 doi:10.20944/preprints202208.0321.v1

Article

Effects of Exogenous 5-Aminolevulinic Acid on the Biosynthetic and Recycling Metabolism of GSH in Loquat Leaves under Low-Temperature Stress

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Abstract: Reduced glutathione (GSH) is an antioxidant in plants and is one of the important ways for plants to combat low-temperature stress. In this paper, *Eriobotrya japonica* Lindl. cv. Zaozhong No. 6 seedlings were used to study the effects of exogenous 5-aminolevulinic acid (ALA) application on glutathione synthesis and cyclic metabolism of loquat seedlings under low-temperature stress and to explore the regulatory mechanism of ALA on loquat cold tolerance. The results showed that ALA treatment could increase the content of GSH and the reduced glutathione/oxidized glutathione (GSH/GSSG) ratio in loquat leaf slices under low-temperature stress; reduce the electrolyte leakage rate and GSSG, H_2O_2 and MDA contents in leaf tissues; and alleviate the peroxidation damage caused by low temperature. ALA treatment increased the activity of γ-glutamine synthetase (γ-ECS) in loquat leaf slices under low-temperature stress and promoted the biosynthesis of reduced glutathione, thereby increasing the GSH content in leaf tissues. On the other hand, ALA treatment could also improve the activities of glutathione reductase (GR), glutathione S-transferase (GST) and glutathione peroxidase (GPX) and promote the cyclic regeneration of GSH, accordingly maintaining a high GSH/GSSG ratio, promoting the removal of reactive oxygen species (ROS), and enhancing the antioxidant capacity of leaves. The regulatory effect of ALA on enhancing the antioxidant capacity of loquat seedlings under low-temperature stress can be inhibited by L-buthionine-sulfoximine (BSO, GSH biosynthesis inhibitor). The results showed that ALA improved the antioxidant capacity of loquat seedlings under low-temperature stress, and GSH was involved in the regulation of the antioxidant effect of ALA on loquat seedlings under low-temperature stress.

Keywords: 5-aminolevulinic acid; loquat; low-temperature stress; glutathione

1. Introduction

China as the origin of loquat (*Eriobotrya japonica* Lindl.), which is utilized in a variety of applications. Loquat is a thermophilic fruit tree whose low-temperature tolerance varies by variety. Temperate loquat cultivars cultivated in the northern subtropical region and some frost-snow regions (such as Jiangsu, Zhejiang, Hubei and Anhui) in China have strong cold tolerance, while the southern subtropical and tropical edge areas (such as Fujian, Guangdong, etc.) in China are the producing areas of tropical loquat cultivars with poor cold tolerance. Low-temperature stress is the limiting factor affecting the distribution and yield of tropical loquat cultivars [1]. 'Zaozhong No. 6' loquat (*Eriobotrya japonica* Lindl. cv. Zaozhong No. 6) is a typical tropical loquat cultivar with poor cold resistance. This cultivar often suffers from severe freezing injury during winter or early spring in loquat-producing areas of southern China. In some years, it even has no harvest, resulting in serious economic losses [2]. Therefore, it is of great scientific and practical significance

 doi:10.20944/preprints202208.0321.v1

to explore ways to mitigate the damage of low-temperature stress on loquat to realize safe loquat production over winter.

The damage of low-temperature stress to plants is mainly manifested in the oxidative stress on cells, so the important mechanism for plants to adapt to low-temperature stress is to enhance the cell oxidation resistance. There are two types of antioxidant protection systems in plants: enzymatic and nonenzymatic. Glutathione (GSH) is an important component of the nonenzymatic antioxidant protection system. After direct involvement in the removal of intracellular reactive oxygen species, GSH itself is transformed into oxidized glutathione (GSSG), which loses the antioxidant effect of scavenging ROS [3]. However, on the one hand, plants can restore GSSG through the intracellular ASA-GSH circulatory system so that GSH can be recycled. On the other hand, GSH can be supplemented by intracellular resynthesis [4]. Changes in the intracellular GSH pool level and GSH/GSSG ratio are important parameters for measuring plant low-temperature, salt, drought and other stress resistance [5,6]. Under stress, the cyclic regeneration and synthesis ability of GSH depends on plant species, which reflects the difference in stress resistance among plants to some extent [7,8]. Related studies have shown that promoting the efficient circulation or biosynthesis of GSH will increase the content of intracellular GSH, and the ROS will be eliminated in time to avoid oxidative damage, further enhancing the stress resistance of plants; in contrast, hindering the cyclic regeneration or synthesis of GSH will reduce the content of intracellular GSH, and the ROS cannot be eliminated in time, resulting in excessive accumulation, which will lead to oxidative damage in plants [9]. Therefore, cell GSH recycling and biosynthesis play important roles in regulating plant stress resistance [10].

Although it is not involved in protein synthesis, 5-aminolevulinic acid (ALA), as an amino acid that widely exists in organisms, has extensive regulatory effects on plant growth and development, such as promoting seed germination [11], regulating stomatal movement [6,12], improving antioxidant capacity, increasing chlorophyll content, and promoting photosynthesis and other biological functions [13-15]. ALA enhances the plant antioxidant capacity under stress and reduces ROS accumulation, improving plant stress resistance, which is a prominent physiological function [13,16,17]. There are many studies on the plant physiological regulation of ALA under salt stress [17-21], but the regulatory mechanism of ALA on GSH synthesis and cyclic metabolism in loquat under low-temperature stress has not been reported. In this study, 'Zaozhong 6' loquat container seedlings with poor cold tolerance were used to study the effects of exogenous ALA application on the recycling and biosynthesis of GSH in loquat seedling leaves under low-temperature stress to explore the regulatory mechanism of exogenous ALA application on loquat cold tolerance from the perspective of the nonenzymatic antioxidant system.

