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Full Length Article

The Effect of Glutathione in the Regulation of the Degradation of Residual Fungicide in Tomato

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Abstract

It is a threat to human health that pesticide residues in vegetables worldwide. Glutathione plays an important role in the degradation of pesticides in plant. In order to explore the role of glutathione in the regulation of pesticide metabolism in plant, tomato plants were pretreated with reduced glutathione (GSH), oxidized glutathione disulfide (GSSG), glutathione synthesis inhibitor (BSO) and H_2O(CK) respectively, and glutathione related detoxification system were investigated. The results show that the increase of reduced glutathione could induce the ratio of GSH/GSSG and the activity of glutathione-related detoxification enzyme (GST) and antioxidant enzymes (GPX and GR) directly or indirectly, and contribute to the formation of non-protein thiol. Glutathione not only works as a substrate for CHT conjugation, but also induces detoxification enzyme and antioxidant enzymes associated with Chlorothalonil (CHT) metabolism and detoxification. Consequently, glutathione promoted the CHT metabolism processes and finally reduced CHT residue in plant. These observations provide evidences for the involvement of the glutathione and glutathione-dependent redox signal are critical for the induction of detoxifying response against pesticides in tomato plants. Our study provides strong evidences for understanding the mechanism of glutathione in the detoxification of CHT *in vivo*.

Keywords: Glutathione; Pesticide; Redox; Detoxification; Antioxidant

Introduction

It is an important and effective measure to control pathogen, pest and weed with pesticide in modern agriculture. And the crop yield may be lost by 80% without pesticides Zhou *et al.* (2015). However, in the production of vegetable, it is common that the use of pesticide is excessive or not suitable, which causes a series of changes in physiological and biochemical reactions in the vegetable, eventually leading to a decline in plant yield, quality, and resistance. And the excessive application of pesticides also results in pollution to agricultural products and the environment in most countries in the world, which has become a serious problem of food safety and could directly endanger human health Bucker-Neto *et al.* (2017). Excessive residual pesticide in vegetable will not only cause acute and chronic poisoning, but also may cause cancer and other chronic diseases. In addition, it is harmful for human health that long-term uptake of low-dose residual pesticides and even human reproduction by disrupting endocrine balance Alavanja *et al.* (2013). Therefore, the problem of pesticide residues in vegetables poses a serious threat to food safety and people's health. Simultaneously, the problem of pesticide residues in vegetables has also affected the international competitiveness of agricultural products. Therefore, it has become an urgent problem to reduce pesticide residues in vegetables and explore the mechanism of degradation and metabolism of residual pesticides in plants. Until now, there are only few studies focused on the degradation metabolism of pesticides in plants, especially on the degradation of fungicides.

In fact, plants have developed some detoxification mechanism to relieve the negative impacts of herbicide. The detoxification of herbicides in plant could be divided into diverse processes: (Phase I) transformation mainly catalyzed by peroxidases or P450 enzymes, (Phase II) conjugation with glucose or glutathione primarily catalyzed by UGT or GST Rouhier *et al.* (2008), and (Phase III) further degradation of metabolites are transported to apoplast or vacuoles Huber *et al.* (2009).Glutathione is involved not only in the detoxification of herbicides, but also in cellular reactions related to redox homeostasis and signal. Glutathione can endure reversible oxidation and reduction in cells to protect cells against lesion from free radicals Geu-Flores *et al.* (2011), and oxidized glutathione disulfide (GSSG) can be restored into reduced glutathione (GSH) catalyzed by glutathione reducase (GR) Martya *et al.* (2009).

It has been demonstrated that glutathione plays a critical role in the regulation of chlorothalonil (2,4,5,6-tetrachloroisophthalonitrile, CHT) detoxification metabolism with brassinosteroids in our previous study

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Zhou *et al*. (2015). However, the participation mechanism of glutathione is still not clear. Therefore, it is worth to study the pesticide degradation metabolism in plant related to glutathione. Chlorothalonil, widely used in agriculture for pathogen control, was taken as experiment material in the present study, and reduced glutathione (GSH), oxidized glutathione (GSSG), and glutathione synthesis inhibitor (BSO) were used as pretreatments. The objective of this study is to explore the mechanism of glutathione related degradation of CHT in tomato, in order to provide strong evidences for understanding the mechanism of glutathione in the detoxification of CHT *in vivo*.

