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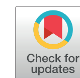
Glutaredoxin GRXS16 mediates brassinosteroid-induced apoplastic H_2O_2 production to promote pesticide metabolism in tomato☆



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Brassinosteroids (BRs), a group of steroid phytohormones, are involved in multiple aspects of plant growth, development and stress responses. Despite recent studies on BRs-promoted pesticide metabolism in plants, the underlying mechanisms remain poorly understood. Here, we showed that 24-epibrassinolide (EBR) significantly enhanced the expression of *RESPIRATORY BURST OXIDASE HOMOLOG1* (*RBOH1*) and H_2O_2 accumulation in the apoplast of chlorothalonil (CHT, a broad spectrum nonsystemic fungicide)-treated tomato plants. Silencing of *RBOH1* significantly decreased the efficiency of EBR-induced CHT metabolism. Moreover, the EBR-induced upregulation in the transcripts of glutaredoxin gene *GRXS16* was suppressed in *RBOH1*-silenced plants. Further studies indicated that silencing of *GRXS16* compromised EBR-induced increases in glutathione content, activity of glutathione *S*-transferase (GST) and transcript of *GST1*, leading to an increase in CHT residue. By contrast, overexpression of tomato *GRXS16* enhanced the basal levels of glutathione content and GST activity that eventually decreased CHT residues in transgenic plants. Our results reveal that BR-mediated induction of a modest oxidative burst is essential for the acceleration of glutathione-dependent pesticide metabolism via redox modulators, such as GRXS16. These findings shed new light on the mechanisms of BR-induced pesticide metabolism and thus have important implication in reducing pesticide residues in agricultural products.
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1. Introduction

Since the “Green Revolution”, pesticide application has become an integral part of common agricultural practices for increasing the yield and quality of crops throughout the world (Jez et al., 2016; Van Lenteren, 2000). However, the inappropriate use of pesticides has raised a huge public concern regarding pesticide residues in food crops, especially in greenhouse-grown vegetables. Because the temperature and relative humidity in the greenhouse are favorable for the outburst of plant diseases, fungicides are frequently applied (every 7–10 d) to protect crops from phytopathogens (Jin et al., 2014). Notably, the pesticide residues in vegetable crops dissipate at a slow rate as a result of the reduced airflow and rainfall, limited volatilization and leaching, and retarded photodegradation due to filtration of sunlight by a cover layer in the greenhouse (Rial-Otero et al., 2005; Fang et al., 2006). In addition, the release of pesticides into the environment has substantially increased health problems, such as chronic neurotoxicity, endocrine disruption, immune impacts, genotoxicity, mutagenicity and carcinogenesis (Dabrowski et al., 2014; Blair et al., 2015).

Pesticides are not only toxic to the environment and human health but also phytotoxic as they induce oxidative stress through production of reactive oxygen species (ROS), lipid peroxidation, protein damage and DNA breaks (Ramel et al., 2012). Sensing of the oxidative stress is crucial for activating efficient responses towards detoxification of pesticides (Riechers et al., 2010). Generally, the detoxification process can be divided into three to four phases. In phase I, pesticides undergo the cytochrome P450 monooxygenase-catalyzed oxidation, and the esterases- and amidases-catalyzed hydrolysis. In phase II, the resulting products are conjugated to

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glutathione or glucose, involving glutathione *S*-transferases (GSTs) or glucosyl transferases, respectively. In phase III, the conjugates are either secreted and deposited in the cell wall or transported to the vacuole by ATP-binding cassette (ABC) transporters. Finally, pesticide-glutathione conjugates may be further processed, partially degraded or subject to secondary conjugations in phase IV (Coleman et al., 1997; Riechers et al., 2010; Behringer et al., 2011). In tomato plants, when the glutathione metabolism-related and GST-coding genes are silenced, the *in vivo* pesticide degradation capacity of plants is largely compromised (Yu et al., 2013; Zhou et al., 2015). This phenomenon highlights the importance of GSH conjugation-mediated detoxification in reducing pesticide residue in vegetable crops.

During oxidative stress, for example, a situation that occurs after pesticide application, plants use glutathione to scavenge excessive ROS (Riechers et al., 2010). The imbalance in reduced (GSH) to oxidized (GSSG) glutathione enhances glutathione biosynthesis in plants (Hicks et al., 2007; Noctor et al., 2012). As the redox environment becomes more reducing, the GSH/GSSG couple can modify the activity of various compounds (enzymes, regulatory proteins) directly through the reduction/oxidation of their disulfide bridges/sulfhydryl groups and through the (de)glutathionylation of sulfhydryl groups via glutaredoxins (GRXs) (Rouhier et al., 2008; Szalai et al., 2009). The GSH/GSSG couple may also have a direct or indirect regulatory role at the transcriptional level (Ball et al., 2004). However, it is unclear whether the glutathione-mediated redox system contributes to the activation of plant detoxification, leading to a reduction in pesticide residues in vegetables.

