occurrence and high activity as observed in different bioassays [23]. In this study, the potential effects of exogenous trans-zeatin and lovastatin (cytokinin inhibitor) on abortion of small seeds in loquat were studied to provide an important theoretical basis for loquat breeding from the small seeds.

2. Materials and Methods

2.1. Plant Materials and Treatment Conditions

A pre-experiment was conducted first, to screen the suitable concentration of exogenous hormones, in Shimian County, Sichuan Province, China. We found that 20 mg/L trans-zeatin (tZ) and 60 mg/L lovastatin(L) could effectively promote or suppress the development of small seeds, respectively. This experiment was carried out from 16: 00–18: 00 on a sunny and windless day in which the temperature ranged from 21–24 °C in March 2019. The fruits of the 'Dawuxing' were sprayed with 20 mg/L trans-zeatin (CAS:1637-39-4), 60 mg/L lovastatin (CAS: 75330-75-5) and clean water (CK) three times, once a day. The hormones were sprayed onto the surface of the fruit until they dropped from the surface to the ground. Fruits were bagged in Kraft paper bags on the tree after being sprayed with

hormones. Three of the trees at the same growth status were selected for each treatment regimen. Each tree with 200 fruits and at the same maturity level was treated and each treatment group was treated in three identical replicates. The fruits of the control and treatment groups were collected every five days. After sampling, the residual of trans-zeatin and lovastatin on the surface of the fruits was washed off, the stem of the fruit was removed, and the various morphological indexes of 50 fruits on each tree were measured, including the vertical and horizontal diameter, weight of a single fruit and the weight of the seed. The seeds at specific development stages were observed and collected. The seeds with complete embryos were considered as normal, while those without embryos or with embryo development malformation were considered as small aborted seeds. The samples were divided into four different groups, CKN: the group of normal seeds with clean water treatment; CKA: the group of small aborted seeds with clean water treatment; tZA and LA: the group of small aborted seeds treated with 20 mg/L trans-zeatin and 60 mg/L lovastatin, respectively. They were weighed and immediately frozen in liquid nitrogen and cryopreserved at −80 °C.

2.2. Extraction and Determination of Soluble Sugar

The calibration curve preparation: 0–1 mL of 100 mg/L standard sucrose solution (prepared using 100 mg of sucrose dissolved with 1 L distilled water) was added in a test tube. The volume of solution in the test tube was adjusted to 2 mL with distilled water. Then, 0.5 mL anthrone-ethyl acetate reagent (prepared using 1 g of anthrone dissolved in 50 mL ethyl acetate) was added to each tube, and then 5 mL of concentrated sulfuric acid was slowly added. The tubes were then vigorously shaken several times, and immediately placed in a water bath for 1 min at 100 °C. After cooling, the absorbance of the solutions in each tube was measured at 630 nm. The content of sucrose was plotted against the corresponding absorbance, resulting in a standard curve used to determine the soluble sugar in unknown samples.

Extraction and determination of soluble sugar: 0.3 g (W_1) samples were ground, and then 10 mL distilled water was added. The seeds were thereafter placed in the water bath at 100 °C for 30 min (twice), and then filtrated and diluted with distilled water to a volume of 25 mL (V_{T1}). Afterwards, 0–1 mL (V_{S1}) of aliquot of extract were put into 20 mL graduated test tubes (three times repetitions). It would be diluted with distilled water before determination if the sugar content was too high. The volume of solution in the test tube was adjusted to 2 mL with distilled water. Then 0.5 mL anthrone-ethyl acetate reagent was added to each tube, and then 5 mL of concentrated sulfuric acid was slowly added. The tubes were then vigorously shaken several times, and immediately placed in a water bath for 1 min at 100 °C. After cooling, the absorbance of the sample was measured at 630 nm to calculate the soluble sugar content [24]. The soluble sugar content (%) = ($C_1 \times V_{T1} \times N_1$) $\times$ 100%/($W_1 \times V_{S1} \times 10^{6)}$. Where C_1 (μg) was the content of soluble sugar calculated by standard curve, and N_1 was the dilution ratio of supernatant before determination.

2.3. Extraction and Determination of Starch

The method of calibration curve preparation for determination of starch was the same as the method described in calibration curve preparation for soluble sugar. A 100μg/mL standard starch solution was used as the standard reagent for the determination of the calibration curves. The starch was dissolved in distilled water by placing it in the water bath at 100 °C for 30 min.

Extraction and determination of starch: 0.5 g (W_2) samples were ground, and then 50 mL distilled water was added into it. The solid residues were transferred to a 15 mL centrifuge tube and, after centrifugation, 10 mL hot distilled water was added into it. 2 mL of 9.2 mol/L perchloric acid was added after that, and then the extraction was placed in the water bath at 100 °C for 15 min. After centrifugation, the supernatant was diluted with distilled water to a volume of 50 mL (V_{T2}). The contents of starch were thereafter determined based on anthrone–H_2SO_4 colorimetry as described previously [25]. Each

sample was determined with three replicates. The starch content (%) = ($C_2 \times V_{T2} \times N_2$) $\times$ 100% $\times$ 0.9/($W_2 \times V_{S2} \times 10^6$). Where C_2 (μg) was the content of starch calculated by standard curve, V_{S2} (mL) was the volume of determined supernatant, and N_2 was the dilution ratio of supernatant before determination.

2.4. Extraction and Determination of Soluble Protein

The calibration curve preparation: 0–1 mL of 100 μg/mL Bovine serum albumin reagent (prepared using 100 mg of Bovine serum albumin dissolved in 1 L distilled water) was added to a test tube for the determination of the calibration curves. The volume of solution in the test tube was adjusted to 1 mL with distilled water. 5 mL of 0.01% (*w*/*v*) Coomassie brilliant blue G-250 reagent was added to the test tube. The contents were mixed by inversion and incubated for 2 min after which absorbance was measured at 595 nm with the spectrophotometer. The content of protein was plotted against the corresponding absorbance, resulting in a standard curve used to determine the protein in unknown samples.

Extraction and determination of soluble protein: 0.5 g (W_3) samples of seeds were ground in 5 mL distilled water. The homogenate was then centrifuged at 9600× *g* for 20 min, and then the supernatant was diluted with distilled water to a volume of 10 mL (V_{T3}). 0–1 mL (V_{S3}) of supernatant from the samples was placed into a graduated test tube (three repetitions). It was diluted with distilled water before determining if the protein content was too high. The volume in the test tube was adjusted to 1 mL with distilled water, and then 5 mL of 0.01% (*w*/*v*) Coomassie brilliant blue G-250 reagent was added into it. It was incubated for 2 min after which absorbance was measured at 595 nm with the spectrophotometer [26]. The soluble protein content (mg/g) = ($C_3 \times V_{T3} \times N_3$)/($W_3 \times V_{S3} \times 10^3$). Where C_3 (μg) was the content of protein calculated by standard curve, and the N_3 was the dilution ratio of supernatant before determination.