Materials and Methods

Plant Materials

Tomato (Zhongza No. 4 bought from China Agricultural University) seeds were germinated and plants were grown in a mixture (peat:vermiculite 7:3). The tomato plants were raised under following growth conditions: the temperature was kept at 25°C and 120 mol m^{-2} s^{-1} light for 14 h in the day, and the temperature was kept at 20°C and dark for 10h in the night.

Chemical Treatments and Sample Harvesting

Tomato plants with 6 true leaves fully expanded were pretreated with GSH (5mM), GSSG (5 m*M*), BSO (1 m*M*) as treatments (Sigma, USA), respectively and deionized water as control to explore the role of glutathione in the regulation of pesticide degradation. After 24 h, tomato leaves were sprayed with chlorothalonil (CHT) at 11.2 m*M* with 30 mL per plant (commercial CHT 75% active ingredient). The plants were sampled at 0d, 1d, 3d, 5d, 7d after pesticide treatment for biochemical analysis. And plant samples were harvested 7d after CHT application to analyze the residue of CHT in tomato plants.

Measurement of the Content of GSH and GSSG, the Content of DHA and ASA, and Estimation of the Concentration of Non-Protein Thiol

The level of GSH and GSSG were investigated according to the method of Rahman *et al.* (2007). The content of DHA and ASA were analyzed according to Foyer & Halliwell. The concentration of non-protein thiol (NPT) were determined according to the method of Qian *et al*. (2005).

Assay of GST and GR activity

0.3 g leaf samples were extracted in 2 mL 50 m*M* PBS buffer (pH 7.5) with 10 m*M* KCl, 1 m*M* EDTA, 5 m*M* DTT, 0.5 m*M* AEBSF and 1:4 insoluble PVPP to determine the activity of glutathione reductase (GR) and glutathione S-transferase (GST). The homogenates of plant tissue were centrifuged at 14000 rpm for 20 min, and the supernatants were used to analyze the activity of enzyme. All procedures were maintained between 0°C to 4°C. The activity of GST was measured at the absorbance of 412 nm as described by Wang *et al.* (2010). The activity of GR was assayed dependent on the rate of decrease at the absorbance of 340 nm according to Foyer & Halliwell.

Quantification of CHT in Plant Tissue

To assay the level of CHT residue in tomato, 10 g tomato leaves were homogenized 80 mL petroleum ether and 40 gNa_2SO_4 for 12 h. The mixture was filtered, and the filtrates were collected and dried with rotary evaporators. To analyze the level of CHT, N-hexane was used to dissolve pesticide, and the volume was adjusted to 5 mL, and gas chromatography (GC) with ECD and a capillary column (30 m length, 0.32 mm internal diameter and 0.25 μm film thickness) (Agilent, Santa Clara, CA, USA) was applied. Nitrogen (3.3 mL min^{-1}) was employed as carrier gas, and the injector port temperature was set at 250℃, while the detector temperature was set at 300℃, and column temperature was raised from 80℃ to 260℃ (25℃ min^{-1}), and then maintained for 3.8 min. CHT (Institute for the Control of Agrochemicals, Ministry of Agriculture, Beijing, China) was applied was used as standard. The level of residual CHT in plant tissue was determined according to the method of Kurz *et al*. (2008).

Statistical Analysis

Assay was performed with three replicates. SPSS was used to analyze the statistic. The data were subjected to analysis of variance, and the means were compared using Tukey's test at the 5% level.

Results

The level of Chlorothalonil Residue in Tomato

Chlorothalonil (CHT) is a chlorinated fungicide used extensively in pathogens control in agriculture. To determine whether glutathione participates in the pesticide degradation metabolism in plants, the level of CHT residue in tomato with glutathione related compounds application were investigated tested. As shown in Fig. 1, exogenous pretreatment with reduced glutathione (GSH) significantly reduced the residue of CHT in the tomato plants and the level of CHT residue reduced by 23.9% compared with the control. In contrast, the concentration of CHT residues in the plants with application of oxidized glutathione (GSSG) and glutathione synthesis inhibitor (BSO) were 44.6% and 22.9% higher than the control plants, respectively. The results revealed that glutathione and related redox signal were involved in the regulation of CHT degradation in tomato.

