DOI:10.1021/jf901915a

JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY
ARTICLE

Brassinosteroids Promote Metabolism of Pesticides in Cucumber

Xiao Jian Xia,[†] Yun Zhang,[†] Jing Xue Wu,[†] Ji Tao Wang,[†] Yan Hong Zhou,[†] Kai Shi,[†] Yun Long Yu,[§] and Jing Quan Yu*,[†,‡]

[†]Department of Horticulture, Zhejiang University, Kaixuan Road 268, Hangzhou 310029, China, [§]Department of Plant Protection, Zhejiang University, Hangzhou 310029, China, and [‡]Key Laboratory of Horticultural Plants Growth, Development and Quality Improvement, Agricultural Ministry of China, Hangzhou 310029, China

Brassinosteroids (BRs) are known to protect crops from the toxicity of herbicides, fungicides and insecticides. It is shown here that application of 24-epibrassinolide (EBR) accelerated metabolism of various pesticides and consequently reduced their residual levels in cucumber (*Cucumis sativus* L). Chlorpyrifos, a widely used insecticide, caused significant reductions of net photosynthetic rate (Pn) and quantum yield of PSII (Φ_{PSII}) in cucumber leaves. EBR pretreatment alleviated the declines of Pn and Φ_{PSII} caused by chlorpyrifos application, and this effect of EBR was associated with reductions of chlorpyrifos residues. To understand how EBR promotes chlorpyrifos metabolism, the effects of EBR on activity and expression of enzymes involved in pesticide metabolism were analyzed. EBR had a positive effect on the activation of glutathione *S*-transferase (GST), peroxidase (POD), and glutathione reductase (GR) after treatment with chlorpyrifos, although the effect on GR was attenuated at later time points when plants were treated with 1 mM chlorpyrifos. In addition, EBR enhanced the expression of *P450* and *MRP*, which encode P450 monooxygenase and ABC-type transporter, respectively. However, the expression of *GST* was consistently lower than that of plants treated with only chlorpyrifos. Importantly, the stimulatory effect of EBR on pesticide metabolism was also observed for cypermethrin, chlorothalonil, and carbendazim, which was attributed to the enhanced activity and genes involved in pesticide metabolism. The results suggest that BRs may be promising, environmentally friendly, natural substances suitable for wide application to reduce the risks of human and environment exposure to pesticides.

KEYWORDS: Brassinosteroids; *Cucumis sativus*; degradation; glutathione; enzyme

INTRODUCTION

Biotic stresses caused by herbivores or pathogens lead to major losses in crop productivity worldwide. Over the past several decades, our understanding of the molecular mechanisms by which plants respond and adapt to biotic stresses has been enhanced drastically, and this burst of knowledge has already started to generate innovative approaches in developing transgenic resistant crop varieties through manipulation of genes involved in plant biotic stress responses. However, the use of pesticides is still the most common and effective strategy for controlling pests and for achieving high crop yields for a rapidly growing population. It is difficult to completely imagine the levels and security of today's yields without the use of synthetic pesticides. An estimated 2.5 million tons of pesticides are used annually in the world (*1*). The excessive use of pesticide results in pollution to nontarget agricultural produces and has raised worldwide concerns on food safety (*2*).

*Corresponding author (telephone +86 571 86971120; fax +86 571 86049815; e-mail jqyu@zju.edu.cn).

Extensive research has focused on bioremediation, which uses microorganisms to break down soil pollutants into harmless products (*3*). By contrast, there is only very limited information about cost-effective strategies that reduce pesticide residues in agricultural produces. Many developed countries have set maximum residue limits (MRL) for agricultural produces. To meet these MRLs, many countries rely on optimizing the input and timing of pesticide applications. However, these empirical methods, which are developed from field and glasshouse trials, may not be very reliable and can fail and cause public health problems due to a variety of reasons. Understanding the factors that affect pesticide metabolism can help develop new methods that can provide maximal crop protection and cause minimal public health problems.

Plants are able to transform pesticides by using a phased detoxification system (*4*, *5*). Absorbed pesticides are first metabolically activated by "phase 1" enzymes, such as P450 monooxygenase, peroxidase, and carboxylesterases. The second phase involves conjugation to glutathione (GSH) and glucose catalyzed by glutathione *S*-transferase (GST) and UDP-glycosyltransferase (UGT), respectively. The third phase of pesticide metabolism is

 Published on Web 08/20/2009

sequestration and storage of soluble metabolites either in vacuoles or in apoplast. The glutathione *S*-conjugates are actively transported to the vacuoles or apoplast by ATP-dependent membrane pumps (*6*). It has been shown that overexpressions of endogenous plant genes or transgenic expression of bacterial or animal genes can enhance pesticide metabolism in many plants (*5*). However, the upstream mechanisms controlling the expression of plant detoxification genes are largely unknown.

Brassinosteroids (BRs) play an essential role in plant growth and development (*7*). BRs have also been implicated in plant responses to environmental stresses and in plant defense against bacterial, fungal, and viral pathogens (*8*). However, little is known about the involvement of BRs in plant responses to organic pollutants such as pesticides. We have previously shown that exogenous application of BRs reduced the phytotoxic effect of herbicides, fungicides, and insecticides on cucumber leaves (*9*). In addition, BRs can reduce the damage caused by simazine, butachlor, or pretilachlo in rice (*10*). How BRs reduce the toxicity of various pesticides, however, is unclear.

On the basis of their effects on the toxicity of pesticide, BRs can be considered as safeners, which are known to induce the activity of numerous plant P450s and enhance glutathione conjugation involved in the biodegradation of herbicides (*11*). Indeed, Hatzios and Burgos (*11*) have previously suggested that plant hormones are involved in the induction of plant defense and detoxification genes in response to pesticides. Furthermore, transcriptional analyses of BR-deficient or BR-treated *Arabidopsis* and cucumber plants have shown that BR-regulated genes include those pesticide detoxification genes encoding P450 monooxygenase, glutathione *S*-transferase, and UDP-glycosyltransferase (*12*−*14*). Here we showed that application of 24-epibrassinolide (EBR) accelerated the degradation of various fungicides and insecticides and that this effect was associated with the enhanced expression of detoxification genes. These results strongly suggest that BRs enhance plant tolerance to pesticides by modulating the metabolic process of these pesticides