2.5. Extraction and Determination of Trans-Zeatin

About 0.5 g of fine powder of seeds per sample was weighed in a 50-mL centrifuge tube, and then 20 mL of pre-cooled 80% methanol solution was added. The seeds were then extracted overnight at 4 °C. The supernatant was obtained by centrifuge at 16,000× *g* for 15 min at 4 °C. The extraction procedure was repeated twice and the filtrate was collected. The filtrate was evaporated at a constant temperature of 38 °C and incubated at −20 °C for 30 min. After defrosting and centrifugation purification, the solution was re-dissolved with 1.0 mL acetonitrile, ultrasonicated for 30 s, filtered by 0.22 μm membrane. The filtrate was then subjected to analysis by high performance liquid chromatography (HPLC) using an Agilent 1260 Infinity System with an Agilent ZORBAX Eclipse C18 column (150 × 4.6 mm, 5 μm). The chromatographic conditions used were as follows: flow rate: 0.5 mL/min; sample loading: 10 μL; wavelength: 270 nm. The program of mobile phase gradient elution is shown in supplement Table S1. Each sample was measured with three replicates. The unit of trans-zeatin content was expressed as ng/g, which means the content of trans-zeatin(ng) in 1 g sample.

2.6. Extraction and Determination of Antioxidant Enzymes

Extraction: 0.5 g (W) sample was weighed and ground into homogenate with 6 mL of 0.05 mol/L phosphoric acid buffer (pH 7.8) on an ice bath. The supernatant was thereafter collected into a 10 mL centrifuge tube after centrifugation at 9600× *g* for 15 min at 4 °C, and diluted with phosphoric acid buffer to a volume of 10 mL (V).

Activity assays: Each sample was measured with three replicates. The activity of superoxide dismutase (SOD) was determined by the NBT (nitro-blue tetrazolium) method [27]. Briefly, 0.3 mL (V_t) of the enzyme extract was mixed with 1.5 mL of 50 mmol/L phosphate buffer (pH 7.8), 0.3 mL of 130 mmol/L methionine, 0.3 mL of 750 μmol/L nitro-blue tetrazolium (NBT), 0.3 mL of 100 μmol/L EDTA-Na_2 and 0.3 mL of 20 μmol/L riboflavin in a test tube. The reaction was carried out under 4000 lux for 20 min. The blanks and

controls were run in the same manner but without illumination and enzyme, respectively. The absorbance was measured at an absorbance of 560 nm. In this assay, 1 unit of SOD was defined as the amount required to inhibit the photoreduction of NBT by 50%. The SOD activity(U/g) = $(A_{ck} - A_E) \times V/(0.5 \times A_{CK} \times W \times V_t)$. Where A_{ck} was the absorbance of the extract without enzyme at 560 nm and A_E was the absorbance of reaction mixture containing enzyme at 560 nm.

The activity of peroxidase (POD) was determined by the Guaiacol method [28]. The reaction mixture consisted of 50 mL of 50 mmol/L phosphate buffer (pH 7.8), 28 µL guaiacol and 19 µL of 30% H_2O_2. The assay mixture consisted of 2.9 mL of reaction mixture and 0.1 mL of the enzyme extract (V_S) was measured at 470 nm for 3 min at 37 °C for the measurement of enzyme activity. In this assay, 1 unit of POD was defined as absorbance at 470 nm increased 0.01 per minute. The SOD activity(U/(g.min)) = $(C \times V)/(V_S \times W \times 0.01)$. Where C was the variation rate of an enzyme at 470 nm.

Catalase (CAT) activity was determined by the Potassium Permanganate titration method [29]. Briefly, 1.5 mL (V_d) of enzyme solution was added to the test flask. Used as the control was 1.5 mL of inactivate enzyme solution placed in the water bath at 100 °C for 5 min. Added into the flasks were 1.5 mL of 0.1 mol/L H_2O_2, and the flasks were kept in the water bath at 30 °C for 10 min(t), then 1.5 mL of 10% H_2SO_4 was added immediately. The reaction mixture was titrated with 0.1 mol/L potassium permanganate for the measurement of CAT activity. The CAT activity(mg/(g.min)) was expressed as the weight of H_2O_2 (mg) dissolved by 1 g fresh sample in 1 min. A 1 mL 0.1 mol/L potassium permanganate standard solution was equivalent to 1.7 mg H2O2. The CAT activity(mg/(g.min)) = $(A - B) \times V \times 1.7/(W \times V_d \times t)$. Where A(mL) was the volume of potassium permanganate for titration of inactivate enzyme solution. B(mL) was the volume of potassium permanganate for titration of activate enzyme solution.

2.7. Total RNA Extraction, cDNA Synthesis and Relative Expression Analysis

Total RNA was extracted from loquat seeds using an RNAprep Pure Plant Kit (DP441 Tiangen, China). The quality of extracted RNA was detected by 1% agarose gel electrophoresis and its concentration and OD (OD260/OD280) were determined.

The single-stranded cDNAs were synthesized using a PrimeScriptTM RT Reagent Kit with gDNA Eraser (TaKaRa, Japan) and stored at −20 °C. A g DNA-Eraser was used to remove genomic DNA with reaction details as indicated in Table S2. The condition used was as follows: 42 °C for 2 min. After removal of genomic DNA, a reaction system of reverse transcription was performed as described in Table S3. The conditions used were as follows: 37 °C for 15 min; 85 °C for 5 sec; 12 °C for 5 min. The product was stored at −20 °C.

The cDNA of each sample was used as a template for real-time quantitative PCR on a CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, California, USA). The primers used for Real-time quantitative PCR were designed by Primer 5.0 software (Premier, Canada). The gene sequences for qRT-PCR were obtained by searching on the full-length transcriptome database (accession number PRJNA623262; https://www.ncbi.nlm.nih.gov/bioproject/PRJNA623262; accessed on 6 April 2020). Open reading frame analysis was performed on the NCBI ORF Finder, and BLAST alignment was performed on the NCBI to determine the specific sequence. The primers were synthesized by TSINGKE Biological Technology Company (Beijing, China) and were shown in Table S4. The reaction system used was shown in Table S5. The reaction procedure used was as follows: 95 °C for 30 s; 95 °C for 5 s, 56 °C for 30 s, for 40 cycles. In order to verify the specificity of the primer, the melting curve was inserted and analyzed after the completion of each reaction. The $2^{-\Delta\Delta Ct}$ values were computed to quantify the levels of relative gene expression. Every qRT-PCR assay was performed in three replicates. Loquat Actin (Fu et al. 2012) [30] was used as an internal control to normalize the gene expression levels for each sample.

3. Results

3.1. Morphological Indexes of Loquat Treated with Exogenous Hormones

The vertical and horizontal diameter, weight of a single fruit and the weight of per normal seeds in CK group, lovastatin and trans-zeatin treatment groups showed an increasing trend in the development of loquat fruit, whereas the weight of aborted small seeds showed an increasing trend at first and decreased later (Figure 1).