2. Results

2.1. Effects of Exogenous ALA Application on H_2O_2, REC and MDA Contents in Loquat Leaves under Low-Temperature Stress

Low-temperature stress induces excessive accumulation of H_2O_2 in plant cells, resulting in membrane lipid peroxidation damage [22]. Figure 1a shows that the H_2O_2 content of T1 loquat leaves treated with low temperature alone was significantly higher than that of CK leaves ($P<0.05$), indicating that low temperature induces the accumulation of H_2O_2 in leaf cells. Under low-temperature stress, the H_2O_2 content of T2 loquat leaves pretreated with ALA was lower than that of T1 loquat leaves treated with low temperature alone, which indicates that ALA pretreatment reduced the accumulation of H_2O_2 in leaf cells under low-temperature stress. Compared with T1, the H_2O_2 content of T3 loquat leaves pretreated with GSH was 50.65% lower, which demonstrated that GSH pretreatment reduced cell H_2O_2 accumulation under low-temperature stress. Compared with T2, the H_2O_2 content of T4 leaves pretreated with BSO and ALA increased by 41.99%. BSO pretreatment significantly inhibited the physiological effect of ALA on reducing H_2O_2 accumulation in cells under low-temperature stress.

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Peroxidation of the plasma membrane induced an increase in MDA content and membrane permeability. The REC value and MDA content are the main indexes used to measure the degree of membrane oxidative damage [23]. Figure 1b and 1c show that the REC value and MDA content of T1 loquat leaves treated with low temperature were 1.43 and 1.78 times higher than those of CK leaves, respectively (P<0.05). Low temperature caused peroxidative damage in loquat leaves. Under low-temperature stress, REC and MDA of ALA pretreated T2 and GSH pretreated T3 decreased by 31.83%, 47.06%, 46.40% and 55.65%, respectively, compared with T1, indicating that ALA and GSH pretreatments alleviated membrane lipid peroxidation damage. The REC and MDA of T4 leaf cells were 12.18% and 103.92% higher than those of T2, respectively, which indicates that BSO pretreatment inhibited the physiological effect of ALA on reducing cell peroxidation damage under low-temperature stress.

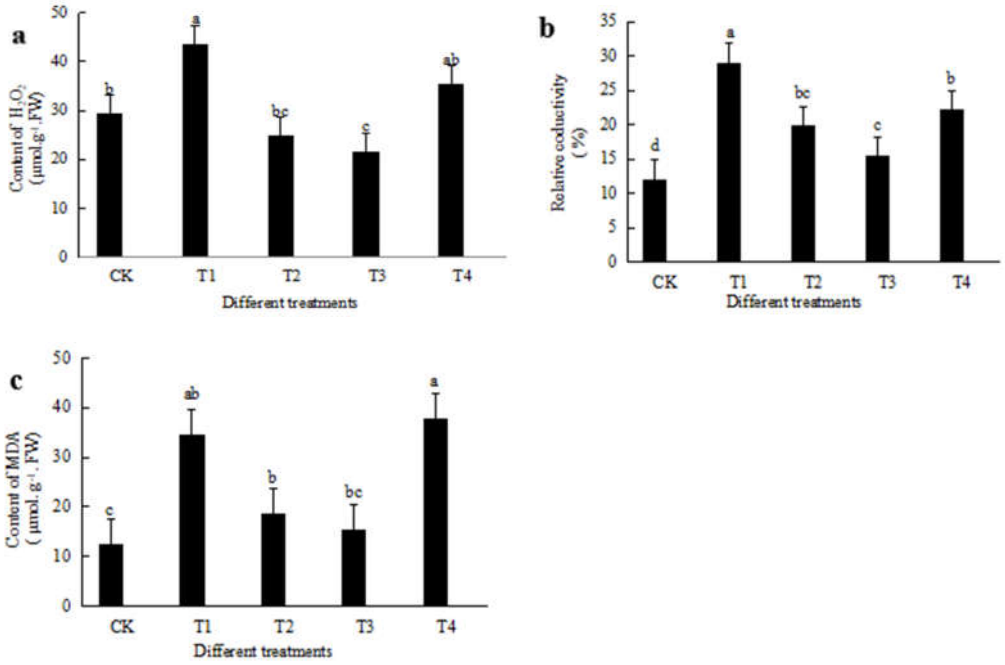

Figure 1. Effect of different treatments on H_2O_2 content (a), REC (b) and MDA content (c) in loquat leaves under low temperature stress. Note: CK: H_2O+RT, T1: H_2O+LT, T2: ALA+LT, T3: GSH+LT, T4: BSO+ALA+LT. Different lowercase letters indicate significant differences (P<0.05). The same applies to subsequent figures.

2.2. Effects of Exogenous ALA Application on GSH and GSSG Contents and GSH/GSSG Ratio in Loquat Leaves under Low-Temperature Stress

GSH is considered to be one of the important members of the second line of defense of the thiol-based antioxidant system, which plays a crucial role in the adaptive response of plants to abiotic stress by scavenging ROS. At the same time, GSH is oxidized into GSSG while scavenging ROS, thus losing its scavenging ability [24,25]. As shown in Figure 2a and 2b, the GSH content of T1 loquat leaves treated with low temperature alone was 30.3% lower than that of CK leaves, which indicates that low-temperature stress inhibited the formation of GSH in cells. Both T2 and T3 leaves showed significantly higher GSH contents than T1 leaves (P<0.05). It was obvious that ALA and GSH pretreatment promoted the production of GSH in leaf cells under low-temperature stress. However, the GSSG content in T2 leaves was lower than that in T1 leaves. This result suggests that ALA pretreatment promoted an increase in cell GSH content while reducing the accumulation of cell GSSG. The GSH content of T4 cells was 67.11% lower than that of T2 cells, indicating that the physiological effect of ALA on GSH production under low-temperature stress may be related to the inhibition of BSO. In addition, the GSSG content of T4 cells was significantly higher than that of T1 and T2 cells (P<0.05). Thus, BSO pretreatment may

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hinder the process of GSSG reduction and GSH regeneration under cellular catalysis, resulting in increased accumulation of GSSG.

