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Induction of pumpkin glutathione *S*-transferases by different stresses and its possible mechanisms

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Abstract

Induction of pumpkin (*Cucurbita maxima* Duch.) glutathione *S*-transferases (GSTs) by different stresses and endogenous *trans*-2-hexenal content were determined in search of a common signal for GST induction. All of the stresses showed significant induction, As_2O_3 causing the highest induction followed by *trans*-2-hexenal. The *trans*-2-hexenal content was highest in *trans*-2-hexenal-treated seedlings and next-highest in methyl jasmonate-treated seedlings, whereas high temperature- and As_2O_3-treated seedlings had *trans*-2-hexenal contents lower than that of control seedlings. Induction of GST, lipoxygenase (LOX) and hydroperoxide lyase (HPL) was compared, since *trans*-2-hexenal and methyl jasmonate are the products of the LOX pathway. All four stresses showed weak LOX induction, high temperature causing the highest induction. However, only methyl jasmonate caused weak HPL induction. Both antioxidants or oxidants induced GST to different degrees. Glutathione contents of reduced glutathione (GSH) or oxidized glutathione (GSSG)-treated seedlings were significantly higher than the content of control seedlings, whereas those treated with other antioxidants or oxidants had contents similar to or less than control seedlings. The GSH:GSSG ratio was lowest in GSSG-treated seedlings and next-lowest in GSH-treated seedlings. The results of this study suggest that pumpkin GSTs are not induced through a common signalling pathway and that redox perturbation plays a role in pumpkin GST induction.

Additional key words: antioxidants, *Cucurbita maxima*, glutathione, lipoxygenase pathway, oxidants, redox homeostasis, *trans*-2-hexenal.

Introduction

Glutathione *S*-transferases (GSTs) are a family of enzymes catalyzing the conjugation of reduced glutathione (GSH) with a wide variety of electrophilic substrates (Mannervik and Danielson 1988, Wilce and Parker 1994). GSTs have been found in almost all species examined, including plants, animals, and bacteria (Hayes and Pulford 1995). The first report of GST activity in plants was that of an enzyme in maize responsible for detoxification of the herbicide atrazine (Frear and Swanson 1970). Although the role of plant GSTs in detoxification of several classes of herbicides is well documented, their role in metabolism has not been fully elucidated. Previously, we found that aldehydes, particularly α,β-unsaturated aldehydes, strongly induce pumpkin GSTs and we hypothesized that plant GSTs play an important role in the detoxification of toxic aldehydes (Fujita and Hossain 2003). In an attempt to determine the possible relationship between GST induction and cellular aldehyde content, we compared content of a model compound, *trans*-2-hexenal and GST induction in control and stressed pumpkin seedlings. This compound strongly induces pumpkin GSTs and is produced in large quantity in stressed plant tissue through the hydroperoxide lyase (HPL) branch of the lipoxygenase (LOX) pathway. C_6 aldehydes, together with their corresponding reduced alcohols, are important volatile constituents of fruits, vegetables, and green leaves. Recent findings have indicated the involvement of short-chain aldehyde

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Abbreviations: ADH - alcohol dehydrogenase; DDT - *threo*-1,4-dimercapto-2,3-butanediol; EDTA - ethylenediaminetetraacetic acid; GSH - reduced glutathione; GSSG - oxidized glutathione; GST - glutathione *S*-transferase; HPL - hydroperoxide lyase; JA - jasmonic acid; LOX - lipoxygenase; MeJA - methyl jasmonate; NADH - nicotinamide adenine dinucleotide reduced form; SDS-PAGE - sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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products of the 13-HPL pathway, including *trans*-2-hexenal, in defenses against microbial pathogens and insects (Bate and Rothstein 1998, Blee 1998, Vancanneyt *et al*. 2001). Activities of lipoxygenase as well as GST have been reported to be induced by pathogen attack (Croft *et al*. 1990, Mauch and Dudler 1993). A comparison of the induction of GST, LOX and HPL by different stresses would therefore be helpful in explaining the cause of pumpkin GST induction by aldehydes. Among the various products of the LOX pathway in higher plants, the biological activities of the jasmonate family, which includes jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), have been extensively studied. These compounds play important roles in the regulation of stress-induced gene expression (Howe and Schilmiller 2002). A subset of JA-induced genes associated with defense response in *Arabidopsis* has been reported to be induced by *trans*-2-hexenal (Bate and Rothstein 1998). It is therefore possible that *trans*-2-hexenal-induced pumpkin *GST* genes are responsive to MJ. High temperature as an environmental stress and As_2O_3 as a metal stress were also included in the study to compare GST induction and aldehyde content under stress conditions.