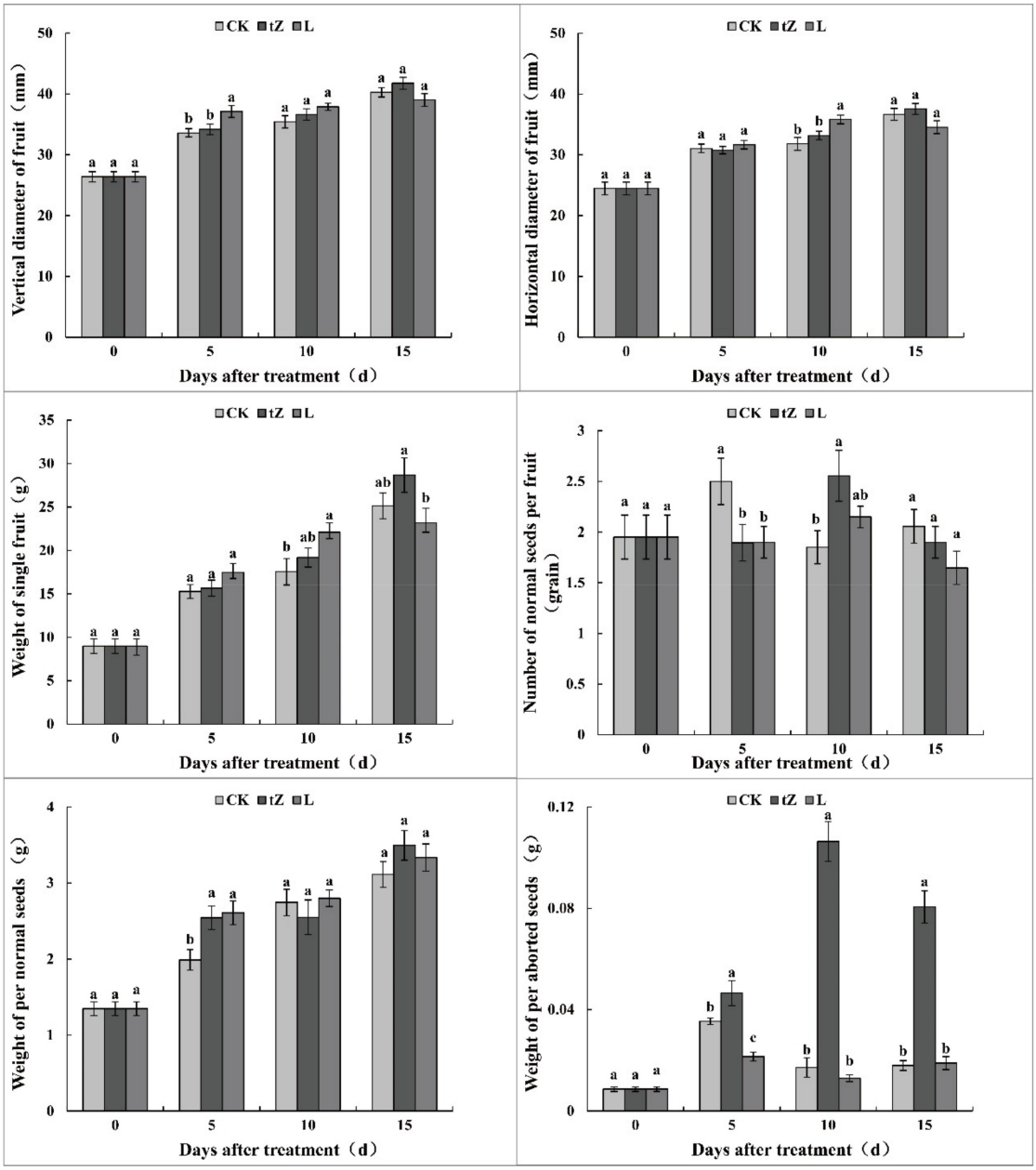

Figure 1. Morphological indexes of loquat fruit treated with CK (clean water), tZ(trans-zeatin) and L(lovastatin). Different lower-case letters among different treatment groups indicate significant differences based on one-way analysis of variance in SPSS 21.0 followed by the Duncan's test ($p < 0.05$).

The morphological indexes of loquat fruit treated with trans-zeatin were analyzed. The weight of aborted small seeds in the trans-zeatin group was found to be significantly higher than that of the CK and lovastatin groups with the development of loquat fruit (Table 1), thereby indicating trans-zeatin treatment could promote the development of small loquat seeds. Vertical and horizontal diameter, weight of single fruit and per normal

seeds in the trans-zeatin treatment group were higher than that of the CK and lovastatin treatment group at 15 DAT (days after treatment), but not significantly different (Figure 1).

The morphological indexes of loquat treated with lovastatin were also analyzed. The weight of small seeds in the lovastatin group was significantly lower than that in the CK group at 5 DAT (Figure 1). However, at 15 DAT, there were no significant differences in the vertical and horizontal diameter, single fruit weight, weight and number of normal seeds as well as the weight of aborted small seeds between the lovastatin and CK groups (Figure 1).

Table 1. The weight of per aborted small seeds.

Treatment	0 Days after Treatment (mg)	5 Days after Treatment (mg)	10 Days after Treatment (mg)	15 Days after Treatment (mg)
CK	8.6 ± 0.9 aA	35.4 ± 1.3 bAB	17.2 ± 3.9 bB	17.9 ± 1.9 bB
tZ	8.6 ± 0.9 aA	46.5 ± 4.9 aA	106.4 ± 7.9 aA	80.6 ± 6.4 aA
L	8.6 ± 0.9 aA	21.5 ± 1.8 cB	12.9 ± 1.3 bB	18.9 ± 2.6 bB

The statistical analysis of the data is based on one-way analysis of variance in SPSS 21.0. The different lower-case and upper-case letters among different treatment groups indicate significant differences followed by Duncan's test with $p < 0.05$ and $p < 0.01$ respectively. The data are expressed as mean ± standard deviation (SD).

3.2. Effects of Exogenous Trans-Zeatin and Lovastatin on Nutrients during Seed Abortion in Loquat

3.2.1. Effects of Exogenous Trans-Zeatin and Lovastatin on Soluble Sugar during Seed Abortion in Loquat

With the development of loquat seeds, the soluble sugar of aborted small seeds in the CKA, lovastatin and trans-zeatin treatment groups showed the same trend (Figure 2). The sugar content showed a trend of decreasing to the lowest level on the 5th DAT and then increased gradually (Figure 2). However, at 15 DAT, the soluble sugar content of aborted small seeds in the trans-zeatin treatment group was significantly higher than that in the CKA and LA groups (Figure 2). It was significantly lower in the lovastatin treatment group than that of the CKA and tZA groups at 15 DAT (Figure 2). The soluble sugar content of normal seeds in the CKN group increased gradually, and was significantly higher than that in all other treatment groups (Figure 2).

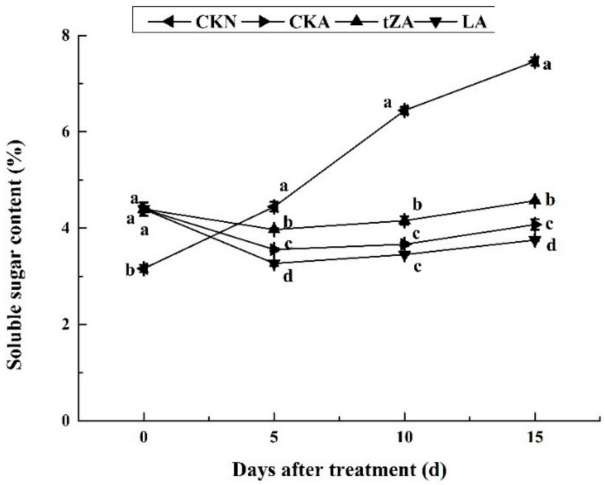

Figure 2. The graph representing changes in soluble sugar content in CKN, CKA, tZA and LA groups after treatment. CKN: the group of normal seeds with clean water treatment; CKA: the group of small aborted seeds with clean water treatment; tZA and LA: the group of small aborted seeds treated with trans-zeatin and lovastatin treatment, respectively. Different lower-case letters among different treatment groups indicate significant differences based on one-way analysis of variance in SPSS 21.0 followed by the Duncan's test ($p < 0.05$).