GSH and GSSG Content in Tomato in Response to Different Pretreatments with the Application of CHT

Reduced glutathione in tomato plant was induced by the application of GSH, and the content of reduced glutathione was significantly higher than control pretreated with water between 0-7 days (Fig. 2a). Contrary to the GSH-induced sharp increase in the content of reduced glutathione, there was a decrease in the content of oxidized glutathione in CHT exposed plants. However, no significant difference in the content of reducing glutathione in tomato with the pretreatment of GSSG between 0-3 days compared with the control, while the content of reduced glutathione was significantly lower than the control since the 3rd day after CHT treatment. But the oxidized glutathione content in tomato treated with GSSG was increased since CHT treatment (Fig. 2b). The content of GSH and GSSG in tomato plants pretreated with BSO were all significantly lower than those of control, indicating that BSO inhibited the synthesis of GSH and GSSG significantly.

ASA and DHA Content in Tomato in Response to Different Pretreatments with the Application of CHT

With the pretreatment of GSH, reduced ascorbic acid (ASA) in tomato plant was induced between 0-1 days after CHT application compared with the control (Fig. 3a), while the content of oxidized ascorbic acid (DHA) decreased significantly between 0-7 days (Fig. 3b). Compared to the control, ASA and DHA were both induced by the pretreatment of GSSG, and the difference was significant except for the 3rd day after CHT treatment. With the pretreatment of BSO, the content of ASA was significantly higher than the control between 0-1 days and the 7th day after the application of CHT. However, the content of DHA increased significantly in tomato plants pretreated with BSO all the time after the application of CHT compared with the control and other pretreatments. All these results suggested that glutathione could induce the metabolism of ascorbic acid to participate in the CHT degradation in tomato plants.

Non-protein Thiol Content in Tomato in Response to Different Pretreatments with the Application of CHT

The content of non-protein thiol in tomato is associated with glutathione conjugates level, which could reflect the decomposition products of pesticide to a certain extent. The content of non-protein thiol in tomato was significantly up-regulated with the pretreatment of GSH compared with the control after the application of CHT (Fig. 4). Contrary to the GSH-induced sharp increase in the content of non-protein thiol, there was a decrease in the level of non-protein thiol with the pretreatment of GSSG in CHT exposed plants, and the difference become significant since the 1st day after the application of CHT. The content of non-protein thiol in tomato was also down-regulated with the pretreatment of BSO, and the content of non-protein thiol in tomato was