GSH is an important component of the ASA-GSH cycle in plant cells. GSH is oxidized to GSSG while scavenging ROS, so GSH/GSSG is an indicator of intracellular GSH activity [26]. As shown in Figure 2c, the GSH/GSSG ratio of T2 leaf cells was the highest among all treatments, which indicates that ALA pretreatment significantly increased the GSH/GSSG ratio of cells under low-temperature stress and enhanced the GSH-dependent capacity of cells to scavenge ROS. Although the GSH/GSSG ratio of T3 leaf cells was significantly higher than that of T1 leaf cells, it was significantly lower than that of T2 leaf cells, indicating that the physiological effect of ALA pretreatment on the increase in the GSH/GSSG ratio was stronger than that of GSH pretreatment. In addition, the GSH/GSSG ratio of T4 leaf cells was significantly lower than that of T1 and T2 (P<0.05), which suggests that BSO pretreatment significantly reduced the GSH/GSSG ratio of loquat leaves at low temperature and inhibited the effect of ALA pretreatment on the increase in the GSH/GSSG ratio in loquat leaves.

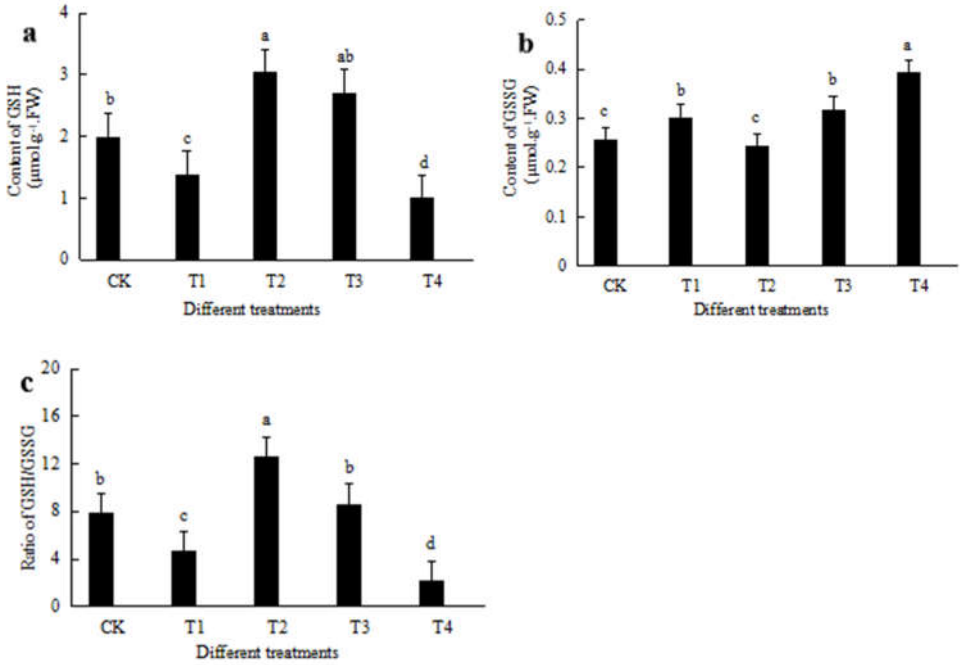

Figure 2. Effect of different treatments on GSH content (a), GSSG content (b) and GSH/GSSG ratio (c) in loquat leaves under low temperature stress.

2.3. Effects of Exogenous ALA Application on GR, GST, GPX and γ-ECS Activities in Loquat Seedling Leaves under Low-Temperature Stress

Under stress, intracellular GSH is oxidized into GSSG by scavenging ROS, while GR, which plays an important role in cell GSH recycling, has the ability to reduce GSSG to GSH. Therefore, GR activity in plant cells is closely related to the level of the GSH pool, affecting the ability of cells to scavenge ROS [27]. Figure 3a shows that the GR activity of T1 loquat leaf cells was significantly lower than that of CK leaf cells, indicating that low temperature had a significant inhibitory effect on GR activity. The GR activity of T2 cells was increased by 77.14% compared with that of T1 cells, which suggests that ALA treatment increased GR activity under low-temperature stress. Compared with that of CK, T1, T2 and T4 cells, the GR activity of T3 cells was decreased by 77.36%, 65.71%, 80.65% and 69.23%, respectively (P<0.05). This result shows that GSH treatment significantly inhibited the GR activity of cells. Additionally, the GR activity of T4 cells was lower than that of T2 cells, indicating that BSO may have a certain antagonistic effect on the GR activity of ALA-activated cells.

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The ability of GST and GPX to scavenge ROS depends on the participation of GSH. They simultaneously promote the transformation of GSH into GSSG and consequently affect the intracellular GSH pool [28,29]. Figure 3b and 3c show that the GST activity of T1 loquat leaf cells was slightly lower than that of CK leaf cells, while its GPX activity was slightly higher. However, the differences were not significant (P>0.05), which indicates that low-temperature stress had little effect on the GST and GPX activities of cells. The GST activity of T2 and T3 cells was 1.82 and 2.72 times higher than that of T1 cells, respectively, while the GPX activity was increased by 94.26% and 33.63%, respectively. The difference was significant (P<0.05) or extremely significant (P<0.01), indicating that ALA and GSH pretreatment had activation effects on the GST and GPX activity of loquat leaf cells under low-temperature stress. In addition, the activities of GST and GPX in T4 cells were significantly lower than those in T2 cells (P<0.05). BSO treatment has a certain inhibitory effect on the activities of GST and GPX in T4 cells. Therefore, the inhibitory effect of BSO treatment on GST activity was comparatively more obvious.