3.2.2. Effects of Exogenous Trans-Zeatin and Lovastatin on Starch during Seed Abortion in Loquat

As shown in Figure 3, within 10 days after treatment, the starch content of aborted small seeds in the lovastatin and trans-zeatin treatment group showed a similar trend of decreasing first and then increasing with the development of seeds, whereas a gradually decreasing trend was noticed in the CKA group. At 10–15 DAT, the starch content of aborted small seeds in the lovastatin and CKA groups decreased, while it gradually rose to the highest level in the trans-zeatin treatment group. At 15 DAT, the starch content in the tZA group was significantly higher than that in the LA and CKA groups. The starch content of the normal seeds in the CKN group gradually increased during the development process initially, and then decreased after reaching the highest level at 10 DAT. It was significantly higher in the CKN group as compared to other treatment groups during the entire development process.

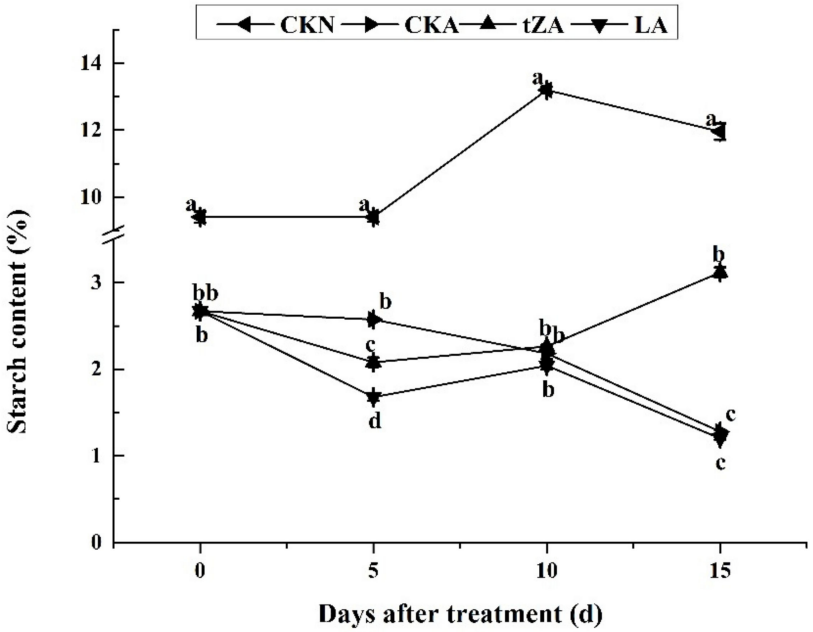

Figure 3. The changes in the starch content in CKN, CKA, tZA and LA groups after treatment. Different lower-case letters among different treatment groups indicate significant differences based on one-way analysis of variance in SPSS 21.0 followed by the Duncan's test ($p < 0.05$).

3.2.3. Effects of Exogenous Trans-Zeatin and Lovastatin on the Soluble Protein during Seed Abortion in Loquat

The soluble protein content of aborted small seeds in the CKA and lovastatin groups showed a similar trend (Figure 4). Its concentration increased to the highest level after 10 DAT and then declined gradually, while it remained at a relatively stable level in the tZA group during the development (Figure 4). The soluble protein content of normal seeds in the CKN group was observed to be significantly lower than that in the other treatment groups during the whole development process (Figure 4).

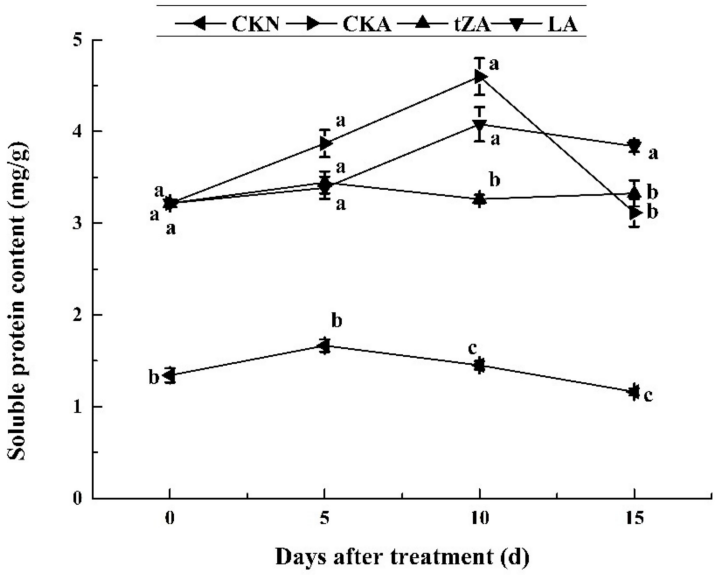

Figure 4. The changes in the soluble protein content in CKN, CKA, tZA and LA groups after treatment. Different lower-case letters among different treatment groups indicate significant differences based on one-way analysis of variance in SPSS 21.0 followed by the Duncan's test ($p < 0.05$).

3.3. The Effects of Exogenous Trans-Zeatin and Lovastatin on the Content of Endogenous Trans-Zeatin and Expression Pattern of Related Genes during Seed Abortion of Loquat

With the development of loquat seeds, the variation trend of endogenous trans-zeatin content in the CKA and LA groups was basically similar: it gradually increased to the highest point at 10 DAT and then declined gradually (Figure 5). However, the trans-zeatin content in the tZA group decreased on the fifth day after treatment, and then showed a trend of gradual increase (Figure 5). It reached the highest level at the 15th day after treatment, which was significantly higher than that in the CKA and LA groups (Figure 5). The trans-zeatin content of normal seeds in the CKN group decreased slowly, and was always significantly higher than that of aborted seeds (Figure 5).

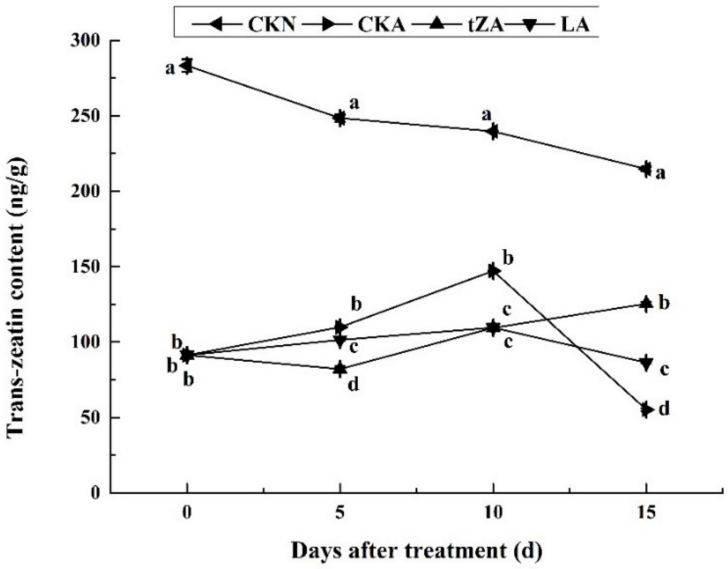

Figure 5. The Changes of trans-zeatin content in CKN, CKA, tZA and LA groups after treatment. Different lower-case letters among different treatment groups indicate significant differences based on one-way analysis of variance in SPSS 21.0 followed by the Duncan's test ($p < 0.05$).