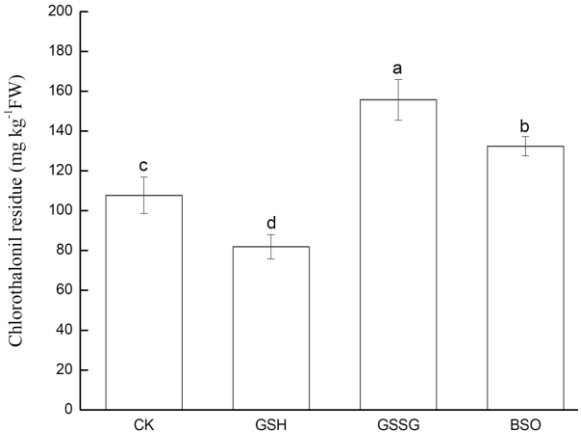

Fig. 1: CHT residue in response to glutathione in tomato

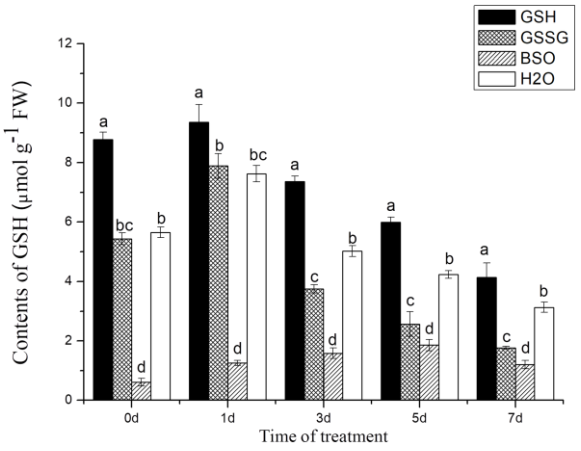

Fig. 2a: Reduced glutathione of tomato in response to different pretreatments

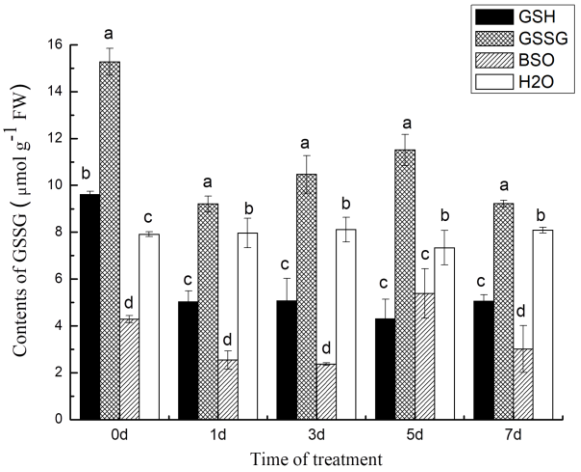

Fig. 2b: Oxidized glutathione of tomato in response to different pretreatments

significantly lower than the control, although there was no significant difference between BSO pretreated plants and the control at the 5th day after the application of CHT.

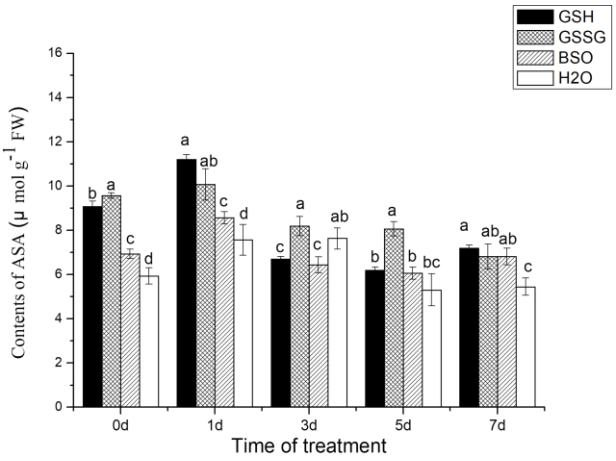

Fig. 3a: ASA of tomato in response to different pretreatments

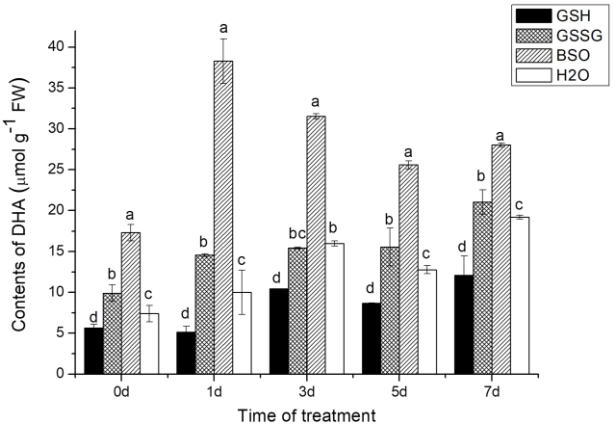

Fig. 3b: DHA of tomato in response to different pretreatments

All the results indicated that GSH could enhance the metabolism of CHT in tomato plant, and increased the content of non-protein thiol, while the inhibition of biosynthesis and regeneration of glutathione weaken the degradation of CHT.

The Activity of GST in Tomato in Response to Different Pretreatments with the Application of CHT

It is well known that glutathione S-transferase (GST) plays an important role in the detoxification of xenobiotics in plant, and participates in the Phase II detoxification reaction of herbicides in plant. The change of GST activity was shown in Fig. 5. The results showed that the activity of GST was induced significantly by GSH pretreatment since the application of CHT compared with the control. However, there was no significant difference between GSSG pretreatment and the control, and the activity of GST was significantly lower than the control since the 3rd day after the application of CHT. Contrary to the GSH-induced sharp