MATERIALS AND METHODS

Plant Materials. Cucumber (*Cucumis sativus* L. cv. Jinyan No. 4) seeds were sown in a mixture of vermiculite and perlite (3:1, v/v) in a growth chamber with a photoperiod of 12 h, temperature of 25/17 °C (day/night), and light intensity of 600 μmol m^{-2} s^{-1}. When the cotyledons fully expanded, groups of six plants were transplanted into a 10 L container filled with Hoagland nutrient solution. Plants at the four-leaf stage were used for the experiments. Plants were first treated with 0.1 μM 24-epibrassinolide (EBR) and 24 h later treated with the following commercial formulations of pesticides: chlorpyrifos (48% active ingredient, Deju Trading Co., Ltd., Nanjing, China), β-cypermethrin (4.5% active ingredient, Deju Trading Co., Ltd.), chlorothalonil (75% active ingredient, Agrovance Chemical Industry Ltd., Nanjing, China), and carbendazim (45% active ingredient, Deju Trading Co., Ltd.). The pesticides were applied using a stainless steel sprayer. The third leaf of each plant was used for analysis of photosynthesis, pesticide residues, enzyme activities, and gene expression after the BR and pesticide treatments.

Gas Exchange and Chlorophyll Fluorescence Measurements. Net photosynthetic rates (Pn) were determined by using an infrared gas analyzer based portable photosynthesis system (LI-6400, LI-COR Biosciences). The air temperature, relative humidity, CO_2 concentration, and PPFD were maintained at 25 °C, 85%, 360 μmol mol^{-1}, and 1000 μmol m^{-2} s^{-1}, respectively.

Chlorophyll fluorescence was measured with a pulse amplitude fluorometer (Hansatech Instruments, Kings Lynn, Norfolk, U.K.) in the same leaves used for gas exchange measurements. Leaves were maintained in darkness for 30 min before determination of maximal photochemical yield of PSII (Fv/Fm). Minimal fluorescence (Fo) was measured under a weak pulse of modulating light over 0.8 s, and maximal fluorescence (Fm) was induced by a saturating pulse of light (4000 μmol m^{-2} s^{-1}) applied over 0.8 s. The experimentally determined Fo and Fm were then used to calculate Fv/Fm, where Fv is the difference between Fo and Fm. For determining Fs (steady-state fluorescence yield), an actinic light source (600 μmol m^{-2} s^{-1}) was applied to achieve steady state photosynthesis, after which a second saturation pulse was applied for 0.7 s to obtain Fm′ (light-adapted maximum fluorescence). Fluorescence parameters were calculated by FMS-2 on the basis of the dark-adapted and light-adapted fluorescence measurements. The quantum yield of PSII (Φ_{PSII}) was calculated as (Fm′ − Fs)/Fm′ (*15*). The photochemical quenching coefficient (qP) was calculated as (Fm′ − Fs)/(Fm′ − Fo) (*16*).

Determination of Pesticide Residues. Pure chlorpyrifos, cypermethrin, chlorothalonil, and carbendazim compounds were purchased from the Institute for the Control of Agrochemicals, Ministry of Agriculture, Beijing, China. For determination of chlorpyrifos, cypermethrin, and chlorothalonil residues, 10 g of chopped cucumber leaves was homogenized in a glass jar containing 80 mL of petroleum ether (60−90 °C) and 80 g of anhydrous sodium sulfate at the speed of 10000 rpm for 2 min with a high-speed disperser. The mixture was decanted and filtered through a 7 cm Büchner funnel with 10 g of anhydrous sodium sulfate, and the filter cake was washed successively three times with 25 mL of redistilled petroleum ether. The filtrates were combined in a 250 mL flat-bottom flask and dried under a N_2 stream. Redistilled petroleum ether was added to dissolve pesticides, and the volume was adjusted to 5 mL for analysis by gas chromatography with a flame photometric detector (GC-FPD). Quantitative analysis was performed with a Fuli GC9790 gas chromatograph equipped with a FPD and a phosphorus filter. A fused silica capillary column (Pesticide Residue-I, Lanzhou Institute of Chemical Physics, Chinese Academy of Sciences, Lanzhou, China) (30 m length, 0.32 mm internal diameter and 0.25 μm film thickness) was employed for the separation in GC. Operating conditions were as follows: injector port temperature, 230 °C; column temperature, 230 °C; detector temperature, 250 °C; carrier gas, nitrogen, 50 mL min^{-1}; air, 80 mL min^{-1}; hydrogen, 125 mL min^{-1}.

For the determination of carbendazim residues, 10 g of chopped cucumber leaves was transferred to a 250 mL Erlenmeyer flask, followed by the addition of 80 mL of methanol and 10 mL of 0.2 M HCl. The samples were homogenized in a glass jar at high speed of 10000 rpm for 2 min and then filtered through a 7 cm Büchner funnel. The filter cake was washed twice with 50 mL of methanol. The filtrates were collected in a 250 mL Erlenmeyer flask and concentrated on a rotary evaporator to about 10 mL. The concentrated extract was transferred into 10 mL of 0.2 M HCl and 50 mL of distilled water in a separatory funnel and then washed with 50 mL of petroleum ether (60−90 °C). After the organic phase had been discharged, the pH of the remaining aqueous phase was adjusted to 7.0. After the addition of 20 mL of saturated NaCl, the solution was extracted three times with 50, 40, and 30 mL of methane dichloride, respectively. All extracts were then passed through a layer of anhydrous sodium sulfate and collected in a flat-bottom flask. The sample extract was dried on a rotary evaporator and dissolved in *N*,*N*-dimethylformamide prior to HPLC analysis. The HPLC (1200 series, Agilent Technologies) was equipped with diode array detector (DAD) set to 281 nm. A Hewlett-Packard stainless steel analytical column (Zorbax SB-C_{18}, 25 cm × 4.6 mm, 5 μm) was used. A Hewlett-Packard stainless steel guard column packed with Zorbax SB-C_{18} (4.6 cm × 12.5 mm, 5 μm) preceded the analytical column. The mobile phase consisted of methanol and water with a ratio of 65:35 (v/v), and all analyses were performed at a flow rate of 0.8 mL min^{-1}. Injection of 10 μL of extract sample was performed in HPLC for quantitative analysis. Under the used experimental conditions, the HPLC retention time for carbendazim was about 5 min.

To determine the extraction and recovery efficiency, cucumber leaves (10 g) were spiked with 0.1, 1.0, and 10.0 mg kg^{-1} of pesticides. Extraction and analysis were performed in triplicate.