As shown in Figure 6, the expression of the cytokinin oxidase/dehydrogenase 5 related gene (F01_i3_LQ_F01 | c18178/f1p13/2960) showed a similar trend in the CKA and LA groups, which decreased to the lowest point at 5 DAT and then increased. However, the variation trend of its expression in the tZA group was opposite to that in the CKA and LA groups. Its expression level reached the peak at five DAT, and then decreased significantly to the lowest point at 15 DAT, showing a 9.8-fold decrease compared with that in CKA at 15 DAT. The expression of the cytokinin oxidase/dehydrogenase 7 related gene (F01_i2_LQ_F01 | c23747/f1p8/2141) showed a substantially higher level in the CKA and LA groups at most of the time points. However, its expression level in the tZA group showed a trend of gradual down-regulation, and it was a 3.7-fold decrease compared with that in CKA at 15 DAT, which was significantly lower than that in the CKA group. During the process of normal seed development in the CKN group, the expression of cytokinin oxidase/dehydrogenase related genes (F01_i3_LQ_F01 | c18178/f1p13/2960, F01_i2_LQ_F01 | c23747/f1p8/2141) was at a relatively lower level.

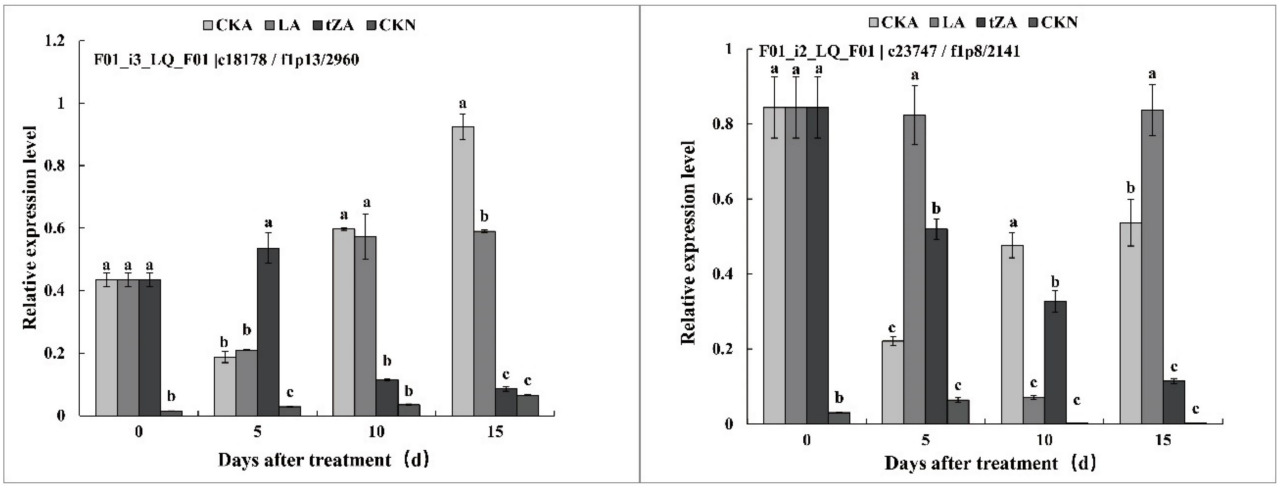

Figure 6. The relative expression level of trans-zeatin related genes in CKN, CKA, tZA and LA groups after the treatment. Different lower-case letters among different treatment groups indicate significant differences based on one-way analysis of variance in SPSS 21.0 followed by the Duncan's test ($p < 0.05$).

3.4. Effects of Exogenous Trans-Zeatin and Lovastatin on Antioxidant Enzymes Activity and Expression Pattern of Related Genes during Seed Abortion of Loquat

3.4.1. Effects of Exogenous Trans-Zeatin and Lovastatin on SOD Activity and Expression Pattern of Related Genes during Seed Abortion of Loquat

With the development of seeds, the SOD activity of the aborted small seeds after clean water treatment (CKA) reached a peak on the 5th DAT, and then decreased significantly (Figure 7). The observed change in the trend of SOD activity in the LA group was consistent with that in the CKA group (Figure 7). The activity level of SOD in the tZA group showed a trend of gradual increase, and in the last period, its activity level was significantly higher than that in the CKA, LA and CKN groups (Figure 7). The SOD activity level of normal seeds after clean water treatment (CKN) almost reached a stable level during the development process (Figure 7).

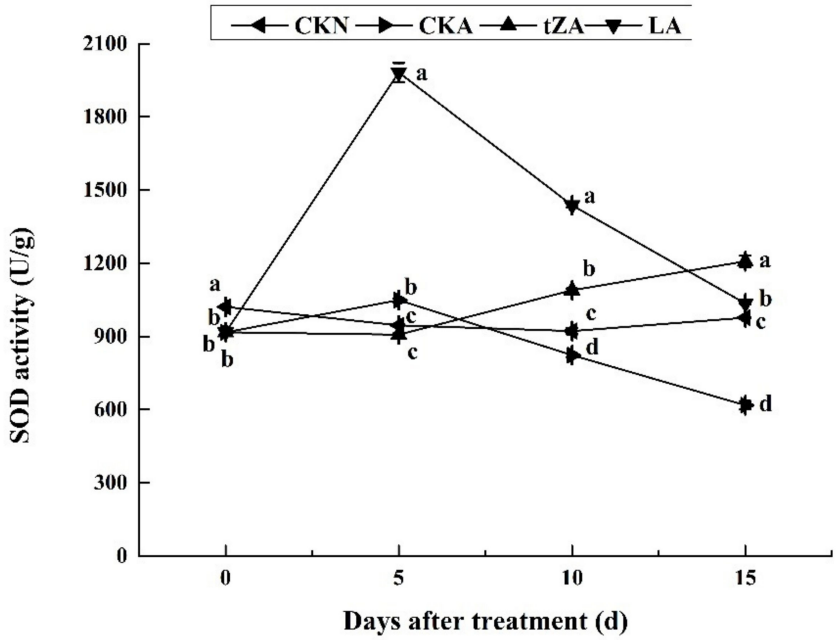

Figure 7. The changes of SOD activity in CKN, CKA, tZA and LA groups after treatment. Different lower-case letters among different treatment groups indicate significant differences based on one-way analysis of variance in SPSS 21.0 followed by the Duncan's test ($p < 0.05$).

As shown in Figure 8, the expression of SOD related genes (F01_i0_LQ_F01 | c1183/f1p0/969, F01_i0_LQ_F01 | c117/f2p1/933) also showed a decreasing trend after initially showing an increase in both the CKA and LA groups, and a trend of increase in the tZA group. At 15 days after treatment, the relative expression levels of these genes in the tZA group were close to those in normal seeds (only 0.2-fold decrease on average), while those in the CKA and LA groups were almost 2.8-fold lower than those in the normal seeds.

