Comparison of the Induction of Glutathione *S*-Transferase in Plants by Treatment with Substrates*

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Abstract: The relationship between the applied chemicals and the substrates were investigated when glutathione *S*-transferase (GST) activities were induced with chemicals (pretilachlor and CDNB) used as substrates in 4 grasses (rice, barnyardgrass, corn, and large crabgrass). The relation between tolerance to chemicals and GST activities was also studied with an eye to the applied chemical and substrate. The GST activities after treating the roots of 4 plants with pretilachlor or CDNB varied with plant species. The GST activities using the applied chemical as substrate were enhanced more highly than the other chemical in rice and barnyardgrass, and treatment with CDNB enhanced GST(CDNB) activities more than the other in rice, barnyardgrass, and corn. On the whole, however, the patterns of GST induction in 4 plants were specific rather than general. From the point of view of applied chemical and substrate, the tolerance to a chemical seemed more correlated with the GST activity using the chemical as substrate than another substrate. In other words, the difference in activity of GST(CDNB) or GST(pretilachlor) induced by CDNB or pretilachlor, respectively, caused the difference in growth.

Key words: glutathione *S*-transferase (GST), induction, substrate, pretilachlor, CDNB

Abbreviations: GST(pretilachlor); GST using pretilachlor as substrate, GST(CDNB); using CDNB as substrate, GST(fencloirim); GST using fencloirim as substrate, GST(metolachlor); GST using metolachlor as substrate, GSH; reduced glutathione

Introduction

Glutathione *S*-transferases (GSTs; EC2.5.1.18) are widely distributed in bacteria, fungi, animals, and plant^{10,13,15,21}. There are many studies on the detoxication and the conjugation of a number of chemicals and xenobiotics^{10,13}. Plant GSTs were intensively studied in relation to the detoxication, safen-ing and selectivity of thiocarbamate and chloroacetanilide herbicides^{7,8,18}. GST activity was shown to increase in response to herbicide and safener treatments and as a result may enhance their metabolic activity in plants⁸. Dean *et al.* reported that treating etiolated sorghum seedlings with a safener like oxabetrinil increased GST(CDNB) and GST(metolachlor) activities². Maximum expression of their activities was noted at 24 ~36 hr after treatment. Treating etiolated sorghum shoots with metolachlor also induced GST(metolachlor) activity. In addition, treating rice seedlings with pretilachlor and fencloirim was also found to enhance not only GST(pretilachlor) activity but also GST(fencloirim) activity within 24 hrs⁵. Wu *et al.*

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reported that microsomal GST activity as well as soluble activity was enhanced by treatment with pretilachlor and fenclorim²⁴).

We previously investigated the induction of GST activities in rice or barnyardgrass after treatment with three kinds of chemicals such as chloroacetanilide herbicide and its safener¹⁷). Induction of GST activities varied depending on plant species, parts extracted, applied chemicals, and enzyme substrates. This result is in agreement with other studies reporting the effect of chloroacetanilide herbicides and their safeners on GST activities^{4,9,11,20,24}).

GST consists of many isozymes (multiple forms)²²), and induction of total GST activity is thought to be influenced by the induction of one or more of these isozyme activities. Each isozyme has substrate specificity though it somewhat overlaps with the others^{6,23}). There are both qualitative and quantitative differences in the pattern of the GST isozymes induced by chemicals^{1,2}), however, little is known about the relation between induction and substrate specificity of GST.

The purpose of this study was: (a) to determine whether there is a relationship between the treated chemicals and the substrates when GST activities are induced; we treated 4 grasses (rice, barnyardgrass, corn, and large crabgrass) with chemicals (pretilachlor and CDNB) used as GST substrates; (b) to compare GST activities of the 4 grasses with regard to their chemical tolerance.

Materials and Methods

1. Chemicals

Technical-grade (96.7% purity) pretilachlor [2-chloro 2', 6'-diethyl-*N*-(2-propoxyethyl) acetanilide] was a gift from Ciba-Geigy Japan, Ltd.. CDNB [1-chloro-2, 4-dinitrobenzene] was purchased from Wako Pure Chemical Industries Ltd. Each com-

pound was dissolved in DMSO (dimethyl sulfoxide). All other chemicals were obtained commercially.