γ-ECS is a rate-limiting enzyme involved in the GSH synthesis pathway [30,31]. As shown in Figure 3d, the γ-ECS activity of T1 leaf cells was 48.39% higher compared with the control CK, which suggests that low temperature had an activation effect on the γ-ECS activity of cells. The γ-ECS activity of T2 and T3 cells was significantly higher than that of T1 cells, indicating that ALA and GSH pretreatment increased γ-ECS activity under low-temperature stress. Moreover, the γ-ECS activity of T4 cells was lower than that of T2 cells, which reveals that BSO pretreatment inhibited γ-ECS activity under low-temperature stress.

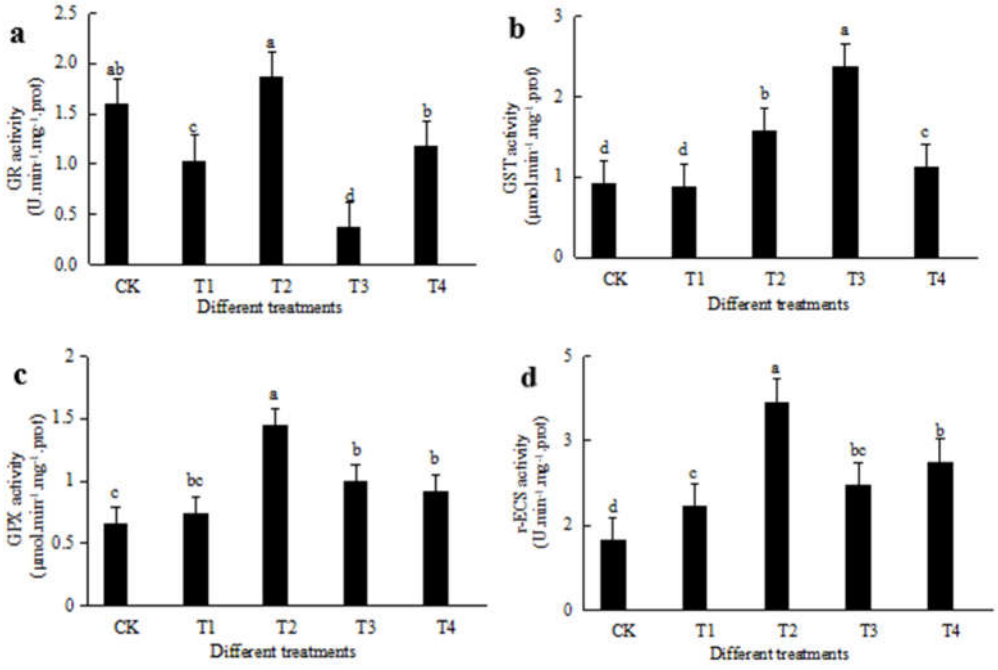

Figure 3. Effect of different treatments on GR activity (a), GST activity (b), GPX activity (c) and γ-ECS activity (d) in loquat leaves under low temperature stress.

3. Discussion

Low-temperature stress can cause excessive accumulation of reactive oxygen species (ROS) in fruit trees, leading to oxidative damage. Oxidative damage is caused by insufficient antioxidant potential of fruit trees or excessive oxidative stress. Fruit trees can mobilize enzymatic and nonenzymatic defense systems to protect cells from oxidative damage. Generally, REC and MDA are used as important physical and chemical indicators to evaluate the damage degree of membrane peroxidation [32-34]. ALA, a nonprotein amino acid, is a new plant growth regulator. Many studies have reported that ALA plays an important role in the regulation of the low-temperature tolerance of pepper, cucumber,

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watermelon, melon and rape [35,36]. For example, ALA treatment could reduce the MDA content and REC value of maize seedling leaves under low-temperature stress and the superoxide anion free radical and H_2O_2 content of sweet cherry style and ovary cells for similarity, which alleviated the cell oxidative damage and the low temperature damage to plants [37,38]. This study showed that low temperature induced the abnormal accumulation of H_2O_2 in the leaf cells of loquat seedlings, while ALA and GSH pretreatment reduced the accumulation of H_2O_2, manifesting as decreased MDA content and REC values, which effectively alleviated the oxidative damage of ROS to the cell membrane. Therefore, it is speculated that exogenous ALA and GSH application may improve the low-temperature resistance of loquat seedlings by enhancing the ability of cells to scavenge ROS in the antioxidant system under low-temperature stress and reducing peroxidation damage to the plasma membrane, which is consistent with the results of research on soybean [14] and pepper [38].