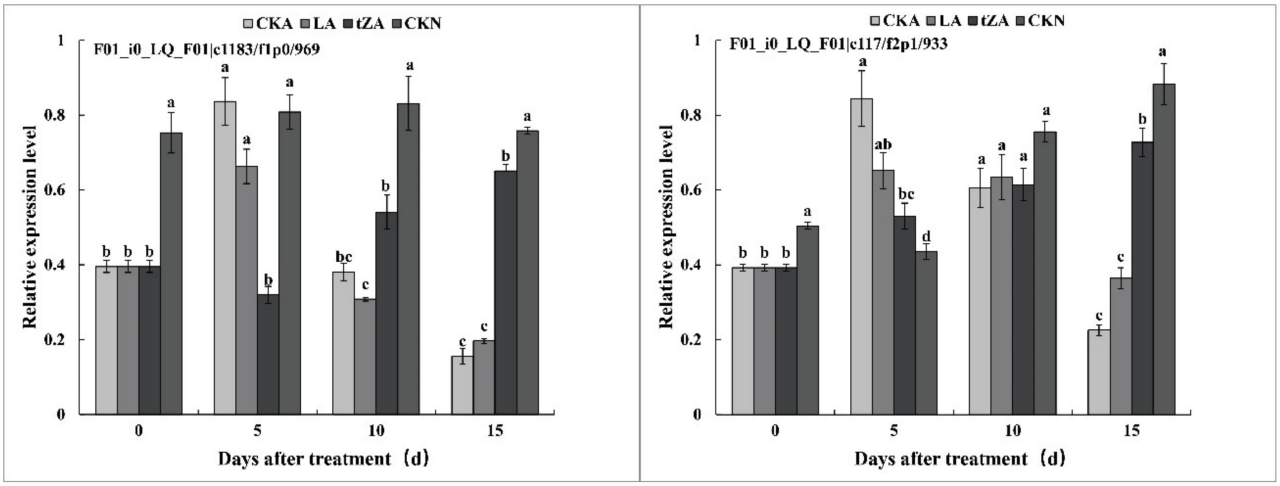

Figure 8. The relative expression level of SOD related genes in CKN, CKA, tZA and LA groups after the treatment. Different lower-case letters among different treatment groups indicate significant differences based on one-way analysis of variance in SPSS 21.0 followed by the Duncan's test ($p < 0.05$).

3.4.2. Effects of Exogenous Trans-Zeatin and Lovastatin on POD Activity and Expression Pattern of Related Genes during Seed Abortion of Loquat

Interestingly, the POD activity of the CKN and CKA groups showed an opposite trend during the seed development (Figure 9). It was significantly lower in the CKA group than that of the normal seeds on the 15th day after the treatment (Figure 9). The activity level of

POD in the CKA and LA groups reached the highest level on the 5th day after the treatment and then decreased gradually (Figure 9). However, in the tZA group, it increased with the development of seeds, and later increased sharply on the 15th day after the treatment (Figure 9). It reached the highest level on the 15th day after the treatment, which was significantly higher as compared to the other groups (Figure 9).

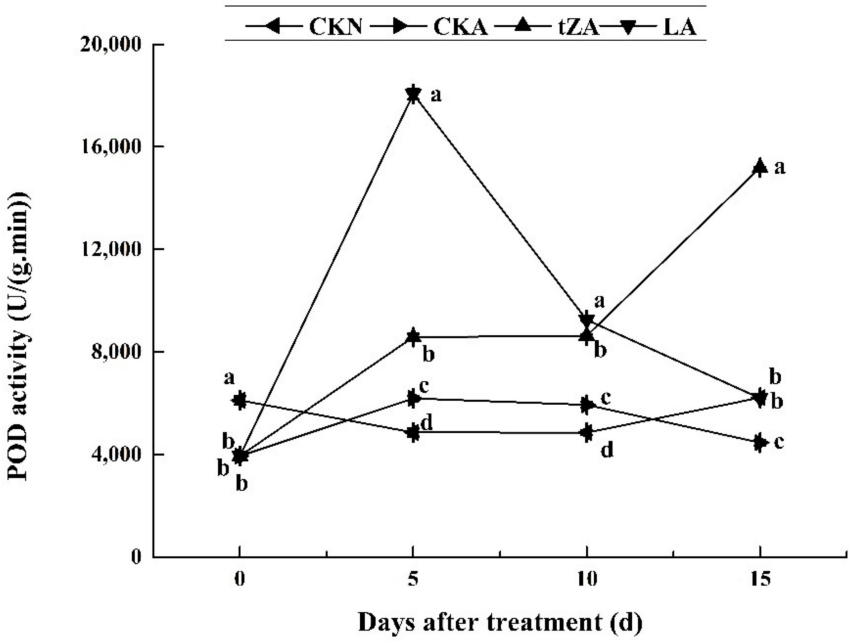

Figure 9. The changes of POD activity in CKN, CKA, tZA and LA groups after the treatment. Different lower-case letters among different treatment groups indicate significant differences based on one-way analysis of variance in SPSS 21.0 followed by the Duncan's test ($p < 0.05$).

As shown in Figure 10, the expression of various POD related genes (F01_i1_LQ_F01 | c13651/f1p1/1333, F01_i1_LQ_F01 | c5575/f1p19/1450, F01_i1_LQ_F01 | c8178/f1p1/1241 and F01_i1_LQ_F01 | c2634/f1p0/1258) showed a similar trend in CKA and LA groups, which increased initially and then decreased slowly. Their expression increased rapidly in the LA group and showed an average 2.3-fold increase per gene compared with those of the CKA group during the second period (the 5th day after the treatment), which was consistent with the alteration trend of POD activity in the LA group. The expression level of POD-related genes in the tZA group increased during the development of the small seeds, and it was significantly higher than that in the CKA, LA and CKN groups at 15 days after the treatment. The expression level of F01_i1_LQ_F01 | c13651/f1p1/1333, F01_i1_LQ_F01 | c5575/f1p19/1450, F01_i1_LQ_F01 | c8178/f1p1/1241 and F01_i1_LQ_F01 | c2634/f1p0/1258 increased 2.0, 18.4, 1.7 and 75-fold compared with those of the CKA group, respectively.