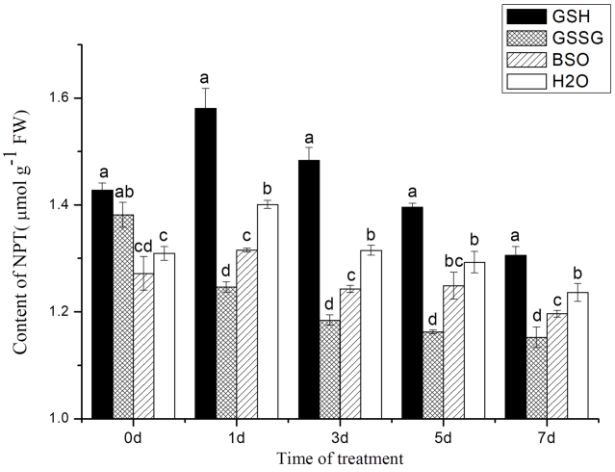

Fig. 4: The content of non-protein thiol in tomato in response to different pretreatments

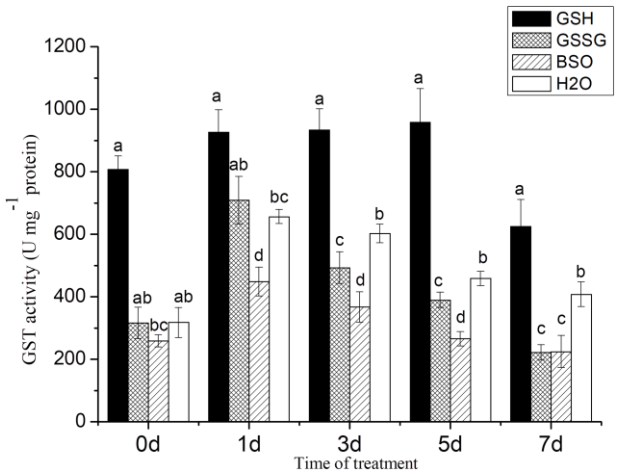

Fig. 5: The activity of GSTs in tomato in response to different pretreatments

increase of the GST activity, there was a decrease in the GST activity with the pretreatment of BSO in CHT exposed plants, and the difference between BSO pretreatment and the control become significant since the 1st day after the application of CHT. Taken together, glutathione enhanced CHT degradation by the increase of GST activity.

The Activity of GPX in Tomato in Response to Different Pretreatments with the Application of CHT

Glutathione peroxidase (GPX) is one of the important antioxidant enzymes associated with stress in plant. The results showed that the activity of GPX was also induced by GSH pretreatment since the application of CHT compared with the control, and the difference was significant except for the 3rd day after the application of CHT (Fig. 6). However, there was almost no significant difference of the activity of GPX between GSSG