Antioxidant enzyme activity is greatly affected by treatments that caused oxidative stress (Bernardi *et al*. 2004). Similarly, plant *GST* gene expression is induced by conditions that lead to oxidative stress (Polidoros and Scandalios 1999). It is known that the encoded GST proteins play an important role in plant responses to stresses, but the principle is not well understood. A review of available literature suggests that plant stresses induce *GST* gene expression by influencing metabolism of ROS or by chemically modifying thiol compounds, leading to transiently reduced GSH levels and change in the intracellular redox equilibrium toward a more oxidized state (Kömives *et al.* 1997). Several research groups have reported the induction of plant GST isoenzymes by antioxidants, namely, GSH, ascorbic acid and threo-1,4-dimercapto-2,3-butanediol (DTT) (Ulmasov *et al*. 1995, DeRidder *et al*. 2002, Dixon *et al*. 2002). Moreover, *Arabidopsis* GSTs have been reported to be induced by the oxidant *t*-butyl hydroperoxide (Dixon *et al*. 2002). Thus, GSTs would be induced by antagonistic signals, such as oxidative signals and antioxidant compounds. Dixon *et al*. (2002) proposed distinct recognition/signalling pathways for *Arabidopsis* GST induction by herbicide safeners and xenobiotic compounds that are different from the regulatory system responsive to antioxidants/oxidants. These contradicting observations led us to hypothesize that pumpkin GST may be sensitive to cellular redox changes. To test this idea, we examined the effects of a range of antioxidants/oxidants, *trans*-2-hexenal, methyl jasmonate, As_2O_3 and high temperature on pumpkin GST induction and redox status. In addition, Mn^{2+}, Cr^{2+}, L-cysteine, ruthenium oxide and 2,3-dichloro-5,6-dicyano-*ρ*-benzo-quinone were used to help elucidating the mechanism of induction.

Materials and methods

Plants and stress treatments: To raise seedling, mature pumpkin (*Cucurbita maxima* Duch.) seeds were sown in vermiculite saturated with deionized water and incubated in the dark at 25 ºC. Roots of 6-d-old seedlings were detached, and the shoot was used as plant material for various stress treatments.

First, *trans*-2-hexenal and methyl jasmonate, each at a concentration of 0.5 mM, were used to determine the reason(s) for pumpkin GST induction by aldehydes. Seedlings were also exposed to a high temperature (42 ºC) and 0.1 mM As_2O_3 as a metal stress for the same purpose. Next agents that directly perturb the redox potential in cells, namely, the oxidants H_2O_2, glutathione disulphide (GSSG), L-cystine, $KMnO_4$, K_2CrO_4, cumene hydroperoxide and *t*-butyl hydroperoxide and the reductants GSH, ascorbic acid, β-mercaptoethanol, *threo*-1,4-dimercapto-2,3-butanediol (DTT) and L-cysteine, were used to treat the seedlings. Additionally, seedlings were treated with Mn^{2+}, Cr^{2+}, ruthenium oxide and 2,3-dichloro-5,6-dicyano-*ρ*-benzoquinone. All of the agents except $KMnO_4$ and K_2CrO_4 were used at concentrations of 1 mM. For both $KMnO_4$ and K_2CrO_4, 1 and 10 mM solutions were used. For each treatment, three seedlings were placed in separate glass cups that each contained 20 cm^3 of solution, and each cup was covered with a polyethylene tube to prevent undesirable mixing of the vapour of different chemicals. The cups containing seedlings were then incubated at 25 °C for 24 h in the dark. Seedlings incubated with 20 cm^3 of distilled water were used as controls.

Preparation of crude enzyme solution: For a GST assay, treated hypocotyls were homogenized in an equal volume of 25 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA and 1 % (m/v) ascorbate with a mortar and pestle. The homogenate was centrifuged at 14 500 *g* for 10 min, and the supernatant was used as a crude enzyme solution. All procedures were performed at 0 to 4 °C. The same solution was used for a lipoxygenase assay.

For a hydroperoxide lyase assay, crude enzyme extracts were prepared by homogenizing hypocotyl samples with equal volumes of 0.1 M potassium phosphate buffer (pH 6.5) containing 3 mM DTT and 0.5 % Triton X-100 using a mortar and pestle. The crude homogenate was centrifuged at 14 500 *g* for 15 min, and the supernatant was used for enzyme activity and protein determinations.

Assay of enzyme activities and protein quantitation: GST activity was determined spectrophotometrically (*Hitachi UV 2000* double beam spectrophotometer, Tokyo, Japan) by measuring the absorbance at 340 nm as described previously (Fujita and Hossain 2003).

The LOX activity was determined at 25 ºC by measuring the increase in absorbance at 234 nm for 1 min, and linoleic acid was used as a substrate. The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 0.25 mM linoleic acid, and enzyme solution in a final volume of 0.7 cm^3. The reaction was initiated by the addition of enzyme solution.