The above results showed that exogenous ALA and GSH application had similar effects on the resistance to low-temperature stress in loquat, which could not help but was associated with their effects on low-temperature stress; either the regulation of ALA depends on GSH, or GSH participates in the synergistic regulation of ALA. GSH, known as one of the core members of nonenzymatic redox regulation in organisms, scavenges H_2O_2 through the Halliwell-Asada pathway, which plays an important role in maintaining the redox balance in plants and preventing and repairing the peroxidation damage of membrane lipids by free radicals. The GSH/GSSG ratio is a marker used to measure glutathione redox potential, which can reflect the level of cell antioxidant capacity to some extent [39,40]. The results of this study indicated that low temperature induced the accumulation of GSH in loquat leaf cells, resulting in defensive response signals to low temperature. Under low-temperature stress, ALA and GSH treatments significantly increased GSH content and reduced GSSG content in leaf cells of loquat seedlings, contributing to an increased GSH/GSSG ratio. Both treatments may directly or indirectly promote the perception and response to low temperature in loquat, stimulate the biosynthesis and recycling of downstream GSH, and improve the antioxidant capacity of loquat. BSO pretreatment inhibited the accumulation of GSH induced by ALA, which may be related to the weakened perception and response of loquat leaves to low temperature and thus inhibited the biosynthesis and recycling of downstream GSH. The above research results are also supported by related studies on grapes, sweet cherry and maize [35,37,41-42].

GSH can be directly oxidized to GSSG, depending on GST and GPX, while scavenging intracellular ROS. GR can catalyze GSSG to restore GSH. Therefore, the level of the intracellular GSH pool is closely related to the enzyme activities of GST, GPX and GR [40]. Roxas et al. [43] found that the GSSG content of transgenic tobacco seedlings overexpressing the GST/GPX gene under cold stress was higher than that of wild-type seedlings, and Qi Yuancheng et al. [28] also found that the GSSG content of *Arabidopsis thaliana* overexpressing the GST gene from Suaeda salsa was higher than that of wild-type seedlings. Lin et al. [44] found that the reductive regeneration of GSH was blocked by the inhibition of GR activity in young fruit cells of loquat. Tian Yongqiang et al. [37] suggested that ALA promoted GSH content and the GSH/GSSG ratio by increasing GPX and GR activities in floral organs of sweet cherry under low-temperature stress. The abovementioned studies clarified the correlation between GSH pool levels and the activities of GST, GPX and GR in different plant cells from molecular, physiological and biochemical perspectives. The results indicated that on the one hand, ALA treatment significantly increased the activities of GR and γ-ECS in loquat leaf cells under low-temperature stress, promoted GSH biosynthesis and recycling, and then was beneficial to the increase in cell GSH content; on the other hand, ALA increased the activities of GST and GPX and accelerated the oxidation of GSH to GSSG. The above two aspects jointly lead to an increase in the GSH/GSSG ratio, which may be due to the dominant role of ALA in promoting GSH recycling and biosynthesis. Under low-temperature stress, GSH pretreatment increased the activities of GST and GPX but inhibited GR activity, which was lower than ALA pretreatment, hindering GR-catalyzed GSSG reduction to GSH. This may be the reason why the GSSG

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content in GSH-pretreated cells was higher than that in ALA-pretreated cells. Compared with ALA pretreatment, GSH pretreatment had a weaker effect on the activation of γ-ECS activity in cells. In the above two aspects of comprehensive regulation, the GSH content and GSH/GSSG ratio of GSH-pretreated cells were lower than those of ALA-pretreated cells under low-temperature stress.

Related studies have pointed out that intracellular GSH can not only eliminate ROS but also act as a signal molecule, affecting the intensity of signals in cells and transmitting stress signals to downstream target signal molecules, which ultimately acts on target enzymes or proteins [40,45]. In this experiment, ALA and GSH were applied exogenously to improve the antioxidant capacity of loquat seedlings under low-temperature stress. However, after endogenous GSH was removed by BSO pretreatment, the ALA-induced antioxidant capacity of loquat seedlings was greatly weakened, as the MDA and H_2O_2 contents in cells and the REC value significantly increased. This is not only directly related to the decrease in the total amount of intracellular antioxidants attributed to the elimination of endogenous GSH by BSO but also may be related to the weakening of GSH signal intensity or blocked signal transduction, resulting in an insufficient response or inhibition of the downstream antioxidant system. Then, target enzymes, such as GR, GST, GPX and γ-ECS, cannot be effectively activated, and GSH synthesis and recycling are blocked. The ALA-induced enhancement of antioxidant capacity in loquat seedlings under low-temperature stress may occur through enhancing the signal intensity of intracellular GSH, participating in the effective activation of target enzymes related to intracellular GSH synthesis and recycling, improving the ability to scavenge ROS, and further enhancing the cold tolerance of loquat seedlings. In addition, BSO pretreatment did not completely inhibit the physiological effect of ALA on improving the antioxidant capacity of loquat seedlings, indicating that ALA may also regulate the antioxidant capacity of loquat seedlings through signaling pathways other than GSH signaling in response to low-temperature stress.

4. Materials and Methods

4.1. Plant Material and Treatments

Three-year-old (*Eriobotrya japonica* Lindl. cv. Zaozhong No. 6) free stock grafted loquat container seedlings growing uniformly and well (provided by Fujian Putian Institute of Fruit Trees) were selected as test materials. The container seedlings were randomly divided into 5 groups, with H_2O+RT (room temperature) as the control (CK), H_2O+LT (low temperature) as treatment 1 (T1), ALA+LT as treatment 2 (T2), GSH+LT as treatment 3 (T3), and BSO+ALA+LT as treatment 4 (T4). Each treatment had three replications, with three container seedlings as a repeat, randomly arranged. Referring to the method of Zhao Baolong et al. [35], the container seedling leaves of loquat were rinsed one day before treatment. In the abovementioned treatments, 175 mg·L^{-1} ALA, 1 mM BSO and H_2O were sprayed with 1% Tween-20, and the solution was sprayed evenly on the front and back of the leaf until it dripped at room temperature (25°C). Forty-eight hours after spraying, the container seedlings of T1, T2, T3 and T4 were transferred into the artificial climate chamber (relative humidity was 70%, light intensity was 2000 lx), cooled to -3°C for 3 h and equilibrated at 25°C for 12 h by the instrument control system, according to the method of Wu et al. [46]. After that, sampling was carried out by referring to the method of Wu et al. [46], and 3-5 leaves from the top to the bottom of the branch were selected for mixed sampling [1]. The samples were stored at -80°C after being quickly frozen in liquid nitrogen for measuring related indexes.