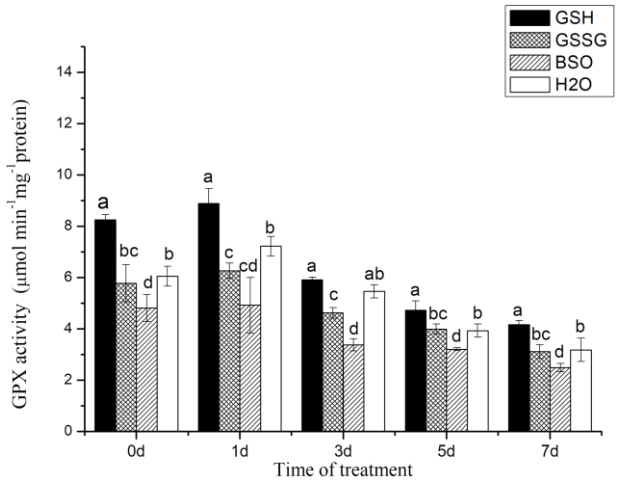

Fig. 6: The activity of GPX in tomato in response to different pretreatments

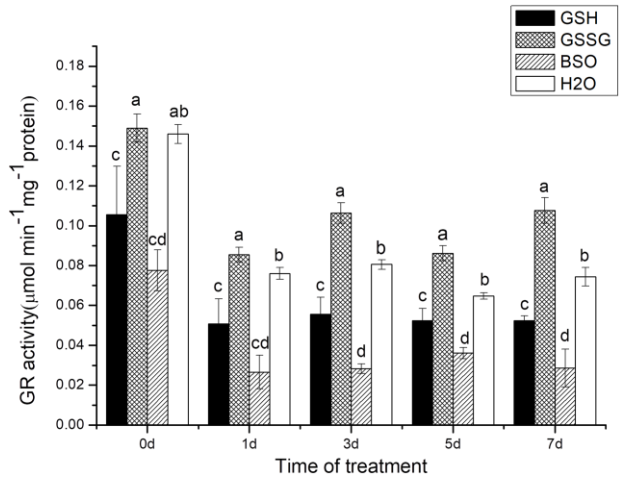

Fig. 7: The activity of GR in tomato in response to different pretreatments

pretreatment and the control. Contrary to the GSH-induced sharp enhancement of the GPX activity, there was a weaken effect in the GPX activity with the pretreatment of BSO in CHT exposed plants, and the difference between BSO pretreatment and the control was significant since the application of CHT. The results showed that the change tendency of GPX activity was similar to GST activity, which was also associated in the degradation of CHT.

The Activity of GR in Tomato in Response to Different Pretreatments with the Application of CHT

Glutathione reductase (GR) catalyzes oxidized glutathione into reduced form, which regulates the redox status of glutathione in plant. The activity of GR enzyme in tomato was significantly down-regulated with the pretreatment of GSH after the application of CHT, due to the inhibition of reaction products catalyzed by GR enzyme (Fig. 7). Contrary to the GSH-induced sharp decrease of the GR activity, there was an significant increase in the GR activity with the pretreatment of GSSG since the application of CHT. Pretreatment with BSO inhibited the activity of GR significantly compared with the control, and the activity of GR was the lowest in tomato compared to the control and other pretreatments since CHT treatment. The results provided strong evidence that glutathione regeneration was involved in the degradation metabolism of CHT *in vivo*.

Discussion

The results of our study revealed that reduced glutathione (GSH) pretreatment could promote the degradation of chlorothalonil in tomato plants. While oxidized glutathione (GSSG) pretreatment and glutathione synthesis inhibitors (BSO) had inhibitory effects on the metabolism of chlorothalonil. Especially, a significant increase in the CHT residue was observed in GSSG treated plants. However, The content of non-protein thiol, associated with the content of glutathione conjugates, which was significantly down-regulated in plants with GSSG or BSO pretreatment. The results provided an important evidence to confirm that glutathione participated in CHT degradation metabolism in plant *in vivo*. In agreement with this, the phenomena also reveals the detoxification of CHT through glutathione conjugation *in vitro* reaction Wang *et al.* (2010). Due to pesticides with electrophilic centers could be conjugated to the nucleophilic thiol and the cysteine residue of glutathione by the catalysis of GST and other enzymes or non-enzymatic action Geu-Flores *et al*. (2011). And then the glutathione conjugates are degraded into the Cys conjugates via the sequential action in vacuole Ohkama-Ohtsu *et al*. (2007).

On the base of this study, the different effect of glutathione content and its mediated redox signal on the degradation of CHT were discussed. The changes in the redox system have a greater effect than the changes in the antioxidant content in plant Kocsy *et al.* (2009). It was found that pretreatment with GSH could increase the content of reduced glutathione and non-protein thiol, while decrease the content of oxidized glutathione in tomato. Simultaneously, the degradation of CHT was enhanced significantly in the tomato plants. To the opposite, BSO inhibited the synthesis of GSH and GSSG significantly, and also restrained the formation of non-protein thiol in tomato, and declined the CHT degradation in tomato. However, pretreatment with GSSG down-regulated the content of reduced glutathione and non-protein thiol to a certain degree, but up-regulated the content of oxidized glutathione, and increased the CHT residue significantly in tomato, which was higher than the BSO pretreatment. The result indicated that GSSG pretreatment was more effective than BSO pretreatment in inhibiting the degradation of CHT. That was, the glutathione related redox signal exerted negligible

influence in the regulation of CHT degradation in plant. Consistent with this, the increase in the content of GSH led to a higher rate of GSH/GSSG, which could regulate the activity of detoxification enzymes and related genes as signal. It means that the pesticide detoxification process is related to the redox homeostasis in plant cell Zhou *et al.* (2015). Recently, it is reported that TGA transcription factors is involved in the detoxification network via activation of redox signal Ramel *et al.* (2012). It has been also demonstrated that glutathione plays an important role in changing the redox state of cell to relieve the harm caused by abiotic stress Maughan and Foyer (2006).

