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Glutathione biosynthesis and regeneration play an important role in the metabolism of chlorothalonil in tomato

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HIGHLIGHTS

- Glutathione is required for detoxification of chlorothelonil (CHT) in tomato *in vivo*.
- Silencing of *GSH1*, *GSH2* and *GR1* all resulted in decreased glutathione synthesis.
- *P450*, *GST* & *ABC* transcripts were downregulated by *GSH1*, *GSH2* & *GR1* gene silencing.
- Gene silencing decreased GST activity but increased CHT residue in plant.
- Glutathione biosynthesis and regeneration are required for CHT metabolism *in vivo*.

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ABSTRACT

Glutathione is one of the major endogenous antioxidants produced by cells. In plants, glutathione is crucial for both abiotic and biotic stress resistance, and also involved in the detoxification of xenobiotics in many organisms. However, as *in vivo* evidences of glutathione function are still lacking so far, its roles in plants are still poorly understood. In this study, we investigated the changes of thiols, glutathione homeostasis and transcripts of genes potentially involved in chlorothalonil (CHT) metabolism in tomato (*Solanum lycopersicum* L.). Two genes (*GSH1*, *GSH2*) encoding γ-glutamylcysteine synthetase and glutathione synthetase, respectively, and a gene for glutathione reductase (*GR1*) involved in glutathione regeneration were silenced by virus induced gene silencing (VIGS) approach. Silencing of *GSH1*, *GSH2* and *GR1* decreased glutathione contents and the ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), but increased CHT residues in plant tissues. The *GSH1* and *GR1* silenced plants showed the lowest GSH level and ratio of GSH/GSSG, respectively. Transcripts of *P450*, *GST* and *ABC* transporter genes as well as glutathione *S*-transferase (GST) activity were induced after CHT treatment. However, the increases of these transcripts were compromised in *GSH1*, *GSH2* and *GR1* silenced plants. This study indicates that glutathione not only serves as a substrate for CHT conjugation, but is also involved in regulation of transcripts of gene in pesticide metabolism via controlling redox homeostasis.

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1. Introduction

Chlorothalonil (CHT, 2,4,5,6-tetrachloroisophthalonitrile) is a broad-spectrum chlorinated fungicide, widely used in agriculture for its high efficacy in the control of pathogens such as blight on potatoes (Pimentel, 1995; Garron et al., 2011). However, the excessive use of pesticide results in pollution of environment and agricultural products, which could directly endanger human health (Pimentel, 2005; Ma and Ren, 2011). Therefore, pesticide residue elimination becomes a serious issue that needs to be handled. Until now, extensive researches have focused on the bioremediation of organic pollutants using enzymes produced from the microorganisms (Singh et al., 2008; Megharaja et al., 2011). In contrast, there are few studies relating the pesticide residue to the metabolism or biotransformation of pesticides in plants.

Abbreviations: ABC, adenosine triphosphate-binding cassette transporter; CHT, chlorothalonil; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; NPT, non-protein thiol; ROS, reactive oxygen species; qRT-PCR, quantitative real-time polymerase chain reaction; TT, total thiol; VIGS, virus-induced gene silencing.

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Plants display either one or a combination of responses to xenobiotics, and develop a positive detoxification mechanism in order to avoid the deleterious effects of xenobiotics. Most often, the biotransformation mechanisms are related to the functional reactive groups or linkages in the compounds which are susceptible to enzymatic or chemical attack, rather than the dependence on the mode of action and physiological lesions involved in the xenobiotics activity (Casida and Lykken, 1969). Detoxification of xenobiotics by plant are composed of diverse processes, which can be divided into three phases viz. transformation (Phase I), conjugation (Phase II), and storage (Phase III) (Van Eerd et al., 2003). Phase I reactions are primarily catalyzed by cytochrome *P450* enzymes and peroxidase (Yun et al., 2005). Conjugations with glutathione and glucose are catalyzed by glutathione *S*-transferase (GST) and UDP-glycosyltransferase (UGT), respectively, referring to Phase II reaction (Korte et al., 2000; Rouhier et al., 2008). Phase III reactions involve sequestration and storage of metabolites in vacuole or apoplast (Huber et al., 2009).

From recent study it's clear that glutathione is involved in the detoxification of xenobiotics in plants (Geu-Flores et al., 2011). Besides detoxification of xenobiotics, glutathione is also involved in numerous cellular processes including redox homeostasis and redox sensing, which protect cells against oxidative stress caused by reactive oxygen species (ROS) and other free radicals (Meyer, 2008). Glutathione is the most abundant low-molecular-weight tripeptide with thiol, composed of glutamic acid, cysteine and glycine. It is synthesized by two enzyme-catalyzed steps in an ATP-dependent process. The enzyme γ-glutamylcysteine synthetase (γ-ECS) encoded by *GSH1* gene (Queval et al., 2009) catalyzed the formation of peptide bond between γ-glutamate and α-cysteine at the first stage, and then glycine is added to the dipeptide (γ-glutamyl-α-cysteine) with the participation of glutathione synthetase enzyme (GS) encoded by *GSH2* (Katerova and Miteva, 2010). Glutathione can be reversibly endured by oxidation and reduction in cells. The reduced glutathione (GSH) could be restored from oxidized glutathione disulfide (GSSG) by the catalysis of glutathione reducase (GR), which is encoded by *GR1* gene (Martya et al., 2009).