4.2. Biochemical Analyses

4.2.1. Measurement of the H_2O_2, MDA, GSH and GSSG Contents

The contents of H_2O_2 and MDA were determined by adopting the method of Zou [47] with slight modifications. Reduced GSH and GSSG were assayed according to Chen and Wang [48].

 doi:10.20944/preprints202208.0321.v1

4.2.2. Measurement of REC Value

The cell membrane permeability was measured by adopting the method of Liu and Zhang [49] and represented by the relative conductivity.

4.2.3. Assay of GST, GPX, GR and γ-ECS Activities

The activities of GST and GPX were determined by adopting the method of Huang et al. [50]. The GR activity was determined by adopting the method of Grace and Logan [51]. The activity of γ-ECS was determined in accordance with the instructions of the detection kits produced by Beijing Baiaolaibo Technology Co., LTD [52].

4.3. Statistical Analysis

The measurement of the above indicators was repeated three times, and the obtained data were the average of the three replications. Microsoft Excel 2003 and SAS 12.0 statistical software were used to test the significance of differences in the obtained data and generate plots.

5. Conclusion

Exogenous ALA treatment could induce and enhance the activities of γ-ECS and GR in loquat leaf cells under low-temperature stress, promoting GSH biosynthesis and recycling and increasing GSH content in cells. Moreover, ALA also increased the activities of GST and GPX in loquat leaf cells under low-temperature stress. However, these enzymes participated in the removal of ROS and promoted the transformation of GSH into GSSG. Despite this, ALA had an advantage in regulating and promoting the biosynthesis and recycling of GSH in cells so that cells could maintain a high GSH/GSSG ratio, further enhancing the antioxidant capacity of leaves to scavenge ROS.

Exogenous GSH treatment increased the activities of γ-ECS, GR, GST and GPX in loquat leaf cells under low-temperature stress, while the GSH biosynthesis inhibitor L-cysteine sulfimide BSO suppressed the activities of γ-ECS, GR, GST and GPX in loquat leaf cells under low-temperature stress. GSH may be involved in the regulation of ALA on antioxidant activity of loquat seedlings under low-temperature stress as a signal molecule. The physiological effect of ALA on advancing the antioxidant capacity of loquat seedlings was not completely inhibited by BSO, and a signaling pathway other than GSH signaling may be involved in the regulation of ALA production in response to low-temperature stress in loquat seedlings.

Author Contributions: He H., Wu J. and Wu M. designed and supervised the experiment. Wu T. mainly performed the research and drafted the manuscript. Ma S. finished specific parts of the experiment. Lin S. carried out the statistical data analysis. He H., Wu J. and Wu T. revised the manuscript. All authors have read and agreed to the publish version of the article.

Funding: This project was funded by the Fujian Provincial Science and Technology Project (2021N5014, 2022N5006).

Acknowledgments: This manuscript was edited by American Journal Experts (AJE) for proper English use.

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

References

1. Wu, J.; Chen, Y.; Wu, B., Huang, L.; Zhang, W.; Zheng, F. Effects calclum on Ca^{2+}-ATPase activity and lipid peroxidation level of loquat seedling under low temperature stress. *J. Northwest A&F Univ.* **2016**, *44*, 121-128.
2. Wu, J.; Chen, J.; Liang, J.; Yang, W.; Wu, J.; Chen, L.; Liu, M.; Chen, L. Effects of exogenous NO on ascorbate-glutathione cycle in loquat leaves under low temperature stress. *Chin. J. Appl. Ecol.* **2009**, *20*, 1395-1400.
3. Jin, Y.; Tao, D.; Hao, Z.; Ye, J.; Du, Y.; Liu, H.; Zhou, Y. Environmental stresses and redox status of ascorbate. *Acta Bot. Sin.* **2003**, *45*, 795-801.
4. Lü, X.; Yang, Y,; Lu, X.; Jin, J.; Bai, R. Effects of $CaCl_2$ on the AsA-GSH cycle of sour Jujube seedlings under NaCl stress. *Acta Hortic. Sin.* **2017**, *44*, 953-962.