Many studies also revealed that ascorbate has an indispensable role in the relief of the harm caused by abiotic stress, so it is important to maintain the stability of ascorbate pool and redox status in plant Gomez *et al.* (2004). In our study DHA accumulation was significantly reduced with pretreatment of GSH in tomato plants treated with CHT. Contrary to this, the increase of ascorbate pool (ASA and DHA) was induced significantly with pretreatment of BSO or GSSG in plants treated with CHT. It seems likely that when the content of glutathione or the ratio of GSH/GSSG reduced in plant, ascorbate was enhanced to work together with glutathione to complete the scavenging function of ROS and maintain the redox balance to reduce the damage caused by ROS in plant. It is also proposed that the ASA could not only induce plant defense against abiotic stress, but also remove ROS directly Hayes *et al.* (2001). The changes in contents of antioxidant GSH and ASA and redox status in plants also reflect their response to abiotic stress.

GST was known as a major enzyme, catalyzing the conjugation of glutathione to pesticide in the detoxification processes of pesticides Kim *et al*. (2004). And the results in our study revealed that glutathione enhanced CHT degradation by the increase of GST activity. GST is known for its conjugate of glutathione and xenobiotics and conversion to nonreactive water-soluble conjugates, which could be easily excreted Muntane (2009). Thus GST is important for the induction of detoxifying response against pesticides involved in glutathione.

Glutathione in plant not only acts as a substrate for the GST detoxification enzyme, but also serves as a substrate or product for the reaction of the antioxidant enzymes, such as GPX and GR, and participates in the clearance of H_2O_2 from plants Li (2002). GPX is a thiol-containing peroxidase that can scavenge H_2O_2, organic hydroperoxides, and lipid peroxides in plant and block further damage to the plant by ROS radicals Margis *et al.* (2008). The results in our study demonstrated that pretreatment with GSH induced a sharp enhancement of the GPX activity, to the opposite, there was a significant weaken effect in the GPX activity with the pretreatment of BSO in CHT exposed plants. It was probably that GPX played an important role in the elimination of ROS caused by CHT application in plant, which is contributed to the induction of detoxifying response of glutathione. It is also confirmed that AtGPX8 is involved in stress tolerance and has the ability to eliminate ROS and prevent DNA damage by gene overexpression and gene silencing mutants Gaber *et al*. (2012). Additionally, both GSH and TRX can act as an electron donor for the GPX in tomato, and GSH can be used to reduce organic hydroperoxides such as alkyls, fatty acids and phospholipids in vitro prokaryotic expression Herbette *et al*. (2002).

Currently, the role of glutathione in pesticide degradation metabolism is still not clear. In this study, the activity of GR enzyme in tomato decreased significantly with the pretreatment of GSH after the application of CHT, due to the inhibition of reaction products catalyzed by GR enzyme, contrary to this, with the pretreatment of GSSG. In plant antioxidant enzymatic systems, glutathione reductase (GR) is also an important enzyme in protecting plant cells from oxidative damage caused by pesticides. Glutathione may influence the content of H_2O_2 through its antioxidation because glutathione status is involved in H_2O_2-triggered signal transduction Mhamdi *et al*. (2010). Accordingly, it is still unclear whether changes of GR related glutathione redox status are themselves sensed or affect gene expression through indirect effects.

Conclusion

In summary, it is shown that the change of glutathione level or glutathione related signal led to significant change in the efficiency of CHT degradation in plants, which was associated with altered detoxification system and antioxidant system. It is strongly suggested that the increase of reduced glutathione could induce the ratio of GSH/GSSG and the activity of glutathione-related detoxification enzymes and antioxidant enzymes directly or indirectly, and contribute to the formation of non-protein thiol. Consequently, glutathione promoted the CHT degradation process and finally reduced the residue of CHT in plant. Thus, glutathione and related redox signal are critical for the induction of detoxifying response and the degradation of residual pesticides in plant. It would be positive to increase the content of glutathione in plant for food safety.

Acknowledgments

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