2. Plants

Rice (*Oryza sativa* L. cv. Nipponbare), barnyardgrass (*Echinochloa oryzicola* Vasing.), corn (*Zea mays* L. cv. Honeybantam) and large crabgrass (*Digitaria adscendens* Henr.) were grown to the 2-leaf stage in a chamber at 25°C during the day (12hr) and at 20°C at night with light intensity of 200 $\mu\text{E m}^{-2}\text{sec}^{-1}$ and relative humidity of 60%.

3. Effect of pretilachlor and CDNB on plant growth

Pretilachlor and CDNB were dissolved in the nutrient solution. Final concentration of the solvent DMSO was 0.02% (v/v). The chemical concentrations in the nutrient solution were 10^{-7} , 10^{-6} , and 10^{-5}M . Solvent control (0.02% DMSO) was also prepared. Plant roots were soaked in 500ml of the solution in a grass beaker; the solutions were renewed every 3 days. The fresh weight of plants was measured after 6 days. Experiments were carried out with 3 replicates.

4. Chemical Treatments for Assays of GST Activity

Pretilachlor at 0, 3×10^{-7} , 10^{-6} , 10^{-5}M and CDNB at 0, 5×10^{-7} , 2×10^{-6} , 10^{-5}M were applied for 60hr as described above. After treatment, plant shoots were excised and stored in a -80°C freezer until extracted. The experiment was performed in triplicate and repeated at least twice.

5. GST extraction

Plant tissues (1g) were ground and homogenized with a homogenizer (Polytoron, 1,000 rpm $\times$ 15 sec and 15,000 rpm $\times$ 30 sec) in 5ml of ice-cold medium containing 0.1 M K-phosphate buffer (pH 7.8) and 0.05 g PVPP (polyvinyl poly-pyrrolidone). The homogenate was centrifuged at $25,000 \times g$ for 20min at 4°C. The supernatant was filtered

through two layers of Miracloth (Calbiochem, CA) and then used for the determination of crude GST activity.

6. Assays of GST activity

GST activity using CDNB as substrate, GST(CDNB) activity, was determined by the method of Gronwald *et al.*⁹⁾ with slight modification. The 3 ml reaction mixture contained 0.76 ml of the enzyme extract, 2 ml of 44 mM potassium phosphate buffer (pH 7.0), 0.12 ml of 10 mM GSH, and 0.12 ml 25 mM CDNB. The mixture was incubated at 30°C for 5 min, and then the reaction was started by the addition of CDNB. The amount of GS-CDNB conjugate was measured by monitoring the change in absorbance at 340 nm, 25°C. A molar absorption coefficient of 9.6/mM/cm¹⁰⁾ was used to calculate enzyme activity, which was corrected for nonenzymatic conjugation.

GST activity using pretilachlor as substrate, GST(pretilachlor) activity, was determined by the method of Shim *et al.*²⁰⁾ with slight modification. The reaction medium consisted of 0.1 ml of 0.1 M GSH, enzyme extracts (0.3 ml), 0.7 ml of 0.1 M K-phosphate buffer (pH 7.6) and ¹⁴C-pretilachlor

(spec. Act. 440 kBq/ μ mol) and was incubated for 1 hr at 25°C. The reaction was stopped by transferring the test tubes to ice and adding 0.1 ml of 32.5% (w/v) trichloroacetic acid. After non-conjugated pretilachlor was removed by extracting three times with 1 ml of petroleum ether (bp 30–60°C), radioactivity in the aqueous phase was determined by liquid scintillation spectrometry (Beckman LS-8100). GST activity values were corrected for non-enzymatic conjugation.

Results and Discussion

1. Effect of pretilachlor or CDNB on plant growth

Figure 1 shows the effect on plant growth of pretilachlor or CDNB treated to roots at the 2nd-leaf stage. Corn was most tolerant to pretilachlor. The ranking of growth inhibition at 10⁻⁵M, from highest to lowest, was barnyardgrass, large crabgrass, rice, and corn. The effect of CDNB on the growth of each plant was less than that of pretilachlor, though at 10⁻⁵M barnyardgrass and large crabgrass were more inhibited than rice and corn.

Fig. 1 Effect of root-applied pretilachlor (left) or CDNB (right) on the growth of plants.

—●— Rice, —▲— Barnyardgrass, —■— Corn, —×— Large crabgrass

Shim *et al.* also reported the effect of pretilachlor on plant growth¹⁹. Their treatment of rice, barnyardgrass, and corn seeds or 3 week old seedlings with pretilachlor (10^{-7} ~ 10^{-5} M) showed the same tendency as this study. The specific difference in sensitivity to pretilachlor was more clear in the case of seed-treatment than seedling-treatment.