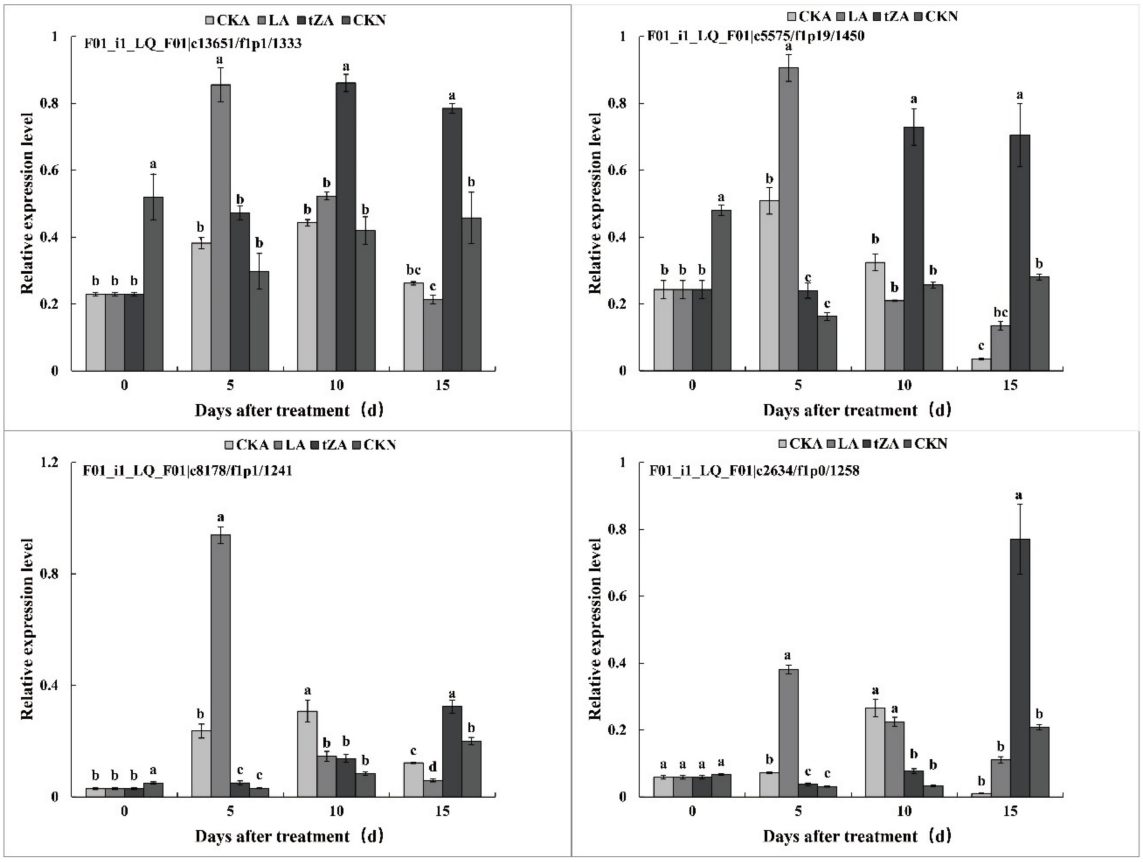

Figure 10. The relative expression level of POD related genes in CKN, CKA, tZA and LA groups after the treatment. Different lower-case letters among different treatment groups indicate significant differences based on one-way analysis of variance in SPSS 21.0 followed by the Duncan's test ($p < 0.05$).

3.4.3. Effects of Exogenous Trans-Zeatin and Lovastatin on CAT Activity and Expression Pattern of Related Gene during Seed Abortion of Loquat

The CAT activity level of aborted small seeds treated with clean water, trans-zeatin and lovastatin showed a similar trend, and reached the highest level on the 10th day after the treatment (Figure 11). It was higher in the tZA group as compared to the CKA and LA groups on the 10th day after treatment (Figure 11). The CAT activity level of the normal seeds was always significantly higher than that of the aborted small seeds during the development process (Figure 11). With the development of seeds, the CAT activity of normal seeds also showed a gradual upward trend, however its content showed a slight decrease on the 15th day after the treatment (Figure 11).

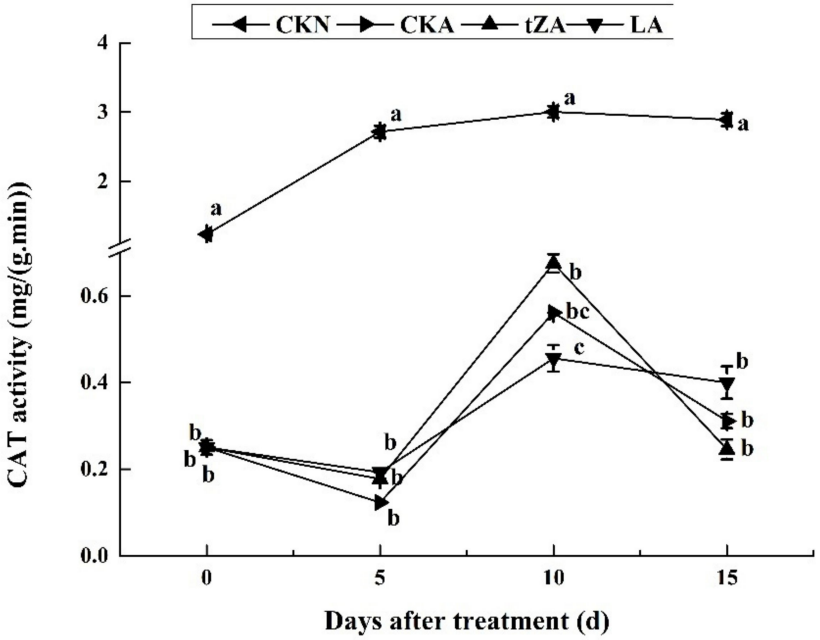

Figure 11. The changes of CAT activity in CKN, CKA, tZA and LA groups after the treatment. Different lower-case letters among different treatment groups indicate significant differences based on one-way analysis of variance in SPSS 21.0 followed by the Duncan's test ($p < 0.05$).

With the development of the loquat seed, the expression of a CAT related gene (F01_i2_LQ_F01 | c39694/f1p0/2142) showed a similar trend of decreasing after rising initially in the CKA, tZA and LA groups, and it reached the highest level at five days day after the treatment (Figure 12). There was no significant difference in gene expression between the tZA, LA and CKA groups (Figure 12). However, during the development of the normal seeds, the CAT related gene was up-regulated in the CKN group and its level was significantly higher than that in aborted seeds (Figure 12).

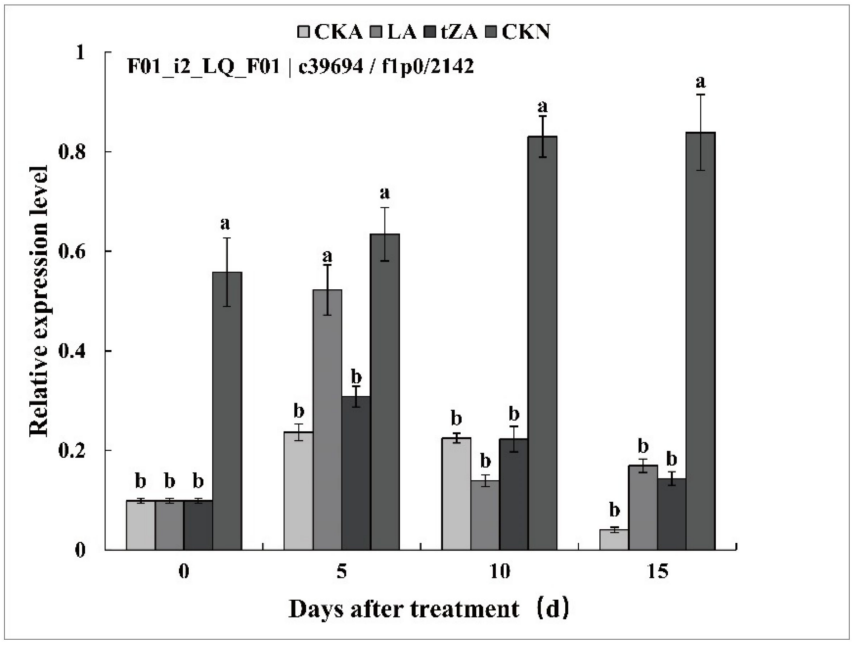

Figure 12. The relative expression level of CAT related gene in CKN, CKA, tZA and LA groups after the treatment. Different lower-case letters among different treatment groups indicate significant differences based on one-way analysis of variance in SPSS 21.0 followed by the Duncan's test ($p < 0.05$).