Determination of Detoxification Enzymes. Approximately 0.3 g of leaf tissue was extracted with 2 mL of extraction buffer of 50 mM potassium phosphate (pH 7.5), 10 mM KCl, 1 mM EDTA, 5 mM dithiothreitol (DTT), 0.5 mM 4-(2-aminoethyl)benzenesulphonyl fluoride hydrochloride (AEBSF, a protease inhibitor), and 1: 4 (w/w) polyvinylpolypyrrolidone (insoluble PVPP). The homogenate was centrifuged for 20 min at 12000 rpm, and the supernatant obtained was used for enzyme assay. All procedures were carried out at 0−4 °C.

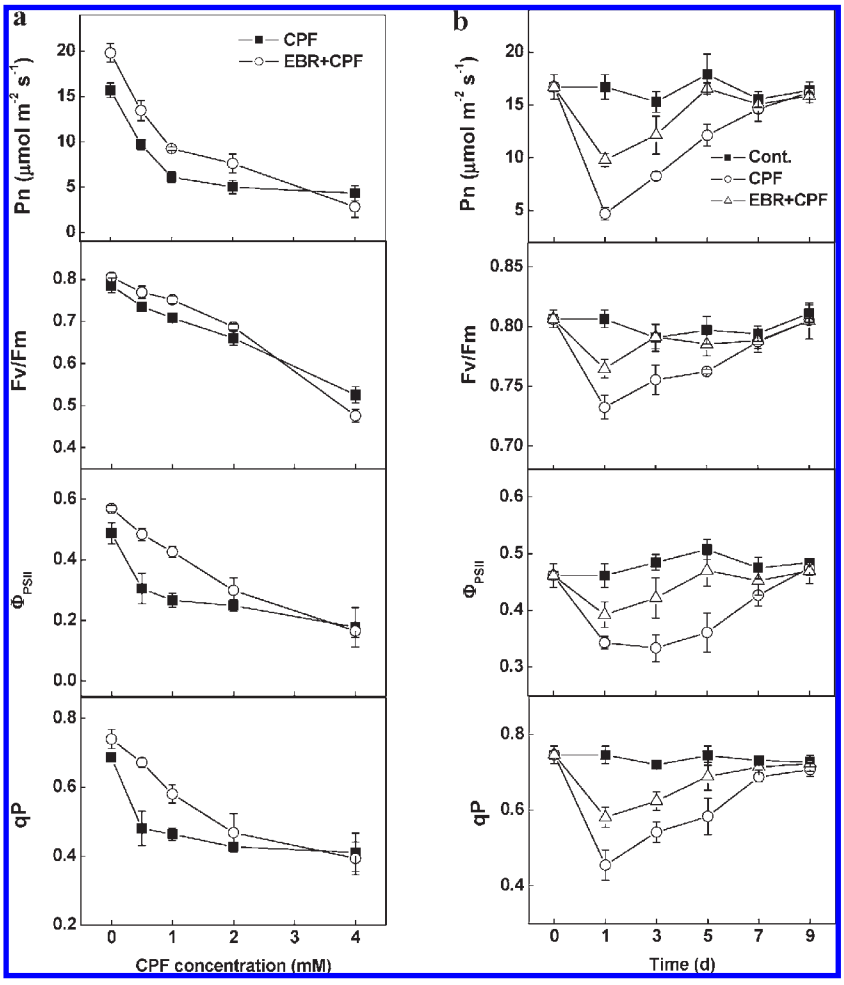

Figure 1. Effects of EBR pretreatment on the concentration response (**a**) or time course (**b**) of Pn, Fv/Fm, Φ_{PSII}, and qP after CPF treatment. Data are means ± SD ($n = 3$).

GST activity was determined using a GST colorimetric activity assay kit (Jiancheng Bio Co., Nanjing, China). The reactions contained 50 mM potassium phosphate (pH 6.5) at 25 °C, aliquots of enzyme extract, 5 mM GSH, 0.4 mM CDNB, and 1% (v/v) ethanol in a final volume of 1 mL. Reactions were initiated with addition of the CDNB substrate in ethanol. Enzymatic formation of 2,4-dinitrophenyl-*S*-glutathione at 340 nm (E = 9.6 mM^{-1} cm^{-1}) was monitored for 5 min and corrected for nonenzymatic controls. The activity of peroxidase (POD) and glutathione reductase (GR) was assayed following the method described by Cakmak and Marschner (*17*) with some modifications. For measurement of POD activity, the reaction mixture contained 25 mM phosphate buffer (pH 7.0), 0.05% guaiacol, 10 mM H_2O_2, and aliquots of enzyme extract. Activity was measured by the increase in absorbance at 470 nm caused by guaiacol oxidation (E = 26.6 mM^{-1} cm^{-1}). GR activity was measured on the basis of the rate of decrease in the absorbance of NADPH at 340 nm. All spectrophotometric analyses were conducted on a Shimadzu UV-2410PC spectrophotometer.

Total RNA Extraction and Gene Expression Analysis. Total RNA was extracted using Trizol according to the supplier's instruction. Contaminated DNA was removed with a purifying column. One microgram of total RNA was reverse-transcribed using 0.5 μg of oligo(dT) 12−18 (Invitrogen) and 200 units of Superscript II (Invitrogen) following the supplier's recommendation. The gene-specific primers were designed according to the EST sequences: 5′-TACTGATTTCCATTGTTGT-3′ and 5′-CTTGTATGTTTTATGCTTT-3′ for *P450*; 5′- TTTGAGGAGGTGAAGGTAA-3′ and 5′-ACGCACAAGAAATGTAGAT-3′ for *GST*; and 5′-TCAGAAAACAATAAGGGAA-3′ and 5′-CCAGAATCGAGTACAAGGA-3′ for *MRP*. For quantitative RT-PCR analysis, we amplified PCR products in triplicate using iQ SYBR Green SuperMix (Bio-Rad) in 25 μL qRT-PCR reactions, an iCycler iQ 96-well real-time PCR detection system (Bio-Rad), iCycler software to calculate threshold cycle values, and cucumber *actin* as an internal control. The quantification of mRNA levels is based on the method of Livak and Schmittgen (*18*). The threshold cycle (Ct) value of *actin* was subtracted from that of the gene of interest to obtain a ΔCt value. The Ct value of untreated control sample was subtracted from the ΔCt value to obtain a ΔΔCt value. The fold changes in expression level relative to the control were expressed as $2^{-\Delta\Delta Ct}$.

Statistical Analysis. Measurements were performed randomly using three replicates. SAS 8.0 (SAS Institute, Cary, NC) for Windows was used for statistical analysis. The data were analyzed with a one-way analysis of variance, and differences between treatments were separated by Tukey's HSD test at the $P < 0.05$ level.