The HPL activity was assayed by the method of Vick (1991), and 13-hydroperoxide of linolenic acid was used as a substrate. The reaction mixture contained, in order of addition, 100 mM potassium phosphate buffer (pH 6.0), 0.1 mM NADH, 50 units per cm^3 yeast ADH, 0.1 mM linolenic acid hydroperoxide, and enzyme solution to a final volume of 0.7 cm^3. The lyase reaction was initiated by the addition of enzyme solution, and the initial rate of decrease in absorbance was measured at 340 nm. After subtraction of the background rate, net enzyme rate was expressed as moles of NADH oxidized per min, based on a coefficient of absorbance of 6.22 $mM^{-1}\,cm^{-1}$ for NADH.

The protein concentration of each sample was determined by the method of Bradford (1976).

SDS-PAGE and Western blotting: SDS-PAGE and Western blot analysis of pumpkin GSTs was carried out by the methods described by Fujita and Hossain (2003).

Northern blot hybridization analysis: Total RNA was extracted using an RNeasy Plant Mini Kit (*Qiagen*, Hilden, Germany), and Northern blot analysis was carried out as described previously (Fujita and Hossain 2003).

Measurement of *trans*-2-hexenal: To determine whether the induction of pumpkin GSTs by aldehydes is due to a rise in contents of aldehydes in plant cells, the concentrations of *trans*-2-hexenal in control and stressed seedlings were measured by gas chromatography. After treatment with selected agents, hypocotyl samples each of approximately 1.0 g (from the same set of seedlings that were used for determination of enzyme activities) were put into 10-cm^3 screwcap vials and stored at -80 ºC for 24 h. For preparation of samples for *trans*-2-hexenal measurement, each vial into which a capillary syringe had been inserted was placed in a 60 ºC water bath for 30 min. After incubation, the syringe was taken out and inserted directly into a *Shimadzu GC-14A* gas chromatograph (Kyoto, Japan) with a 30 m × 0.32 mm × 0.25 µM *Spelcowax-10* fused silica capillary column operated under the following conditions: injector temperature, 220 ºC; oven temperature, 50 ºC for 5 min followed by temperature increase 3 ºC min^{-1} to 110 ºC and then 10 ºC min^{-1} to 250 ºC; flame ionization detector temperature 240 ºC; helium carrier flow rate, 40 $cm^3\,min^{-1}$. All quantitative analyses were repeated at least three times. The identity of the compound was determined by co-chromatography with an authentic standard (*Wako*, Osaka, Japan). The retention time for *trans*-2-hexenal was 4.52 min.

Determination of glutathione: Hypocotyls of pumpkin seedlings were homogenized with 3 volumes of 5 % (m/v) metaphosphoric acid containing 1 mM EDTA. Centrifugation was carried out for 20 min at 15 500 *g*. All operations were carried out at 0 - 4 ºC, and clear supernatant was used for glutathione determination. GSH and GSSG were determined according to Knörzer *et al*. (1996).

Statistics: Data of 9 measurements (6 for *trans*-2-hexenal and for glutathione measurement) from three independent experiments were compared using two-way analysis of variance, and mean differences were compared with least significant difference/Duncan's multiple range test using *MSTAT-C* (Department of Crop and Soil Sciences, Michigan State University, East Lansing).

Results

Induction of pumpkin GSTs by *trans*-2-hexenal, methyl jasmonate, high temperature and As_2O_3: Among the tested stresses, As_2O_3 was found to be the strongest inducer at a concentration of 0.1 mM, and *trans*-2-hexenal was the next-strongest inducer at a concentration of 0.5 mM (Table 1). In addition to activity assays, Northern blot and Western blot analyses also showed similar patterns of induction for different pumpkin GST species (Figs. 1, 2). However, the responses of different pumpkin GSTs to *trans*-2-hexenal and methyl jasmonate were not similar. Northern blot analysis showed that *trans*-2-hexenal was more effective than was MeJA in *Cm*GSTU1 and *Cm*GSTU3 mRNA accumulation, whereas MeJA was more effective in the case of *Cm*GSTU2 and *Cm*GSTF1. In Western blot analysis, *Cm*GSTU1 appeared to be slightly more responsive to *trans*-2-hexenal than MeJA as was found in the activity assays, whereas *Cm*GSTU2 showed similar magnitudes of induction by the two inducers. However, induction of *Cm*GSTF1 mRNA by MeJA is a new finding.

Table 1. Comparison of GST, LOX and HPL activities and *trans*-2-hexenal content in stressed pumpkin seedlings. Seedlings were treated with high temperature (42 °C), 0.5 mM *trans*-2-hexenal (tH), 0.5 mM methyl jasmonate (MeJA) and 0.1 mM As_2O_3 for 24 h. Mean ± SD values of specific activity of 9 measurements from 3 independent experiments are presented. For *trans*-2-hexenal content, mean ± SD values of 6 measurements from 3 independent experiments are presented. Values with different letters are significantly different at $P \leq 0.05$.