5. Shang, C.; Han, R.; Liang, Z. Responses to drought stress of the biosynthetic and recycling metabolis of glutathione and ascorbate in *Agropyron cristatum* leaves on the Loess Plateau of China. *Chin. J. Plant Ecol.* **2011**, *35*, 653-662.
6. Chen, L.; Liu, L.; An, Y.; Zhang, Z.; Wang, L. Preliminary studies on the possible mechanism underlying 5-aminolevulinic acid-induced stomatal opening in apple leaves. *Acta Hortic. Sin.* **2014**, *41*, 1965-1974.
7. Mishra, P.; Bhoomika, K.; Dubey, R.S. Differential responses of antioxidative defense system to prolonged salinity stress in salt-tolerant and salt-sensitive indica rice (*Oryza sativa* L.) seedlings. *Protoplasma.* **2013**, *250*, 3-19.
8. Mohammad, G.M.; Mohammad, A.H.; Masayuki, F. Trehalose pretreatment induces salt tolerance in rice (*Oryza sativa* L.) seedlings: oxidative damage and coinduction of antioxidant defense and glyoxalase systems. *Protoplasma.* **2015**, *252*, 461–475.
9. Amor, N.B.; Jimenez, A.; Lundqvist, M.; Sevilla, F.; Abdelly, C. Response of antioxidant systems to NaCl stress in the halophyte *Cakile maritima*. *Physiol. Plant.* **2006**, *126*, 446-457.
10. Shao, H.; Chu, L.; Shao, M.; Jaleel, C.A.; Mi, H. Higher plant antioxidants and redox signaling under environmental stresses. *C. R. Biol.* **2008**, *331*, 433–441.
11. Wang, L.; Jiang, W.; Liu, H.; Liu, W.; Kang, L.; Hou, X. Promotion by 5-aminolevulinic acid of germination of pakchoi (*Brassica campestris* ssp. *chinensis* var. *communis* Tsen et Lee) seeds under salt stress. *J. Integr. Plant Biol.* **2005**, *47*, 1084-1091.
12. An, Y.; Liu, L.; Chen, L.; Cheng, X.; Wang, L. ALA inhibits abscisic acid-induced stomatal closure via reducing H_2O_2 and Ca^{2+} levels in guard cells. *Front. Plant Sci.* **2016**, *7*, 482.
13. Anwar, A.; Yan, Y.; Liu, Y.; Li, Y.; Yu, X. 5-aminolevulinic acid improves nutrient uptake and endogenous hormone accumulation, enhancing low-temperature stress tolerance in cucumbers. *Int. J. Mol. Sci.* **2018**, *19*, 3379.
14. Balestrasse, K.B.; Tomaro, M.L.; Batlle, A.; Noriega, G.O. The role of 5-aminolevulinic acid in the response to cold stress in soybean plants. *Phytochemistry.* **2010**, *71*, 2038-2045.
15. Korkmaz, A.; Korkmaz, Y.; Demirkıran, A.R. Enhancing chilling stress tolerance of pepper seedlings by exogenous application of 5-aminolevulinic acid. *Environ. Exp. Bot.* **2010**, *67*, 495-501.
16. Li, D.; Zhang, J.; Sun, W.; Li, Q.; Dai, A.; Bai, J. 5-Aminolevulinic acid pretreatment mitigates drought stress of cucumber leaves through altering antioxidant enzyme activity. *Sci. Hortic.* **2011**, *130*, 820-828.
17. Wu, W.; He, S.; An, Y.; Cao, R.; Sun, Y.; Tang, Q.; Wang, L. Hydrogen peroxide as a mediator of 5-aminolevulinic acid (ALA)-induced Na^+ retention in roots for improving salt tolerance of strawberries. *Physiol. Plant.* **2019**, *167*, 5-20.
18. Zhao, Y.; Yan, F.; Hu, L.; Zhou, X.; Zou, Z. Effect of 5-aminolevulinic acid on photosynthetic characteristics of tomato seedlings under NaCl stress. *Chin. J. Appl. Ecol.* **2014**, *25*, 2919-2926.
19. Al-Ghamdi, A.A.; Elansary, H.O. Synergetic effects of 5-aminolevulinic acid and Ascophyllum nodosum seaweed extracts on Asparagus phenolics and stress related genes under saline irrigation. *Plant Physiol. Biochem.* **2018**, *129*, 273-284.
20. Tang, X.; Wang, Y.; Lv, T.; Xiao, Y. Role of 5-aminolevulinic acid on growth, photosynthetic parameters and antioxidant enzyme activity in NaCl-stressed *Isatis indigotica* Fort. *Russ. J. Plant Physiol.* **2017**, *64*, 198-206.
21. Xiong, J.; Wang, H.; Tan, X.; Zhang, C.; Naeem, M.S. 5-aminolevulinic acid improves salt tolerance mediated by regulation of tetrapyrrole and proline metabolism in *Brassica napus* L. seedlings under NaCl stress. *Plant Physiol. Biochem.* **2018**, *124*, 88-99.
22. Okuda, T.; Matsuda, Y.; Yamanaka, A.; Sagisaka, S. Abrupt increase in the level of hydrogen peroxide in leaves of winter wheat is caused by cold tolerance. *Plant Physiol.* **1991**, *97*, 1265-1267.
23. Guo, P.; Qi, Y.; Cai, Y.; Yang, T.; Yang, L.; Huang, Z.; Chen, L. Aluminum effects on photosynthesis, reactive oxygen species and methylglyoxal detoxification in two Citrus species differing in aluminum tolerance. *Tree Physiol.* **2018**, *38*, 1548-1565.
24. Guo, P.; Li, Q.; Qi, Y.; Yang, L.; Ye, X.; Chen, H.; Chen, L. Sulfur-mediated-alleviation of aluminum-toxicity in *Citrus grandis* seedlings. *Int. J. Mol. Sci.* **2017**, *18*, 2570.
25. Capaldi, F.R.; Gratão, P.L.; Reis, A.R.; Lima, L.W.; Azevedo, R.A. Sulfur metabolism and stress defense responses in plants. *Trop. Plant Bio.* **2015**, *8*, 60-73.
26. Ma, J.; Zheng, G.; Pei, C.; Zhang, Z. The function of ascrbate-glutathione cycle in salt tolerance of alfalfa mutant. *Plant physiol. J.* **2015**, *51*, 1749.
27. Mishra, S.; Srivastava, S.; Tripathi, R.D.; Trivedi, P.K. Thiol metabolism and antioxidant systems complement each other during arsenate detoxification in *Ceratophyllum demersum* L. *Aquat. Toxicol.* **2008**, *86*, 205-215.
28. Qi, Y.; Zhang, S.; Wang, L.; Wang, M.; Zhang, H. Overexpresion of GST gene accelerates the growth of transgenic arabidopsis under salt stress. *J. Plant Physiol. Mol. Biol.* **2004**, *30*, 517-522.
29. Gao, F.; Chen, J.; Ma, T.; Li, H.; Wang, N.; Li, Z.; Zhang, Z.; Zhou, Y. The glutathione peroxidase gene family in *Thellungiella salsuginea*: genome-wide identification, classification, and gene and protein expression analysis under stress conditions. *Int. J. Mol. Sci.* **2014**, *15*, 3319- 3335.
30. Yi, H.; Ravilious, G.E.; Galant, A.; Krishnan, H.B.; Jez, J.M. From sulfur to homoglutathione: thiol metabolism in soybean. *Amino Acids*. **2010**, *39*, 963-978.
31. Noctor, G.; Foyer, C.H. Ascorbate and glutathione: keeping active oxygen under control. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 249-279.
32. Bose, J.; Rodrigo-Moreno, A.; Shabala, S. ROS homeostasis in halophytes in the context of salinity stress tolerance. *J. Exp. Bot.* **2014**, *65*, 1241-1257.
33. Knight, M.R.; Knight, H. Low-temperature perception leading to gene expression and cold tolerance in higher plants. *New Phytol.* **2012**, *195*, 737-751.
34. Willems, P.; Mhamdi, A.; Stael, S.; Storme, V.; Kerchev, P.; Noctor, G.; Gevaert, K, Breusegem, F.V. The ROS wheel: refining ROS transcriptional footprints. *Plant Physiol.* **2016**, *171*, 1720-1733.