The concentrations of applied chemicals for subsequent experiments were determined from the result of this experiment. Pretilachlor concentrations were 3×10^{-7} , 10^{-6} , and 10^{-5} M, and CDNB were 5×10^{-7} , 2×10^{-6} , and 10^{-5} M.

2. Effect of treatment of chemicals used as substrate on GST activity

GST (CDNB) activities in rice and corn seedlings were much higher than those in barnyardgrass and large crabgrass (Fig. 2). Treating with CDNB more or less enhanced GST(CDNB) activities in the three species except large crabgrass. The respective percentages of maximal enhancement in rice, barnyardgrass, and corn were 10.8%, 16.7%,

and 27.7%. Treatment with pretilachlor induced GST(CDNB) activities only in rice and corn, with maximal induction percentages of 21.6% and 30.7%, respectively.

GST(pretilachlor) activities of corn were about twice as high as the others (Fig. 3). Treatment with CDNB had a little effects on GST(pretilachlor) activities in all plants. Treatment with pretilachlor enhanced GST (pretilachlor) activities in rice and corn by a maximum of 41.3% and 15.7%, respectively. It is likely that greater GST activities are induced by treatment of substrates.

Taking a look at each plant, in rice seedlings, treatment with pretilachlor enhanced both GST(pretilachlor) and GST(CDNB) activities more than treatment with CDNB (Fig. 2, 3). Apart from CDNB treatment, treating of barnyardgrass with pretilachlor had little effect on the induction of GST activities. Treatment of corn with both pretilachlor and CDNB increased GST (CDNB) activity more than GST(pretilachlor), and treatment of large crabgrass with

Fig. 2 Effect of root-applied pretilachlor (left) or CDNB (right) on GST(CDNB) activity in shoot of plants. pretilachlor concentration; Untreated 3×10^{-7} M 10^{-6} M 10^{-5} M CDNB concentration; Untreated 5×10^{-7} M 2×10^{-6} M 10^{-5} M

either CDNB or pretilachlor had little effect on the enhancement of GST activities.

Han and Hatzios¹¹⁾, Deng *et al.*⁴⁾, and Shim *et al.*²⁰⁾ previously reported on GST activities in plants after treatment with pretila-

chlor. Han treated etiolated 2-day-old rice (cv. Lement) seedlings with 2 μ M pretilachlor. This treatment pretilachlor decreased GST(pretilachlor) activity to a level lower than the untreated. Combined treatment with

Fig. 3 Effect of root-applied pretilachlor (left) or CDNB (right) on GST(pretilachlor) activity in shoot of plants.

pretilachlor concentration; $\square$ Untreated $\square$ 3×10^{-7} M $\blacksquare$ 10^{-6} M $\blacksquare$ 10^{-5} M
CDNB concentration; $\square$ Untreated $\square$ 5×10^{-7} M $\blacksquare$ 2×10^{-6} M $\blacksquare$ 10^{-5} M

Fig. 4 Highest induction rate of plant GST activities induced by pretilachlor or CDNB.

$\square$ GST(pretilachlor) $\blacksquare$ GST(CDNB)

fencloirim greatly induced GST activity. Deng treated seeds of rice (cv. Nipponbare) and barnyardgrass with $10\mu\text{M}$ pretilachlor; GST (pretilachlor) activity in rice was enhanced, and GST(pretilachlor) and GST(CDNB) activities in barnyardgrass were also increased. Shim also treated seeds of rice(cv. Nipponbare), corn (cv. Honey bantam), and barnyardgrass with $5\mu\text{M}$ pretilachlor, which induced GST activities in all plants.

Thus, inductions of GST activities vary greatly. This is probably attributable to the great variation in plant species, cultivar, growth stage, and concentration of treatment chemicals. We initially expected that an appropriate chemical concentration for GST activity induction occurred because growth inhibitions vary with plants. However, there seemed to be no relation between the chemical concentration treated and GST activity.