Treatment	Specific activity [μmol g^{-1}(protein) min^{-1}]			*trans*-2-hexenal content
	GST	LOX	HPL	[μg g^{-1}(f.m.)]
Control	27 ± 3^{d}	1733 ± 370^{b}	62 ± 10^{c}	1.04 ± 0.23^{c}
42 ºC	93 ± 11^{c}	2340 ± 446^{a}	47 ± 1^{d}	0.54 ± 0.11^{d}
tH	132 ± 14^{b}	2213 ± 394^{a}	63 ± 10^{c}	2.54 ± 0.35^{a}
MeJA	101 ± 17^{c}	2245 ± 425^{a}	90 ± 15^{a}	2.03 ± 0.30^{b}
As_2O_3	144 ± 9^{a}	2186 ± 328^{a}	71 ± 10^{b}	0.88 ± 0.13^{c}

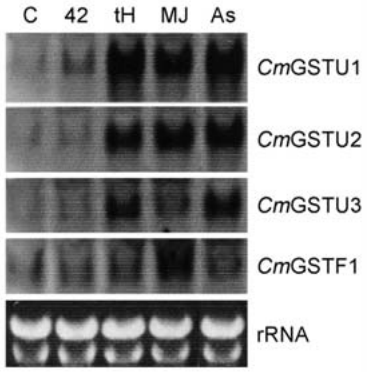

Fig. 1. Northern blot analysis of induction of *Cm*GSTU1, *Cm*GSTU2, *Cm*GSTU3 and *Cm*GSTF1 mRNAs by high temperature (42), 0.5 mM *trans*-2-hexenal (tH), 0.5 mM methyl jasmonate (MJ) and 0.1 mM As_2O_3 (As). For Northern blot analysis, pumpkin seedlings were treated with the above stresses for 6 h. The control is expressed as C. Each lane received 5 μg of total RNA.

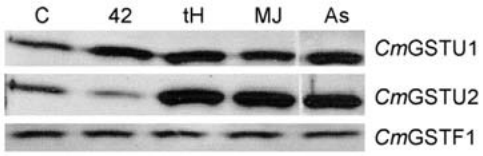

Fig. 2. Western blot analysis of induction of *Cm*GSTU1, *Cm*GSTU2 and *Cm*GSTF1 by high temperature (42), 0.5 mM *trans*-2-hexenal (tH), 0.5 mM methyl jasmonate (MJ) and 0.1 mM As_2O_3 (As). For Western blot analysis, pumpkin seedlings were treated with the above stresses for 24 h. Each lane received 5 μg of protein.

In *trans*-2-hexenal- and MeJA-treated seedlings, considerably larger amounts of *trans*-2-hexenal than those in control seedlings were found (Table 1), while high temperature and As_2O_3 treatment did not increase *trans*-2-hexenal content in pumpkin seedlings. Instead, *trans*-2-hexenal concentration decreased significantly in high temperature-stressed tissues. In addition, induction of two important lipoxygenase (LOX) pathway enzymes, LOX and HPL that play key roles in the generation of *trans*-2-hexenal, was compared with that of GST induction. All of the stresses induced pumpkin LOX weakly (Table 1), and the induction was significantly different to that of the control. However, the scenario was different for HPL. Only MeJA weakly induced HPL activity level (1.45 fold), whereas *trans*-2-hexenal and As_2O_3 showed activities similar to that of control. On the other hand, high temperature caused a reduction in HPL activity in pumpkin hypocotyl, to 75 % that of the control.

Induction of pumpkin GSTs by antioxidants and oxidants: All of the tested oxidants, namely, H_2O_2, glutathione disulfide (GSSG), ruthenium oxide, 2,3-dichloro-5,6-dicyano-*ρ*-benzoquinone, $KMnO_4$, K_2CrO_4, cumene hydroperoxide and *t*-butyl hydroperoxide, increased GST activity (Figs. 3, 5), and the magnitude of the increase was dependent on the oxidants to which the seedlings were exposed. For example, H_2O_2, GSSG, ruthenium oxide, 2,3-dichloro-5,6-dicyano-*ρ*-benzoquinone, and $KMnO_4$ enhanced GST activity to less than 2 fold, whereas cumene hydroperoxide, *t*-butyl hydroperoxide and K_2CrO_4 increased the activity from 2.5 to 5.5 fold. Induction capability of both $KMnO_4$ and K_2CrO_4 was tested at concentrations of 1 mM and 10 mM. At concentrations of both 1 mM and 10 mM, $KMnO_4$ appeared to be a weak inducer, whereas K_2CrO_4 induced pumpkin GSTs significantly (Fig. 3). To explain the different induction capabilities of $KMnO_4$ and K_2CrO_4, the effects of Mn^{2+} and Cr^{2+} on pumpkin GSTs were also tested. Mn^{2+} induced GSTs very weakly, whereas Cr^{2+} exhibited moderate induction (Fig. 3). It is therefore possible that composition of antioxidants/oxidants play a role in the induction process. Induction of *Cm*GSTU1, *Cm*GSTU2 and *Cm*GSTF1 was specifically analyzed through Western blotting using the same set of samples. The expression levels were almost as same as the activity levels except for those of *Cm*GSTF1 (Fig. 3). *Cm*GSTF1