4. Discussion

We have reported previously that seed abortion in the loquat could be primarily attributed to sibling competition, which may be caused by the difference in fertilization time as a result of the unequal length of the stigmas in loquat [31]. Abortion of loquat seeds was similar to intra-pod seed abortion (IPSA), which was due to a dominance hierarchy of fertilized ovules ranging from the distal (near stigma) to the basal end [32]. Additionally, studies in *Dalbergia sissoo* and *Syzygium cuminii* have found that intra-pod seed abortion was caused by the diffusion of certain chemicals by dominant ovules at the distal end, which in turn prevented the subordinate embryos from drawing further resources [33,34]. Sibling competition has also been attributed to the differences in genetics among the developing seeds. Mohana et al. [35] reported that developing seeds could effectively compete intensely when they are genetically less related, but tend to develop together when genetically closely related. This theory also could explain the rich and diverse genetic characteristics among loquat-aborted small seeds. These small seeds in loquat could be utilized for the breeding of novel varieties and germplasm.

The seeds could influence fruit development because of their high metabolic activities and consequent production of phytohormones [36]. Fruits without seeds or with abortive seeds constitute weak sinks that could ultimately fail to develop [37]. In the fruit development of *avocado*, Tomer et al. [38] have reported the cuke structure of underdeveloped fruits in which ovule growth was disrupted and embryo sac was found to be degenerated. Robert C. Hare [39] also found that physical abortion of conelets in *Longleaf pine* was caused by significant loss of seeds in orchards. Therefore, promoting the development of the small seeds of loquat could also ultimately contribute to the normal development of the fruit.

The effects of cytokinins (CKs) such as N6-benzyladenine (BA) [40,41], kinetin (KT) [41], CPPU [42] and N-phenyl-N′-1,2,3-thiadiazol-5-ylurea (thidia-zuron;TDZ) [40] on fruit growth have been studied and it has been reported that all of them could promote fruit growth. Trans-zeatin (tZ) has also been found to significantly increase fruit growth in *Cucumis sativus* L. [41]. In loquat (*Eriobotrya japonica* Lindl.), our study firstly showed that trans-zeatin treatment could cause a substantial increase in the weight of fruit and small seeds (Figure 1).

Cytokinins have been reported to play an important role in the regulation of cell division [43]. However, cytokinin levels have been found to decrease invariably with seed maturation [44]. Many studies have indicated that the level of cytokinins decreased in response to extended stress [45–48]. These findings could explain significantly lower concentration of cytokinins (tZ) in small loquat seeds than normal seeds because of sibling competition in this study (Figure 5). In dicotyledonous plants, several studies have proved that cytokinins could stimulate cotyledon expansion [49,50] and could be transported between the cotyledons and embryonic axis [51]. Hutton and Van Staden [52] reported that when 8[^{14}C]t-zeatin was applied to the tips of the embryonic radicle of *Phaseolus vulgaris*, cytokinins could be transported rapidly from the embryonic axis to the cotyledons where they were metabolized extensively. We firstly found that exogenous trans-zeatin could significantly increase the level of endogenous tZ in the small loquat seeds, which could be possibly used for stimulating cotyledon expansion. Cytokinin oxidase/dehydrogenase (CKX), which catalyzes the irreversible degradation of CKs [53–55], plays an important role in controlling CK levels in the different plant tissues [56]. A number of studies have indicated that the activity of CKX could be induced by both exogenous [57,58] and endogenous [59] CKs. In this study, the CKX related genes, especially cytokinin oxidase/dehydrogenase 5 related gene (F01_i3_LQ_F01 |c18178/f1p13/2960), which significantly down-regulated in small loquat seeds upon treatment with endogenous cytokinin (tZ) (Figure 6), could be a potential key gene involved in the regulation of seed development in loquat.

It has been found that application of cytokinin (BA) before anthesis in soybeans could act to redirect the movement of assimilates into the treated tissues, increase sink strength and prevent abortion of the developing flowers and pod [60]. Dai Yuling et al. [7] reported that application of cytokinin (KT) could effectively promote the unloading assimilates from

the seed coat to the cotyledon during the development of soybean pods. In this study, we found that cytokinin (tZ) treatment promoted the accumulation of various nutrients (soluble sugar and starch) in the small loquat seeds (Figures 2 and 3). Therefore, it can be speculated that the application of cytokinin could promote transfer of assimilates to the seed sink for its development. Besides, it has been demonstrated that various sugars and amino acids can be transported preferentially to the regions of high cytokinin activity [61]. Thus, higher activity of endogenous tZ in the small seeds enhanced by exogenous trans-zeatin in our study may promote the accumulation of different nutrients. Additionally, some studies have found that the content of sugar and starch showed an opposite varying trend during the development of seeds [62–64]. However, our study indicated that the accumulation of sugar and starch was greatly synchronized. We speculated that it could possibly vary in different plant species and seed development stages.

During seed development, reactive oxygen species (ROS) have been reported to be continually produced [65]. For instance, a number of studies have reported that the seed deterioration (loss of seed vigor) was a result of the overaccumulation of ROS and subsequently could result in lipid peroxidation [66–68]. Antioxidant enzymes such as SOD, POD and CAT, which are considered to be the main protective enzymes, could remove the ROS [69]. It was observed that plants treated with dihydrozeatin [70] or zeatin riboside [71] showed elevated CAT and SOD activities. In this study, trans-zeatin treatment could significantly enhance the activities of POD and SOD.

Lovastatin-inhibitor of mevalonic acid synthesis can also affect cytokinin biosynthesis by inhibiting HMG-CoA reductase activity [72]. Our study found for the first time that it could significantly decrease the weight of small loquat seeds (Figure 1) and significantly enhance the activities of both POD and SOD during the early period of treatment in loquat (Figures 7 and 9). However, there may be other potential regulatory mechanisms that can cause the weight of small seeds to recover to the same level as that of the CKA group during the late development stages.

5. Conclusions

Exogenous trans-zeatin can effectively promote small seed development by causing an accumulation of various important nutrients (soluble sugar and starch), enhancing the antioxidant capacity of the small seeds and modulating the level of endogenous trans-zeatin. Additionally, exogenous cytokinin inhibitor (lovastatin) could significantly inhibit the development of small seeds at the early period of treatment. In summary, the findings established a foundation for the rescue of new germplasm resources of loquat by promoting the development of small loquat seeds which contained rich and diverse genetic characteristics.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/agriculture11050409/s1, Table S1: The program of mobile phase gradient elution, Table S2: Reaction system used for removing genomic DNA, Table S3: Reaction system used for reverse transcription, Table S4: The primers used for qRT-PCR analysis, Table S5: Reaction system of qRT-PCR.

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

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