Previously, we have demonstrated *in vitro* evidence that glutathione plays an important role in the detoxification of fungicides via participation of plant GST (Wang et al., 2010). However, no *in vivo* or genetic evidence has been reported that glutathione is required for the detoxification of pesticide in plant. In this regard, further exploration of roles of glutathione *in vivo,* especially the glutathione dependent pesticide metabolism in plant needs in-depth study. Therefore, our study here was aimed to investigate the mechanism of biotransformation and degradation of CHT via regulation of glutathione metabolism components including *GSH1*, *GSH2* and *GR1* in tomato (*Solanum lycopersicum* L.) plants. Strong evidences were demonstrated here for the involvement of glutathione in the detoxification of CHT *in vivo*.

2. Materials and methods

2.1. Plant materials and growth conditions

Tomato (*S. lycopersicum* L. cv. Zheza No. 205) seeds were germinated and grown in a mixture of peat and vermiculite (7:3, v/v) under the following growth conditions: a 16 h light at 120 μmol m^{-2} s^{-1}/8 h dark photoperiod, and a day/night temperature regime of 23/20 °C. Plants were used for virus-induced gene silencing (VIGS), when cotyledonary leaves were fully expanded and the true leaves were not yet appeared.

2.2. VIGS in tomato

Gene silencing was conducted using the pTRV1 and pTRV2 vectors as previously described (Liu et al., 2002). PCR products were cloned with primers introducing unique BamHI and XbaII sites and cloned in geminivirus satellite TRV vectors to generate pTRV2-GSH1, pTRV2-GSH2, pTRV2-GR1. The resulting plasmids were subsequently introduced into *Agrobacterium tumefaciens* strain GV3101. In order to silence *GR1*, *GSH1* and *GSH2* genes in tomato, 474, 455 and 380 bp sequences of corresponding genes were PCR-amplified respectively from tomato cDNA using primers as following:

GR1: 5′-TGCTCTAGAAGCCATAGAGGTTGACGA-3′ and 5′-CGCG GATCCTCTGCTGCTGTAGGGTGA-3′; *GSH1*: 5′-TGCTCTAGAGAAC CCTGCGACCCAT-3′ and 5′-CGCGGATCCAAGACCAGCACGGAAC-3′; *GSH2*: 5′- TGCTCTAGACCAGCCAAACGTAAAG-3′ and 5′-CGCGGAT CCAAGTCCACGAAGAGGG-3′.

For VIGS assay, a mixed culture of *A. tumefaciens* bearing pTRV1: pTRV2-target gene in a 1–1 ratio (OD_{600} = 0.9 for each construct) was co-infiltrated into fully-expanded cotyledonary leaves of tomato plants. *Agrobacterium* cultures carrying TRV empty vector TRV2 were also co-infiltrated to set as a control. Inoculated plants were placed in an insect-free growth room at 22 °C with supplemental light at 120 μmol m^{-2} s^{-1} for 14 h per day. After 6 weeks, quantitative RT-PCR was performed to determine the gene silencing efficiency before using the plants for further research.

2.3. CHT treatment and sample harvesting

Gene silenced plants were verified by quantitative real-time polymerase chain reaction (qRT-PCR) and treated with 11.2 mM commercial CHT (75% active ingredient, Chemical Industry Ltd., Nanjing, China) using sprayer. After about 24 h, samples were harvested for biochemical and gene expression analyses. Samples for pesticide residue analysis were taken after 7 d of CHT treatment.

2.4. Gene transcript analysis

Total RNA was extracted from leaf tissues using the Total RNA Miniprep Kit (Axygen Biosciences, CA, USA) according to the manufacturer's protocol. Total RNA was reverse-transcribed by using ReverTra Ace qPCR RT Kit (Toyobo, Japan) following the supplier's recommendation. In addition to *GSH1*, *GSH2* and *GR1*, one *P450* (*CYP72A*), three GST genes (*GST1*, *GST2* and *GST3*) and three ABC transporter genes (*ABC2*, *ABC3* and *ABC4)* were selected for elucidating the role of glutathione redox homeostasis in the CHT detoxification. The sequences of primers used were listed in Table 1.

For each candidate of gene, the level of transcript was normalized using the housekeeping *Actin* gene. qRT-PCR was performed using iQ SYBR Green SuperMix (Takara, Japan) in an iCycler iQ 96-well real-time PCR detection system (Bio-Rad, Hercules, CA) with iCycler software to calculate threshold cycle values. For each qRT-PCR experiment, three biological repetitions were performed, and the mean values were calculated.

2.5. Measurement of total and oxidized glutathione level and estimation of total and non-protein thiol concentration

The levels of GSH and GSSG were determined according to the method of Rahman et al. (2006) with some modifications (Rahman et al., 2006). To determine GSH and GSSG level in plant, 0.3 g frozen leaf tissue was homogenized and extracted in 2 mL 0.1 M sodium phosphate buffer containing 5 mM EDTA (pH 7.5). After micro-centrifugation (20 min, 12000*g*), total glutathione was measured with the supernatant. The assay was based on the reaction of GSH with DTNB [5,5′-dithio-bis (2-nitrobenzoic acid)] that produces the