RESULTS

EBR Alleviated the Toxicity of CPF. Chlorpyrifos (CPF), an acetylcholinesterase inhibitor, is the most intensively used organophosphate insecticide in agriculture. With the increasing use of CPF, it is of great importance to understand its physiological effects on nontargeted crops. The effect of CPF on cucumber photosynthesis was determined by gas exchange and Chl fluorescence measurements. CPF concentration in the range of 0.5−1 mM causes a 38−61% decrease in net photosynthetic rate (Pn) (**Figure 1a**). The higher concentrations of CPF resulted in further but marginal declines in Pn. The maximum quantum yield of PSII (Fv/Fm) decreased almost linearly with increasing concentrations of CPF. Changes in the quantum yield of PSII (Φ_{PSII}) and photochemical quenching coefficient (qP) were similar to

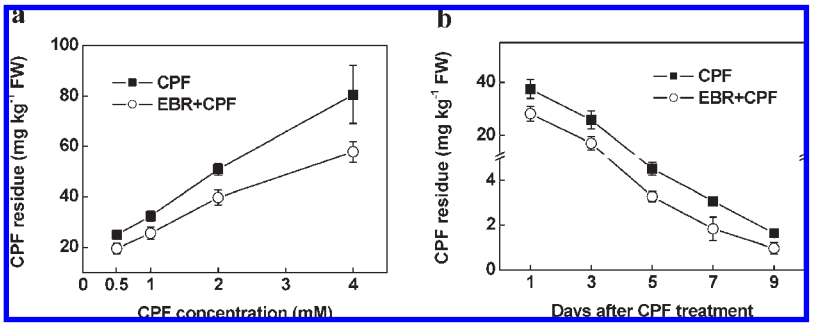

Figure 2. Effects of EBR pretreatment on the pesticide residues after treatment with different concentrations of CPF (**a**) and dissipation of CPF (**b**). Data are means ± SD (n = 3).

that of Pn after treatments of various concentrations of CPF. Consistent with our previous results, pretreatment of EBR alleviated the inhibitory effect of CPF on photosynthesis. EBR pretreatment did not completely prevent the decline of Fv/Fm, Φ_{PSII}, or qP after CPF treatment; however, for all of these photosynthetic parameters, the concentration response curves showed partial effects of EBR pretreatment following treatment of 0.5−2 mM CPF.

The magnitude of CPF-induced toxicity in cucumber was further determined by assaying the recovery of photosynthesis after 1 mM CPF treatment. After a dramatic repression by CPF, the Pn started to recover 3 days after CPF treatment (**Figure 1b**). The Pn increased steadily and recovered to the normal value at 7 days post-treatment. Three days after CPF treatment, the Fv/Fm, Φ_{PSII}, and qP also gradually increased. The Fv/Fm recovered to the normal value at 7 days after treatment, whereas Φ_{PSII} and qP were completely recovered at 9 days after CPF treatment. EBR pretreatment promoted recovery of all these photosynthetic parameters. The Pn of EBR-pretreated plants recovered to control value at 5 days after CPF treatment. Fv/Fm increased to control level as early as 3 days, and Φ_{PSII} and qP recovered close to the control values at 5 days after CPF treatment. The results indicated that EBR could alleviate CPF toxicity and promote recovery from CPF-inflicted injury.

EBR Accelerated Dissipation of CPF. To determine whether difference in tolerance to CPF was related to changes in CPF metabolism, plants with or without EBR pretreatment were treated with increasing concentrations of CPF and the residues were determined at 24 h after treatment. The residues of CPF steadily increased with increased CPF concentration (**Figure 2a**). Importantly, EBR pretreatment reduced the CPF residue at all of the CPF concentrations applied, and the effect was most pronounced with 4 mM CPF. Time course analysis of CPF residue was also performed after treatment with 1 mM CPF. The CPF residues in plants without EBR pretreatment were generally higher than those in EBR-pretreated plants (**Figure 2b**). Notably the CPF residues of EBR-pretreated plants fell below the maximum residue limits (MRL) set by China (1 mg kg^{-1}) at 9 days when the values of plants without EBR pretreatment were still 60% higher than the limit. We found a close relationship between the CPF residue and the net photosynthetic rate ($r = 0.91$, $p < 0.01$). This suggested that EBR reduced the negative effects of CPF on plant photosynthesis by enhancing the dissipation of the pesticide.

EBR Increased Detoxification Enzyme Activities. The effects of CPF on enzymes involved in detoxification pathways were determined. Treatment with 0.5 mM CPF did not have a significant effect on GST activity (**Figure 3a**). A significant increase in GST activity was observed in plants treated with 1 or 2 mM CPF, but application of a high CPF concentration (4 mM) led to a reduction of GST activity. POD activity showed a concentration-dependent increase from 1 to 4 mM CPF. GR activity was increased even by treatment of 0.5 mM CPF and was further elevated by higher concentrations of the pesticide. In EBR-pretreated plants, induction of GST and POD activities was observed even with treatment of 0.5 mM CPF, suggesting that EBR increases the sensitivity of plants to CPF for induction of these enzymes. In addition, CPF-induced activities of GST, POD, and GR were generally higher in EBR-pretreated plants than in unpretreated plants (**Figure 3a**).

The time courses of the activities of detoxification enzymes after 1 mM CPF treatment were also measured (**Figure 3b**). The highest activity of GST was observed at 3 days, and the activity gradually declined to control levels at 9 days after treatment, the last time point for the experiment. POD activity steadily increased from 1 days and reached a plateau by 5 days. GR activity increased 2- and 4-fold at 3 and 5 days after treatment, respectively. After reaching peak activity at 5 days, GR activity gradually declined but was still higher than the control at the end of the experiment. Pretreatment with EBR did not alter the time course of GST and POD induction, but significantly enhanced their activities when compared with those of unpretreated plants. On the other hand, in EBR-pretreated plants, CPF induction of GR activity was faster, although the induced activity was somewhat lower at later time points than that of unpretreated plants. Enhanced induction of detoxification enzymes by EBR may contribute to the enhanced dissipation rate of CPF in EBR-pretreated plants.