induction by different oxidants was negligible. Moreover, enzyme activity and *Cm*GSTU1 expression level of *t*-butyl hydroperoxide treated-samples were higher than those of cumene hydroperoxide-treated samples, whereas the *Cm*GSTU2 expression level of *t*-butyl hydroperoxide was slightly lower than that of cumene hydroperoxide. This indicates that the effects of the inducers toward pumpkin GST species were different and specific.

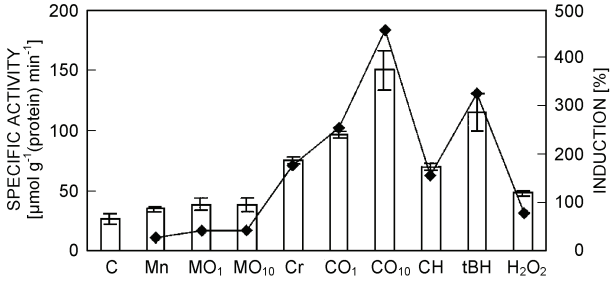

Fig. 3. Induction of pumpkin GSTs by different oxidants [$KMnO_4$ (MO), K_2CrO_4 (CO), cumene hydroperoxide (CH), *t*-butyl hydroperoxide (tBH) and H_2O_2] and metals [Mn^{2+} (Mn) and Cr^{2+} (Cr)]. Six-day-old seedlings were treated with 1 mM solutions of different agents separately except for $KMnO_4$ and K_2CrO_4 for 24 h. Both $KMnO_4$ and K_2CrO_4 were used at 1 and 10 mM concentrations. Mean ± SD values of specific activity of 9 measurements from 3 independent experiments are presented. *Open bars* indicate specific activity and *line graph* indicates induction. Induction is expressed as a percentage of the control value.

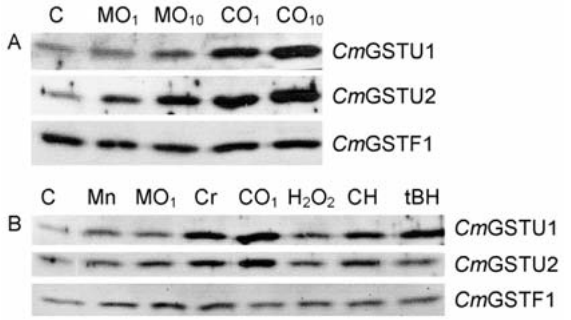

Fig. 4. Western blot analysis of induction of pumpkin GSTs by different oxidants. *A* - Seedlings were treated with 1 and 10 mM solutions of $KMnO_4$ (MO_1, MO_{10}) and K_2CrO_4 (CO_1, CO_{10}) for 24 h. *B* - Seedlings were treated with 1 mM each of Mn^{2+} (Mn), $KMnO_4$ (MO_1), Cr^{2+} (Cr), K_2CrO_4 (CO_1), H_2O_2, cumene hydroperoxide (CH), and *t*-butyl hydroperoxide (tBH) for 24 h. In all cases, 5 µg of protein was loaded in each lane.

The antioxidants, namely, GSH, ascorbic acid, β-mercaptoethanol and L-cysteine, showed weak induction, compared with those of oxidants. L-cysteine and β-mercaptoethanol enhanced GST activity to 2 fold, whereas GSH and ascorbic acid enhanced GST activity to less than 2 fold (Fig. 5). Another antioxidant, DTT, and the oxidized form of L-cysteine, L-cystine, were mostly ineffective as inducers. In Western blot analysis, the expression levels of both *Cm*GSTU1 and *Cm*GSTU2 were almost the same as the activity levels, while *Cm*GSTF1 was almost nonresponsive to the tested antioxidants (Fig. 6).

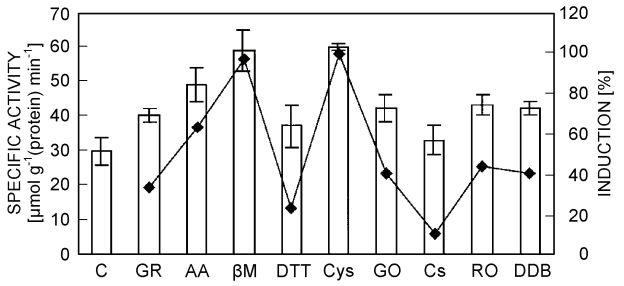

Fig. 5. Effects of antioxidants [glutathione (GR), ascorbic acid (AA), β-mercaptoethanol (βM), DTT and L-cysteine (Cys)] and oxidants [oxidized glutathione (GO), L-cystine (Cs), ruthenium oxide (RO) and 2,3-dichloro-5,6-dicyano-*ρ*-benzoquinone (DDB)] on the induction of pumpkin GSTs. Six-day-old seedlings were treated with 1 mM of each chemical separately for 24 h. Mean ± SD values of specific activity of 9 measurements from 3 independent experiments are presented. *Open bars* indicate specific activity and *line graph* indicates induction. Induction is expressed as a percentage of the control value.