3. Relationship between applied chemical and substrate when GST activity is induced

To look at whether inductions of GST (CDNB) and GST(pretilachlor) activities were affected by applied chemicals (CDNB,

pretilachlor), the highest induction rates of GST activities were summarized (Fig 4). Treatment of rice and barnyardgrass with pretilachlor enhanced GST(pretilachlor) activities more than GST(CDNB), and treatment with CDNB enhanced GST(CDNB) activities more than GST(pretilachlor). That is to say, there was a relationship between the applied chemicals and substrates when GST activities were induced. For corn, on the other hand, treatment with both chemicals always induced higher GST(CDNB) activity than GST(pretilachlor). Treatment of large crabgrass with CDNB and pretilachlor had little effect on the enhancement of GST activities. Therefore, specificity but no general patterns of GST induction were found in the plants.

4. Relationship between growth and GST activity

Figure 5 and 6 show the growth-GST activity relationships at each concentration of pretilachlor or CDNB. The concentrations of pretilachlor are 10^{-5} , 10^{-6} M, and of CDNB is 10^{-5} M. When plants were treated with pretilachlor, growth seemed to be correlated

Fig. 5 Relations between plants' growths and GST activities after pretilachlor application. pretilachlor concentration: ○, △, □, ☆ 10^{-6} M; ●, ▲, ■, ★ 10^{-5} M
○ ● Rice, △ ▲ Barnyardgrass, □ ■ Corn, ☆ ★ Large crabgrass

with GST (pretilachlor) activities more closely than with GST (CDNB) activities. When plants were treated with CDNB, growth seemed to be roughly correlated with both GST activities. It seemed, however, that there was a stronger relation between growth and GST (CDNB) activity.

It is well known that tolerance to an herbicide conjugated with GSH is correlated with GST activity, and that it is the main factor of selectivity of the herbicide^{12,23}. Shim *et al.* also reported that there was a correlation between I_{50} values and GST activities in grasses²⁰.

From this experiment, it was found that tolerance to a chemical conjugated with GSH is correlated with GST activity using the chemical as substrate after treatment with that chemical. In other words, the difference in activity of GST (CDNB) or GST (pretilachlor) induced by CDNB or pretilachlor, respectively, resulted in the difference in growth shown in Fig. 1.

GST consists of many isozymes (multiple forms), and each isozyme has substrate

specificity^{3,6}, but these specificities somewhat overlap one another, and they exhibit activities to a lot of chemicals^{22,23}. There are various substances inducing GST activities: herbicides, safeners, active oxygens, ethylene, pathogens, *etc.*^{8,14,15,16}. There are also a myriad of substances capable of being substrates. For this reason, it is very hard to know the mechanism of induction of GST activities, and little has yet been determined.

Further investigation using more plants and substrates and characterization of the GST isozyme of each plant are required to learn the relation between a chemical inducing GST activity and a substrate, that is, the molecular diversity.

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● Rice   ▲ Barnyardgrass   ■ Corn   ★ Large crabgrass

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基質処理による glutathione S-transferase 誘導の植物種間における比較

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摘 要

Glutathione S-transferase (GST)活性が誘導される際、処理化合物と基質の種類に関連があるのかどうか、イネ科植物4種（イネ、タイヌビエ、トウモロコシ、メヒシバ）を用いて調べた。研究の目的に合わせて、処理化合物は測定に用いる基質2種類（プレチラクロール、CDNB）に限った。また、薬剤耐性とGST活性の誘導の関係においても、処理化合物と基質の種類に注目して検討した。プレチラクロール、CDNB処理後の4種の植物のGST(pretilachlor), GST(CDNB)活性を測定したところ、植物によって処理の影響は様々であった (Fig. 2,

3)。誘導が処理基質の種類により影響を受けているか調べるために、最も大きく誘導された時の誘導率をまとめたところ、イネ、タイヌビエに限れば、処理化合物を基質とするGSTでの誘導の方が大きかった (Fig. 4)。またCDNB処理に限れば、イネ、タイヌビエ、トウモロコシの3種でGST(CDNB)活性の方が大きく誘導された。しかし、全体としては、処理化合物と誘導率の関係は各植物ごとに特異的で、共通性は少なかった。薬剤耐性とGST活性の誘導の関係において、処理化合物と基質の種類という観点から見ると、処理した化合物を基質としたGST活性の方がそうでないものより、その化合物に対する耐性と関連が大きいといえた (Fig. 5)。つまり、プレチラクロール処理により誘導されたGST(pretilachlor)や、CDNB処理により誘導されたGST(CDNB)活性の大きさの違いが Fig. 1に見られるような薬剤耐性の差を生み出していると考えられた。

キーワード: glutathione S-transferase, 誘導, 基質, プレチラクロール, CDNB

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