EBR Induced Expression of Detoxification Genes. To investigate the molecular mechanism of EBR-enhanced CPF dissipation, we analyzed the gene expressions of *P450*, *GST*, and *MRP*, which encode proteins involved in pesticide conversion, conjugation, and transport, respectively. The expressions of *P450* and *GST* were not correlated linearly with the applied CPF concentrations (**Figure 4a**). Expressions of *P450* and *GST* were significantly induced by 0.5 mM CPF, but only slightly induced by higher CPF concentrations (1 and 2 mM). At 4 mM CPF, however, the transcripts of *P450* and *GST* were again significantly increased relative to that of untreated control. In comparison, the expression of *MRP* increased almost linearly with increasing concentrations of CPF and transcript level after treatment of 4 mM CPF increased to 200% of control levels. Consistent with the roles in CPF dissipation, EBR pretreatment significantly increased the induction of *P450* by 0.5−2 mM CPF. However, this effect of EBR was not observed when the concentration of CPF was increased to 4 mM. In the absence of CPF, EBR significantly increased the expression of *GST*. With CPF treatment, however, the transcript level of *GST* in EBR-pretreated plants was significantly lower than that in unpretreated

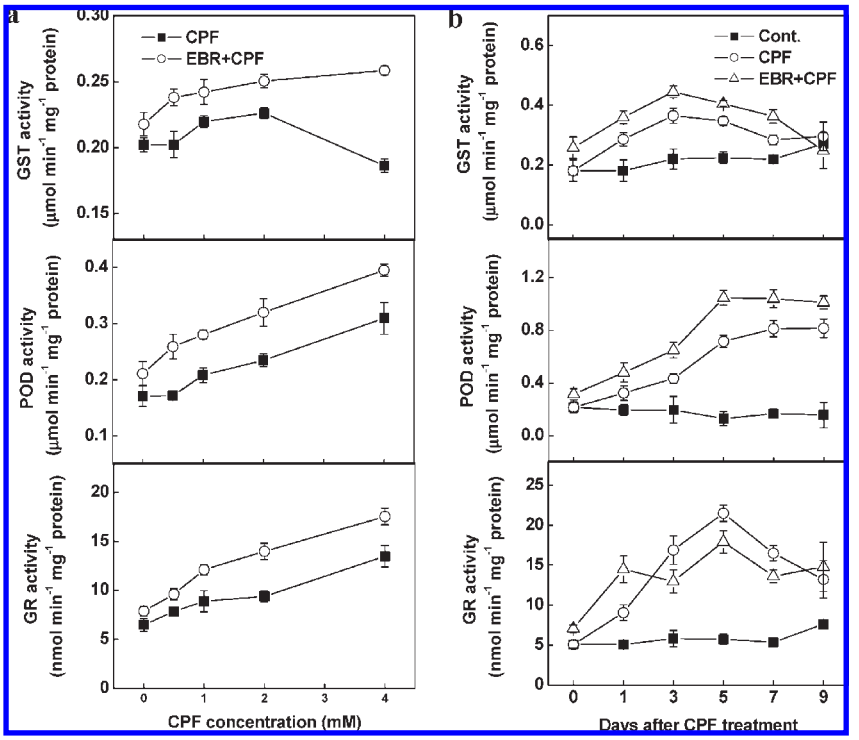

Figure 3. Effects of EBR pretreatment on the concentration response (**a**) or time course (**b**) of GST, POD, and GR activities after CPF treatment. Data are means ± SD (n = 3).

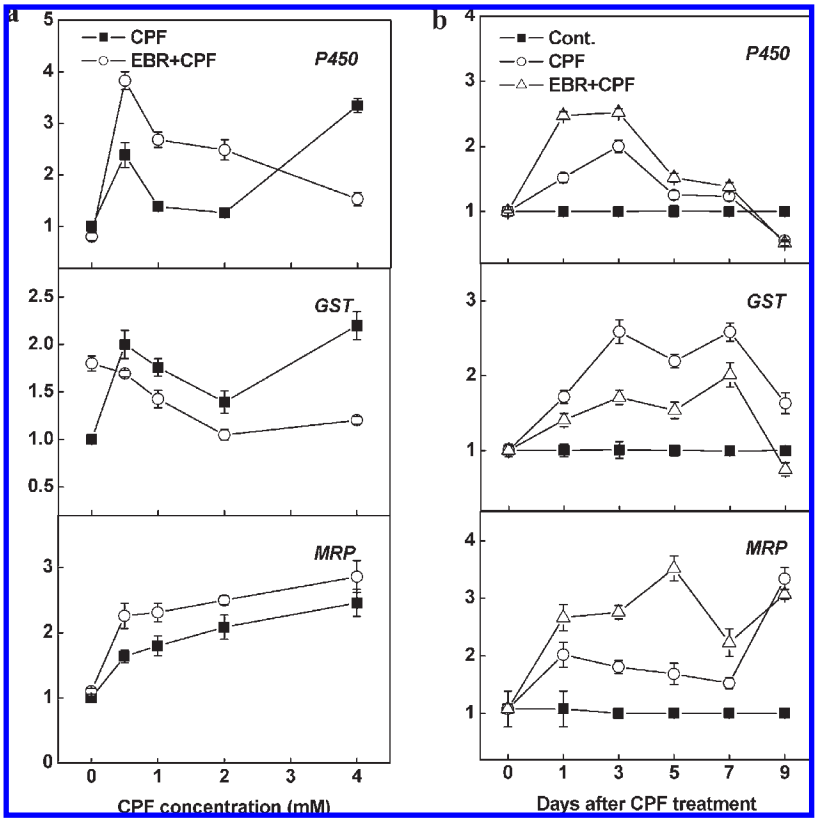

Figure 4. Effects of EBR pretreatment on the concentration response (**a**) or time course (**b**) of *P450*, *GST*, and *MRP* expression after CPF treatment. Data are means ± SD (n = 3).

plants. On the other hand, EBR enhanced induction of *MRP* by all four concentrations of CPF applied.

The time course analysis of gene expression after 1 mM CPF treatment was also performed. The expression of *P450* showed the largest increase at 3 days and then gradually declined below the control level (**Figure 4a**). The expression of *GST* was induced 1.5-fold at 3 days and remained induced for another 4 days before declining to the control level by 9 days after CPF treatment. The

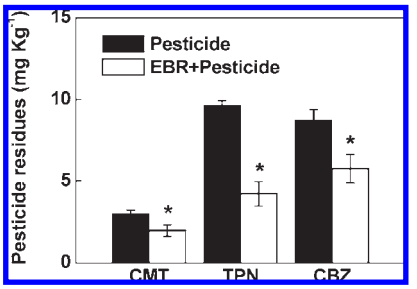

Figure 5. Effects of EBR pretreatment on pesticide residues after treatment with CMT, TPN, and CBZ. Data are means ± SD (n = 3). Asterisks indicate a significant difference between plants with or without EBR pretreatment (P < 0.05).

expression of *MRP* was significantly induced during the first 7 days but was markedly induced at 9 days after CPF treatment. EBR-pretreated plants showed greater induction of *P450* and *MRP* by CPF at most of the time points than plants without EBR pretreatment. On the other hand, unlike GST activity, CPF induction of *GST* gene expression was significantly inhibited by EBR pretreatment.