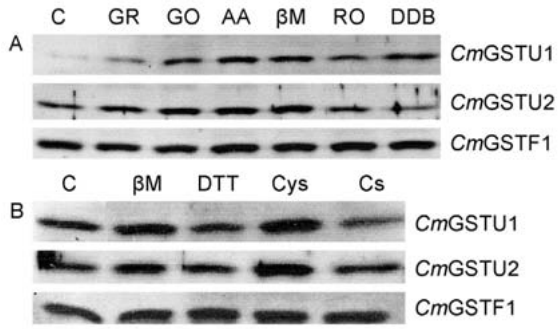

Fig. 6. Western blot analysis of induction of pumpkin GSTs by different oxidants and antioxidants. *A* - Seedlings were treated with 1 mM each of reduced glutathione (GR), oxidized glutathione (GO), ascorbic acid (AA), β-mercaptoethanol (βM), ruthenium oxide (RO) and 2,3-dichloro-5,6-dicyano-*ρ*-benzoquinone (DDB) for 24 h. *B* - Seedlings were treated with 1 mM each of β-mercaptoethanol (βM), DTT, L-cysteine (Cys) and L-cystine (Cs) for 24 h. In all cases, each lane received 5 µg of protein.

Glutathione content and GSH:GSSG ratio in oxidant/antioxidant-treated seedlings: To determine the possible relationship between cellular redox potential and GST induction, pumpkin seedlings were exposed to various oxidants and antioxidants each at a concentration of 1 mM. Total glutathione contents of reduced and oxidized glutathione-treated seedlings increased substantially, and the mean levels were almost 2- and

2.6-fold higher than the control level, respectively (Table 2). Other antioxidants, namely, thiol-containing antioxidants, cysteine and β-mercaptoethanol, as well as ascorbic acid, did not cause an increase in glutathione content. Seedlings treated with another oxidant, ruthenium oxide, showed slightly lower glutathione content than that of the control seedlings. We separated the total glutathione pool into reduced and oxidized forms. Reduced glutathione-treated seedlings showed the highest GSH content (142.31 μg g^{-1} tissue) and GSSG-treated seedlings (138.95 μg g^{-1} tissue) showed the next-highest GSH content, and the levels were 1.9- and 1.85-fold higher than the control level, respectively. Seedlings treated with other oxidants/antioxidants showed GSH contents that were similar to or lower than that of control seedlings (Table 2). For GSSG, GSSG-treated seedlings showed a significantly higher level (61.82 μg g^{-1} tissue) than that of GSH-treated seedlings (8.51 μg g^{-1} tissue), and the levels were 27.0- and 3.72-fold higher than the control level, respectively. In other cases, antioxidants/oxidants did not cause an increase in oxidized glutathione content in pumpkin seedlings (Table 2).

We also estimated the ratios of reduced glutathione and oxidized glutathione to get a picture of the redox status in oxidant/antioxidant-treated seedlings. The ratio was the lowest for oxidized glutathione-treated seedlings (only 2.27), indicating that pumpkin seedlings successfully uptake applied GSSG. The ratio for GSH-treated seedlings was 17.13, significantly lower than that of the control seedlings (Table 2). This indicated that a portion of GSH that had been taken up was converted to GSSG within plant tissue. Two thiol-containing anti-oxidant, cysteine and β-mercaptoethanol, showed significantly higher ratios (46.3 and 46.49, respectively) than that of the control. Another antioxidant, ascorbic acid, and oxidant ruthenium oxide showed ratios almost similar to that of the control. Considering total glutathione content and the ratio of reduced glutathione and oxidized glutathione in oxidant- and antioxidant-treated seedlings, it became clear that there is no common relationship between pumpkin GST induction and total glutathione content and/or GSH/GSSG ratio.

Table 2. Glutathione contents and GSH:GSSG ratios in oxidant/antioxidant-treated pumpkin seedlings. Six-day-old seedlings were treated with 1 mM of each chemical solution separately for 24 h in the dark at 25 °C. Mean ± SD values of 6 measurements from 3 independent experiments are presented. Values with different letters are significantly different at $P \leq 0.05$.