Brassinosteroids (BRs), a group of steroid phytohormones, play critical roles in multiple aspects of plant growth and development (Vriet et al., 2012). In addition, BRs function in plant resistance to different types of diseases (Nakashita et al., 2003) and tolerance to a range of abiotic stresses (Kagale et al., 2007; Bajguz and Hayat, 2009; Divi et al., 2010). The apoplastic H_2O_2 generated by RESPIRATORY BURST OXIDASE HOMOLOG1 (RBOH1) has been shown to be an essential component in BR-induced stress signaling pathway (Xia et al., 2009; Nie et al., 2013; Zhou et al., 2014). In addition, the apoplastic H_2O_2 can mediate the BR-induced pesticide metabolism in tomato plants (Zhou et al., 2015). However, it is unclear whether glutathione-mediated redox system acts downstream of the apoplastic H_2O_2 to regulate the pesticide metabolism. Here, we show that expression of *GRXS16* is regulated by BR in an RBOH1-dependent manner. The transcript levels of *GRXS16* are correlated well with the glutathione content, GST activity and pesticide metabolism in tomato plants. The results also suggest that regulation of GRX activity in plants can be exploited to decrease pesticide residues for assuring food safety in vegetable products.

2. Methods and materials

2.1. Plant growth conditions

Seeds of wild-type (WT) tomato (*Solanum lycopersicum* L. cv. Condine Red) were obtained from the Tomato Genetics Resource Center (http://tgrc.ucdavis.edu). Seeds were sown in a mixture of peat and vermiculite (2:1; v/v) in a growth chamber under a 12 h light (200 μmol m^{-2} s^{-1} at 21 °C) and 12 h dark (at 18 °C) cycle. The relative humidity was kept at 70%. The seedlings were watered with full-strength Hoagland's nutrient solution every two days. When the second true leaf emerged, the plants were transferred to pots (height × diameter, 15 cm × 10 cm).

2.2. Virus-induced gene silencing

The tobacco rattle virus (TRV)-based vector was used for virus-induced gene silencing (VIGS). The cDNA fragments of *RBOH1*, *GRXS14*, *GRXS15*, *GRXS16*, *GRXS17* were PCR amplified using the gene-specific primers (Supplementary Table S1). After digestion with restriction enzymes, the cDNA fragments were ligated into the corresponding sites of the pTRV2 vector. All the constructs were confirmed by sequencing, then transformed to *Agrobacterium tumefaciens* strain GV3101. VIGS was performed by infiltrating a mixture of *A. tumefaciens* carrying pTRV2 and the helper vector pTRV1 (1:1) into the fully expanded cotyledons of 15 d-old tomato seedlings. qPCR assays were performed to ensure VIGS efficiency before experiment.

2.3. Generation of GRXS16-overexpressing plants

The full-length coding sequence of *GRXS16* was obtained by PCR using the following primers: 5′-AGGCGCGCCATGGCGACCTTCAA-CATCTC-3′, and 5′-GCGTCGACGCTTTTGAACAAGCTGGCAA-3’. The PCR product was digested with restriction enzymes *Asc*I and *Sal*I and ligated into the plant transformation vector pFGC1008-HA, using CaMV 35S as the promoter. The *GRXS16*-OE-HA plasmid was transformed into *A. tumefaciens* strain EHA105. The transformation was performed as described previously (Li et al., 2016a). Western blot was used to confirm the stable expression of GRXS16 protein in the transgenic plants.

2.4. Plant treatments

At the five-leaf stage, plants were uniformly sprayed with 11.2 mM chlorothalonil (CHT). Three days after CHT treatment, the seedlings were sprayed with 0.2 μM 24-epibrassinolide (EBR; Sigma-Aldrich, St. Louis, MO, USA). Twenty mL of CHT or EBR solution were applied to each plant. Seedlings sprayed with distilled water containing equal ratio of organic solvent were used as control group. For CHT residues analysis, samples were harvested 7 d after the CHT treatment. The plant samples were harvested at 3 h after EBR treatment for gene expression analysis, and at 24 h for analysis of GST activity and glutathione content.

2.5. Cytochemical localization of H_2O_2

Subcellular localization of H_2O_2 was detected by $CeCl_3$ staining method (Xia et al., 2009). For each treatment, leaves were cut into small strips (1 mm × 3 mm), then fixed with 5 mM $CeCl_3$ in 50 mM MOPS (pH 7.2) and placed inside a vacuum chamber for 30 min. After release of the vacuum, the leaf strips were transferred to a fixation solution [1.25% (v/v) glutaraldehyde and 1.25% (v/v) paraformaldehyde in 50 mM sodium cacodylate buffer (CAB), pH 7.2] and stored at 4 °C overnight. Leaf strips were washed for 10 min with the CAB buffer, and then fixed in 1% osmium tetroxide in the CAB buffer for 2–3 h. After fixation, the samples were dehydrated with a gradient ethanol series (30–100%), and then treated with Eponaraldite (Agar Aids) for 12 h. Followed by a change of fresh entrapment agent for 4 h, the samples were polymerized at 60 °C for 48 h. Then, the ultra-thin slices with a thickness of 70–90 nm were cut and mounted on uncoated copper grids. The sections were observed and photographed using a transmission electron microscope (H7650 Hitachi, Japan) at an accelerating voltage of 75 kV.

2.6. Detection of GRXS16 protein

To detect GRXS16 protein, western-blot was performed as described previously (Li et al., 2016b). Briefly, total proteins were extracted from 0.3 g leaf samples. The proteins were denatured and separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The proteins were transferred to

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