EBR Influenced the Metabolism of Other Pesticides. To investigate whether EBR-enhanced metabolism of pesticide was specific to CPF, we analyzed changes of residue levels of another insecticide (cypermethrin, CMT) and two fungicides (chlorothalonil, TPN, and carbendazim, CBZ). The pesticides were applied 24 h after EBR pretreatment, and the residues were determined 4 days later. The analysis revealed a strong effect of EBR on pesticide degradation (**Figure 5**). The residues of CMT and CBZ in EBR-pretreated plants were 35 and 34% lower than those in plants without EBR pretreatment, respectively. EBR pretreatment had an even greater effect on degradation of TPN, with a >50% reduction in the pesticide residue in EBR-pretreated plants relative to that in plants without EBR pretreatment. These results suggest a general role of EBR in the metabolism of various pesticides.

Enhanced activities of detoxification enzymes may again be associated with enhanced metabolism of the pesticides in EBR-pretreated plants. Indeed, GST and POD activities in EBR-pretreated plants were increased significantly in response to CMT, TPN, or CBZ when compared with plants without EBR application (**Figure 6**). GR activity was also increased by EBR pretreatment in response to the application of TNP and CBZ but not CMT.

The effect of EBR pretreatment on gene expressions of *P450*, *GST*, and *MRP* was also examined when plants were treated with the three pesticides (**Figure 7**). At 5 days after treatment with CMT, TPN, or CBZ, the transcripts for *P450*, *GST*, and *MRP* were either below or close to the control levels. By contrast, the expressions of the genes after pesticide treatment were all increased in EBR-pretreated plants. Enhanced expression of these genes would likely promote pesticide metabolism and consequently reduce the pesticide residues.

DISCUSSION

Fungicides and insecticides control diseases and insects by inhibiting important biochemical reactions within their target organisms. However, these compounds may also have consequences on crop physiology, such as growth reduction, perturbation of reproductive organ development, and alteration of nitrogen and/or carbon metabolism (*19*). Here, we showed that chlorpyrifos, a broad-spectrum organophosphate insecticide, induced reduction of CO_2 assimilation (Pn) and electron transport

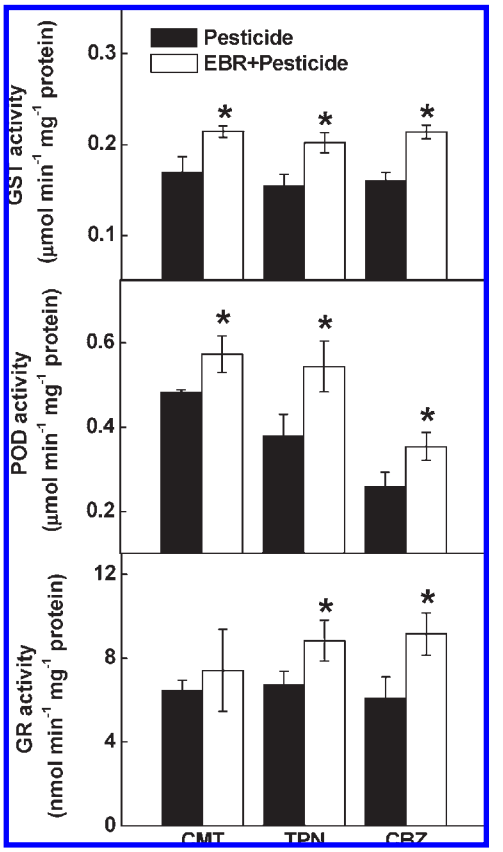

Figure 6. Effects of EBR pretreatment on the GST, POD, and GR activities after treatment with CMT, TPN, and CBZ. Data are means ± SD (n = 3). Asterisks indicate a significant difference between plants with or without EBR pretreatment (P < 0.05).

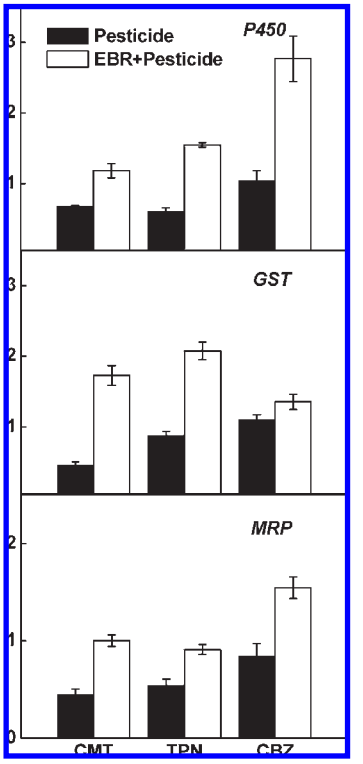

Figure 7. Effects of EBR pretreatment on *P450*, *GST*, and *MRP* expression after treatment with CMT, TPN, and CBZ. Data are means ± SD (n = 3).

(Φ_{PSII}) in cucumber leaves (**Figure 1a**). Although it is not clear whether chlorpyrifos directly targets photosynthetic apparatus, changes in Chl fluorescence parameters suggest strongly that chlorpyrifos causes perturbations of metabolism processes in leaves (*20*). Chlorpyrifos also induced significant declines in Fv/Fm (**Figure 1a**), which is often associated with oxidative stress. Previous study (*21*) has suggested that insecticides in combination with fungicides cause uncoupling of the photosynthetic electron flow from phosphorylation, which would activate pseudocyclic electron transport, result in generation of reactive oxygen species in chloroplast, and cause damage to the photosystems. The recovery of photosynthesis was observed at 3 days after 1 mM chlorpyrifos treatment (**Figure 1b**), indicating that chlorpyrifos did not permanently destroy photosynthetic activity. Pesticide metabolism and transformation in plants usually result in less toxic metabolites (*4*,*5*). The recovery in photosynthesis may result from chlorpyrifos detoxification in treated plant leaves. This possibility is consistent with the observation that the recovery of photosynthesis was accompanied by a decrease of chlorpyrifos residue (**Figure 2b**). EBR decreased chlorpyrifos residues (**Figure 2**) and alleviated the toxic effect of chlorpyrifos on photosynthesis (**Figure 1**). EBR may enhance the metabolism of pesticides into less toxic forms. The protective effect of EBR may also be attributed to the induction of plant antioxidant capacity. However, we observed no significant enhancement of activities of antioxidant enzymes in EBR-pretreated plants (data not shown).