Treatment	Total glutathione [μg g^{-1}(tissue)]	GSH [μg g^{-1}(tissue)]	GSSG [μg g^{-1}(tissue)]	GSH:GSSG
Control	77.30 ± 4.51^{c}	75.01 ± 4.35^{b}	2.29 ± 0.24^{c}	32.94 ± 2.38^{bc}
GSH	150.82 ± 4.94^{b}	142.31 ± 3.48^{a}	8.51 ± 1.49^{b}	17.13 ± 2.34^{d}
GSSG	201.10 ± 18.70^{a}	138.95 ± 10.05^{a}	61.82 ± 8.88^{a}	2.27 ± 0.17^{e}
Cysteine	71.64 ± 6.44^{cd}	70.10 ± 6.22^{b}	1.54 ± 0.27^{c}	46.30 ± 5.92^{a}
β-Mercaptoethanol	75.23 ± 5.35^{cd}	73.80 ± 5.49^{b}	1.42 ± 0.15^{c}	46.49 ± 3.76^{a}
Ascorbic acid	64.03 ± 4.24^{d}	61.99 ± 4.06^{c}	2.03 ± 0.23^{c}	30.73 ± 2.43^{c}
Ruthenium oxide	70.30 ± 3.94^{cd}	68.36 ± 3.70^{bc}	1.95 ± 0.27^{c}	35.57 ± 3.33^{b}

Discussion

In an attempt to determine whether aldehydes act as a common signal in plant GST induction, four different types of stress, *trans*-2-hexenal, methyl jasmonate, high temperature and As_2O_3, were used in this study. All four types of stress induced pumpkin GSTs effectively, but the corresponding contents of *trans*-2-hexenal did not show any synchronized and positive correlation with GST induction. High temperature caused an increase in LOX activity but reductions in the HPL activity and *trans*-2-hexenal content (Tables 1). The reduced HPL activity might be the reason for the lower *trans*-2-hexenal content in high temperature-stressed hypocotyl. As_2O_3-treated samples showed weak induction of both LOX and HPL but had slightly reduced *trans*-2-hexenal content. In contrast, both *trans*-2-hexenal and methyl jasmonate exhibited weak induction both of LOX and HPL as well as causing a significant increase in *trans*-2-hexenal content. It might be mentioned here that *trans*-2-nonenal is an important toxic aldehyde derived through lipid peroxidation. It caused significant induction of pumpkin GST at a concentration of 0.5 mM, but gas chromato-graph analysis showed a negligible amount of *trans*-2-nonenal content in pumpkin seedlings (data not shown). Moreover, *Cm*GSTF1 mRNA was induced by MeJA whereas it was non-responsive to many stresses including a wide range of aldehydes/alcohols (Fujita and Hossain 2003). Considering the observations, we hypothesized that GSTs are not induced through a common signalling pathway, and the results suggested the existence of independent recognition/signalling pathways in pumpkin

GST induction by aldehydes and environmental stresses. Regarding the mechanism by which pumpkin GSTs are induced by aldehydes, it is possible aldehydes are not directly related to GST induction and that they exert their inducibility through some other means, possibly through conferring oxidative stress. Moreover, it is possible that aldehydes might induce GSTs through activation of aldehyde-responsive elements, if any, in the pumpkin GST promoter. This possibility warrants further study.

Plant GSTs have been reported to be induced by both antioxidants and oxidants. To address this apparently paradoxical situation in detail, a range of oxidants and antioxidants were used in this study. The effects of oxidants and antioxidants on pumpkin GST expression were different and specific for each compound. Even compounds for which antioxidant activities are the result of similar chemical properties, such as the -SH-containing compounds β-mercaptoethanol and DTT, affected the same enzyme to different degrees. The similar oxidants cumene hydroperoxide and *t*-butyl hydroperoxide also induced pumpkin GSTs to different degrees. Thus, the responses do not seem to be due solely to the oxidant properties of those compounds. In the present study, a wide range of antioxidants and oxidants were used to determine their effects on pumpkin GST induction. Hydrogen peroxide was an inducer (1.78-fold induction) of pumpkin GST at a concentration of 1 mM. This indicates that oxidative stress directly induces GST expression. Other tested oxidants induced pumpkin GSTs weakly to moderately (Figs. 3-6). Roxas *et al.* (2000) studied the responses of GST-overexpressed transgenic tobacco seedlings to environmental stresses and suggested a role of *in vivo* GSSG in acquisition of oxidative stress tolerance. Several antioxidants such as GSH, ascorbic acid, β-mercaptoethanol and L-cysteine were also weak GST inducers. Several research groups have reported similar results (Ulmasov *et al.* 1995, Polidoros and Scandalios 1999, DeRidder *et al.* 2002, Dixon *et al.* 2002). So, redox potentials of the applied antioxidants/oxidants may be important in the induction process. Among the antioxidants, redox potentials of ascorbic acid, GSH, and L-cysteine at pH 7.0 are 58, -10 and -340 mV, respectively. The reducing capacity of L-cysteine is the highest, and L-cysteine also caused the highest GST induction among the antioxidants tested. However, the reducing capacity of DTT is also high (approximate redox potential at pH 7.0 is -330 mV) but was least effective in induction, though Ulmasov *et al.* (1995) reported that DTT was an efficient GST inducer. These facts suggest that redox potentials of different antioxidants/oxidants play a role in plant GST induction.