35. Zhao, B.; Liu, P.; Wang, W.; Sun, J.; Ma, H. Effects of 5-aminolevulinic acid on the AsA-GSH cycle in grape leaves under salt stress. *Plant Physiol. J.* **2015**, *51*, 385-390.
36. Sun, Y.; Wang, Y.; Qu, D.; Li, J.; Jiao, J.; Cao, X.; Gu, W.; Wei, S. Enhanced low-temperature resistance and physiological mechanism of maize seedlings by exogenous application of 5-aminolevulinic acid. *Chin. J. Ecol.* **2016**, *35*, 1737-1743.
37. Tian, Y.; Nie, G.; Li, K.; Zhang, X.; Dai, L. Ameliorating Effects of 5-aminolevulinic acid damage to sweet cherry floral organ under low temperature stress. *Acta Agric. Boreali-Occident. Sin.* **2020**, *29*, 595-602.
38. Korkmaz, A.; Korkmaz, Y.; Demirkıran, A.R. Enhancing chilling stress tolerance of pepper seedlings by exogenous application of 5-aminolevulinic acid. *Environ. Exp. Bot.* **2010**, *67*, 495-501.
39. Chan, K.X.; Wirtz, M.; Phua, S.Y.; Estavillo, G.M.; Pogson, B.J. Balancing metabolites in drought: the sulfur assimilation conundrum. *Trends Plant Sci.* **2013**, *18*, 18-29.
40. Foyer, C.H.; Noctor, G. Ascorbate and glutathione: the heart of the redox hub. *Plant Physiol.* **2011**, *155*, 2-18.
41. Sun, Y. Physiological effects of ALA on chilling tolerance of maize seedlings. *J. Northeast Agric. Univ.* **2017**, 24.
42. Wu, J.; Liang, J.; Chen, J.; Dai, Q.; Cao, L.; Xu, X.; Xu, J.; Guan, L. Effects of GSH on AsA-GSH circulation metabolism in chloroplasts of young loquat fruits under low temperature stress. *Sci. Silvae Sin.* **2009**, *45*, 15-19.
43. Roxas, V.P.; Smith, R.K.; Allen, E.R.; Allen, R.D. Overexpression of glutathione S-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nat. Biotechnol.* **1997**, *15*, 988-991.
44. Lin, S.; Liang, J.; Huang, Z.; Wang, B.; Chen, D.; Kang, X.; Wu, G.; Wu, J. Effects of calmodulin antagonist TFP on AsA-GSH cycle in young loquat fruits under low temperature stress. *Chin J. Trop. Crops*. **2012**, *33*, 1980-1984.
45. Han, Y.; Mhamdi, A.; Chaouch, S.; Noctor, G. Regulation of basal and oxidative stress-triggered jasmonic acid-related gene expression by glutathione. *Plant Cell Environ.* **2013**, *36*, 1135-1146.
46. Wu, J.; Chen, W.; Cai, L.; Xie, C.; Huang, S.; Lin, L.; Ye, M. Effects of exogenous nitric oxide on anti-oxidation capacities in young loquat fruits under low temperature stress. *Sci. Silvae Sin.* **2010**, *46*, 73-78.
47. Zou, Q. The exprimental guide for plant physiology. *China Agriculture Press.* **2001**, 166-167.
48. Chen, J.; Wang, X. The exprimental guide for plant physiology. *South China University of Technology Press.* **2002**, 123-127.
49. Liu, Z.; Zhang, S. Physiology of plant resistance. *China Agriculture Press.* **1994**, 39-60.
50. Huang, Z.; Wu, J.; Chen, W.; Cai, L.; Xie, C.; Huang, S.; Lin, L.; Ye, M. Effects of SA on enzymes of ascorbate-gluthione cycle in young loquat fruits after low temperature stress. *Sci. Silvae Sin.* **2011**, *47*, 37-42.
51. Grace, S.C.; Logan, B.A. Acclimation of foliar antioxidant system to growth irradiance in three broad-leaved evergreen species . *Plant Physiol.* **1996**, *112*, 1631-1640.
52. Rüegsegger, A.; Brunold, C. Effect of cadmium on γ-glutamylcysteine synthesis in maize seedlings. *Plant Physiol.* **1992**, *99*, 428-433.