The enhanced metabolism of chlorpyrifos was associated with EBR-mediated increases in activities of detoxifying enzymes in cucumber (**Figure 3**). GST is involved in the detoxification of xenobiotic compounds by covalently linking glutathione to hydrophobic substrates, forming less reactive and more polar glutathione *S*-conjugates in plants (*22*). Chlorpyrifos is known to undergo hydroxylation reactions and is detoxified by glutathione conjugation in mammals (*23*). The observation that the activity of GST is induced by chlorpyrifos suggests that plants have a similar mechanism for detoxification of chlorpyrifos. POD, like GST, is also involved in plant response to xenobiotic stress by oxidizing and deactivating chemical xenobiotics (*24*). It has been demonstrated that *ATPA2* and *ATP24a* genes, which encode peroxidases, were regulated by brassinosteroids in *Arabidopsis* (*13*). In this study, EBR significantly increased the POD activity in chlorpyrifos-treated plants (**Figure 3**), supporting the importance of BR in plant detoxification pathways. Because POD is a general defense enzyme and responds to a variety of environmental stresses, it is possible that EBR-mediated induction of detoxification enzymes and chlorpyrifos metabolism may be part of a general stress response. GR is an NADPH-dependent enzyme that catalyzes the reduction of oxidized glutathione (GSSG) to GSH (*25*). In addition, GR is important for the maintenance of cellular glutathione pools. Broadbent et al. (*26*) demonstrated that overexpression of pea *gor1* gene resulted in an increase of glutathione pools up to 50% in tobacco. The enhanced activity of GR may indirectly contribute to the increase levels of GSH and enhance the formation of the GS conjugates of pesticides. However, we observed that the induction of GR activity was most pronounced in plants without EBR pretreatment 3 days after 1 mM chlorpyrifos (**Figure 3b**). In addition to its roles in pesticide detoxification, GR also has a central role in oxidative stress response (*25*). It is likely that CPF caused greater oxidative stress in plants without EBR pretreatment and resulted in higher GR activity.

In addition to GSH conjugation, various enzymes involved in the oxidation, transport, or modification in vacuoles or apoplast could be limiting factors for the detoxification of chlorpyrifos. Plant P450 monooxygenases catalyze an oxidative transformation by incorporating an oxygen atom into an organic molecule to create a hydroxyl side group (*5*). ATP-dependent membrane pumps recognize the glutathione *S*-conjugates and shuttle them into the vacuoles or to the apoplast (*4*). EBR has significant effects on the expression of genes encoding P450 monooxygenase and MRP transporter (**Figure 4**). Differences in the expression levels of *P450* and *MRP* may account for the differences in pesticide residues between EBR-treated and untreated plants. Time course analysis of the gene expression after chlorpyrifos treatment demonstrated that the induction of *P450* preceded that of *GST* and that *MRP* was the last gene to be markedly induced (**Figure 4b**). This pattern of expression may reflect the sequence of events for metabolism of pesticide. In contrast to the GST activity, we observed that plants treated with chlorpyrifos contained a higher transcript level of *GST* than EBR-pretreated plants. Plant *GST* genes are induced by various stresses, such as Al stress, oxidative stress, auxin treatment, and inorganic phosphate starvation (*27*). Cucumber is very sensitive to chlorpyrifos, and oxidative symptoms are always observed in field trials when chlorpyrifos is applied to cucumber. The oxidative stress induced by chlorpyrifos may be also involved in the regulation of *GST* expression. For CMT, TPN, or CBZ treatment, however, no obvious oxidative symptom was observed in cucumber. Accordingly, the EBR-mediated enhancement of pesticide metabolism was correlated with a higher transcript level of *GST* (**Figures 5** and **7**).

It is interesting to note that EBR enhanced the metabolism of another three pesticides tested, which is accompanied by induction of detoxification enzymes and genes (**Figures 5**−**7**). The most pronounced difference in metabolism was found for chlorothalonil. The results from in vitro study suggest that chlorothalonil is detoxified in plants via GSH conjugation (*28*). GST catalyzes a wide variety of substrates (*22*). The general increase in GST activity in EBR-pretreated plants in response to all four pesticides tested confirms that GST plays a role in plant transformation of these compounds. EBR pretreatment enhances induction of *P450*, *GST*, and *MRP* transporter genes (**Figure 4**), suggesting a coordinated detoxification process. How BRs mediate induction of these genes is currently unclear. The generation of ROS is a common event in both pesticide treatment and hormone response (*11*). Recently, we discovered that ROS is involved in BR-induced stress tolerance (*14*). Therefore, ROS may serve as a signal in modulating pesticide metabolism genes. In addition, it is important to identify the intermediate in the biotransformation and determine whether the conjugation with glutathione is the limiting step for the metabolism of these pesticides.

Although "organic farming" may reduce the risk of food contamination by pesticides, the acceptance of this approach in developing countries such as China has been very slow. The positive effect of BRs on pesticide degradation in crops is intriguing. At present, there is no direct evidence for harmful effects of BRs on human health. Moreover, BRs have been shown to inhibit the growth of human cancer cell lines without affecting the growth of normal cells (*29*). Therefore, application of BRs may offer a promising approach for controlling food safety by reducing harmful pesticide residues.

ABBREVIATIONS USED

BRs, brassinosteroids; CBZ, carbendazim; CMT, cypermethrin; CPF, chlorpyrifos; EBR, 24-epibrassinolide; Fv/Fm, maximal photochemical yield of PSII; GR, glutathione reductase; GSH, glutathione; GST, glutathione *S*-transferase; MRL, maximum residue limits; Pn, net photosynthetic rate; POD, peroxidase;

qP, photochemical quenching coefficient; TPN, chlorothalonil; Φ_{PSII}, quantum yield of PSII.

ACKNOWLEDGMENT

We acknowledge Professor Z. X. Chen of Purdue for critical reading of the manuscript.