The importance of the intracellular redox state in GST induction has also been reported by several researchers (Pinkus *et al.* 1996, Roxas *et al.* 1997, Polidoros and Scandalios 1999, Dixon *et al.* 2002). Glutathione is a multifunctional metabolite and a major antioxidant in plants. It has been suggested to play an important role in defense of plants and other organisms against oxidative stress (Alscher 1989, Grant *et al.* 1996). Considering this, the total glutathione content and the ratio of GSH and GSSG were determined in antioxidant/oxidant-treated pumpkin seedlings with a view to determining the possible relationship of GST induction with glutathione status as well as with redox potential. In our study, seedlings exposed to GSH and GSSG showed almost 2-fold and 2.6-fold increases in total glutathione content (Table 2). However, other antioxidants and oxidants did not cause an increase in glutathione level, and ascorbic acid significantly decreased the level. Here, we need to clarify one point. Several research groups have reported that incubation of plant material with cysteine resulted in a substantial increase in glutathione content (Strohm *et al.* 1995, Noctor *et al.* 1996, 1997a). However, those experiments were conducted in the presence of light. In the dark, similar treatments did not cause increased accumulation of GSH (Buwalda *et al.* 1988, Noctor *et al.* 1997a,b). We incubated pumpkin seedlings in antioxidant/oxidant solutions in the dark for 24 h. This dark incubation might be the reason for the lower glutathione content in cysteine-treated seedlings. Skórzyńska-Polit *et al.* (2003/4) also reported strong reduction of ascorbate content in Cd^{2+}-treated *Arabidopsis* leaves whereas same stress caused significant increase in superoxide dismutase, ascorbate peroxidase and glutathione reductase activity. However, the antioxidants and oxidants used in this study appeared to be weak pumpkin GST inducers. Our results revealed that induction of pumpkin GST is not directly related to total glutathione content. Thus, the level of glutathione may not be one of the factors that determine resistance of plants against oxidative stress. Instead, the ratio of GSH and GSSG may play an important role in protection against oxidative stress. Kömives *et al.* (1997) also concluded that the level of antioxidants is not necessarily the determining factor in resistance of plants against paraquat-mediated oxidative stress. We therefore determine the ratios of reduced glutathione and oxidized glutathione in oxidant/antioxidant-treated seedlings. Oxidized glutathione-treated seedlings showed the lowest ratio (2.27:1) of GSH to GSSG, apparently due to active uptake of GSSG. The ratio for GSH-treated seedlings was also significantly lower than that of control seedlings (Table 2), indicating the partial conversion of GSH to GSSG within plant cells. Two antioxidants, cysteine and β-mercaptoethanol, caused an increase in the GSH/GSSG ratio (46.3:1 and 46.49:1, respectively) in pumpkin seedlings. However, the antioxidant, ascorbic acid and the oxidant, ruthenium oxide exhibited ratios similar to that of the control (Table 2). We also determined the ratios of GSH to GSSG in high temperature-, *trans*-2-hexenal-, methyl jasmonate- and As_2O_3-treated pumpkin seedlings but failed to establish a specific relationship between GST induction and GSH/GSSG ratio (data not shown). GSTs are important antioxidant enzymes and

play a role in protection of plants from different types of stress. The induction pattern of pumpkin GSTs by oxidants/antioxidants, the glutathione contents in antioxidant/oxidant-treated seedlings and the GSH/GSSG ratios indicate that pumpkin GST induction is not directly related to intracellular glutathione content or GSH/GSSG ratio. However, the possibility of a role of redox perturbation in plant GST induction can not be excluded.

The glutathione redox state is remarkably constant, but extreme oxidative stress leads to oxidation of the glutathione pool (Noctor *et al*. 2002). Furthermore, the physiologically active, reduced forms of antioxidants are more important than the pool size for the survival of plant cells during severe stress conditions (Knörzer 1996). Thus, maintenance of redox homeostasis is important for normal growth and development of plants. Though we did not find a clear relationship between pumpkin GST induction and redox homeostasis, we can not rule out the possibility that plant GSTs play a role in the maintenance of redox homeostasis in plant cells. It is also possible that antioxidants/oxidants change the cell membrane surface redox potential and thus induce GST indirectly.

Finally, we conclude that pumpkin GSTs are not induced through a common signalling pathway and that any stimulus, chemical, antioxidant or oxidant, that can perturb the redox balance of the cell, may serve as an inducer of plant GST genes.

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