LITERATURE CITED

(1) Pimentel, D. Amounts of pesticides reaching target pests: environmental impacts and ethics. *J. Agric. Environ. Ethics* **1995**, *8*, 17–29.
(2) Pimentel, D. Environmental and economic costs of the application of pesticides primarily in the United States. *Environ. Dev. Sustainability* **2005**, *7*, 229–252.
(3) Singh, S.; Kang, S. H.; Mulchandani, A.; Chen, W. Bioremediation: environmental clean-up through pathway engineering. *Curr. Opin. Biotechnol.* **2008**, *19*, 437–444.
(4) Coleman, J.; Blake-Kalff, M.; Davies, E. Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. *Trends Plant Sci.* **1997**, *2*, 144–151.
(5) Cherian, S.; Oliveira, M. M. Transgenic plants in phytoremediation: recent advances and new possibilities. *Environ. Sci. Technol.* **2005**, *39*, 9377–9390.
(6) Martinoia, E.; Grill, E.; Tomasini, R.; Kreuz, K.; Amrehin, N. ATP-dependent glutathione *S*-conjugate 'export' pump in the vacuolar membrane of plants. *Nature* **1993**, *364*, 247–249.
(7) Clouse, S. D.; Sasse, J. M. Brassinosteroids: essential regulators of plant growth and development. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 427–451.
(8) Krishna, P. Brassinosteroid-mediated stress responses. *J. Plant Growth Regul.* **2003**, *22*, 289–297.
(9) Xia, X. J.; Huang, Y. Y.; Wang, L.; Huang, L. F.; Yu, Y. L.; Zhou, Y. H.; Yu, J. Q. Pesticides induced depression of photosynthesis was alleviated by 24-epibrassinolide pretreatment in *Cucumis sativus* L. *Pest Biochem. Physiol.* **2006**, *86*, 42–48.
(10) Bajguz, A.; Hayat, S. Effects of brassinosteroids on the plant responses to environmental stresses. *Plant Physiol. Biochem.* **2009**, *47*, 1–8.
(11) Hatzios, K. K.; Burgos, N. Metabolism-based herbicide resistance: regulation by safeners. *Weed Sci.* **2004**, *52*, 454–467.
(12) Müssig, C.; Fischer, S.; Altmann, T. Brassinosteroid-regulated gene expression. *Plant Physiol.* **2002**, *129*, 1241–1251.
(13) Goda, H.; Shimada, Y.; Asami, T.; Fujioka, S.; Yoshida, S. Microarray analysis of brassinosteroid-regulated genes in *Arabidopsis*. *Plant Physiol.* **2002**, *130*, 1319–1334.
(14) Xia, X. J.; Wang, Y. J.; Zhou, Y. H.; Tao, Y.; Mao, W. H.; Shi, K.; Asami, T.; Chen, Z.; Yu, J. Q. Reactive oxygen species are involved in brassinosteroids-induced stress tolerance in *Cucumis sativus*. *Plant Physiol.* **2009**, *150*, 801−814.
(15) Genty, B.; Briantais, J. M.; Baker, N. R. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. *Biochim. Biophys. Acta* **1989**, *990*, 87–92.
(16) van Kooten, O.; Snel, J. F. H. The use of chlorophyll fluorescence nomenclature in plant stress physiology. *Photosynth. Res.* **1990**, *25*, 147–150.
(17) Cakmak, I.; Marschner, H. Magnesium deficiency and high light intensity enhance activities of superoxide dismutase ascorbate peroxidase, and glutathione reductase in bean leaves. *Plant Physiol.* **1992**, *98*, 1222–1227.
(18) Livak, K. J.; Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. *Methods* **2001**, *25*, 402–408.
(19) Saladin, G.; Clément, C. Physiological effects of pesticides on cultivated crops. In *Agriculture and Soil Pollution: New Research*; Livingston, J. V., Ed.; Nova Science Publishers: Hauppauge, NY, 2005; pp 53−86.
(20) Barbagallo, R. P.; Oxborough, K.; Pallett, K. E.; Baker, N. R. Rapid, noninvasive screening for perturbations of metabolism and plant growth using chlorophyll fluorescence imaging. *Plant Physiol.* **2003**, *132*, 485–493.
(21) Untiedt, R.; Blanke, M. M. Effects of fungicide and insecticide mixtures on apple tree canopy photosynthesis, dark respiration and carbon economy. *Crop Prot.* **2004**, *23*, 1001–1006.
(22) Edwards, R.; Dixon, D. P.; Walbot, V. Plant glutathione *S*-transferases: enzymes with multiple functions in sickness and in health. *Trends Plant Sci.* **2000**, *5*, 193–198.
(23) Fujioka, K.; Casida, J. E. Glutathione *S*-Transferase conjugation of organophosphorus pesticides yields *S*-phospho-, *S*-aryl-, and *S*-alkylglutathione derivatives. *Chem. Res. Toxicol.* **2007**, *20*, 1211–1217.
(24) Farkas, M. H.; Berry, J. O.; Aga, D. S. Chlortetracycline detoxification in maize via induction of glutathione *S*-transferases after antibiotic exposure. *Environ. Sci. Technol.* **2007**, *41*, 1450–1456.
(25) Foyer, C. H.; Lopez-Delgado, H.; Dat, J. F.; Scott, I. M. Hydrogen peroxide- and glutathione-associated mechanisms of acclamatory stress tolerance and signaling. *Physiol. Plant.* **1997**, *100*, 241–254.
(26) Broadbent, P.; Creissen, G. P.; Kular, B.; Wellburn, A. R.; Mullineaux, P. M. Oxidative stress responses in transgenic tobacco containing altered levels of glutathione reductase activity. *Plant J.* **1995**, *8*, 247–55.
(27) Ezaki, B.; Suzuki, M.; Motoda, H.; Kawamura, M.; Nakashima, S.; Matsumoto, H. Mechanism of gene expression of *Arabidopsis* glutathione *S*-transferase, *AtGST1*, and *AtGST11* in response to aluminum stress. *Plant Physiol.* **2004**, *134*, 1672–1682.
(28) Kim, Y. M.; Park, K.; Joo, G. J.; Jeong, E. M.; Kim, J. E.; Rhee, I. K. Glutathione-dependent biotransformation of the fungicide chlorothalonil. *J. Agric. Food Chem.* **2004**, *52*, 4192–4196.
(29) Malikova, J.; Swaczynova, J.; Kolar, Z.; Strnad, M. Anticancer and antiproliferative activity of natural brassinosteroids. *Phytochemistry* **2008**, *69*, 418–426.

Received June 4, 2009. Accepted July 28, 2009. This work was financially supported by the National Basic Research Program of China (2009CB119000), the National Key Technology R&D Program (2008BADA6B02), and the Major Program of Natural Science Foundation of Zhejiang Province, China (Z307394).