Purification of a Phi-type Glutathione S-Transferase from Pumpkin Flowers, and Molecular Cloning of Its cDNA

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A major species of glutathione S-transferase (GST), Pugf, was highly purified from pumpkin flowers. Two-dimensional electrophoresis of the purified enzyme gave two adjacent protein spots. The specific activity of the purified enzyme was 2.4 μmol min⁻¹ mg⁻¹ protein for 1-chloro-2,4-dinitrobenzene. This value is one to two orders of magnitude lower than that of pumpkin tau-type GSTs.

The expression pattern of Pugf in healthy pumpkin plants and responses to various stresses were examined by western blotting. Pugf was found in high concentrations in petioles, stems, and roots as well as flowers, and was more abundant in expanding young organs than in fully expanded mature organs. Dehydration caused a slight increase in its concentration, but high and low temperatures, salty stress, and 2,4-dichlorophenoxyacetic acid seemed to have no effects.

A cDNA encoding Pugf was cloned and sequenced. Sequence comparison with other plant GSTs suggested that it should be classified as a phi-type GST.

Key words: cDNA cloning; Cucurbita maxima; glutathione S-transferase; pumpkin; enzyme purification

Glutathione S-transferases (GSTs, EC 2.5.1.18) are a family of enzymes that can combine endogenous and exogenous toxic electrophiles to a sulfhydryl group of glutathione. In higher plants, GSTs are key enzymes in the detoxification of herbicides,1) in responses to biotic2) and abiotic stresses,3,4) and in cell responses to plant hormones5–7) and senescence.8,9) Some GSTs have glutathione peroxidase activity10–12) and auxin-binding activity13,14) and GSTs are involved in the transport of anthocyanin from the cytosol to vacuoles.15,16) Structures of plant GSTs that have been elucidated are classified into four kinds, phi, zeta, tau, and theta, and relationships between the kind and functions of plant GSTs have been reviewed.17)

Among vegetables, pumpkin plants have high levels of glutathione.10) We have therefore used pumpkin plants and cultured cells in GST research. Three forms of tau-type GSTs, Puga (CmGSTU1), Pugb (CmGSTU2), and Pugc (CmGSTU3), have been purified to homogeneity from pumpkin callus cultured in a medium containing 2,4-dichlorophenoxyacetic acid (2,4-D),19–21) and the corresponding cDNAs have been cloned. Analysis of their expression suggested that a different GST, Pugf, might be the major GST in pumpkin flowers.22) In this paper, we describe the purification of Pugf (renamed CmGSTF1 as a phi-type GST) from pumpkin flowers and the cloning and characterization of its cDNA. We also describe the expression pattern of the GST in healthy plants and the responses to various stresses.

Materials and Methods

Plant. Callus was induced from sarcocarp tissues of pumpkin (Cucurbita maxima Duch. cv. Ebisu) fruit on Murashige and Skoog’s solid medium that contained 4.5 μM 2,4-D and 0.5 μM kinetin, at 25°C in the dark, as described previously. The callus was then cultivated for more than 50 generations under the same conditions except that 5.3 μM naphthalene-1-acetic acid was used instead of 4.5 μM 2,4-D.

Pumpkin organs were harvested at Kagawa University Farm, rinsed with deionized water, and used promptly.

Stress treatments. For treatment with 2,4-D, the callus was further transferred to the same cultivation medium that contained 4.5 or 180 μM 2,4-D and 0.5 μM kinetin, at 25°C in the dark, as described previously. The callus was then cultivated for more than 50 generations under the same conditions except that 5.3 μM naphthalene-1-acetic acid was used instead of 4.5 μM 2,4-D.

Pumpkin organs were harvested at Kagawa University Farm, rinsed with deionized water, and used promptly.

Stress treatments. For treatment with 2,4-D, the callus was further transferred to the same cultivation medium that contained 4.5 or 180 μM 2,4-D instead of 5.3 μM naphthalene-1-acetic acid, and incubated for various periods.

Mature pumpkin seeds were sown in vermiculite saturated with deionized water and incubated in the dark at 25°C. Five-day-old seedlings were used for various stress treatments. Before use, seedlings were
removed from vermiculite and all traces of vermiculite were washed off carefully with water. Seedlings were placed in cups containing 20 ml of distilled water, and incubated at 25°C as a control or at a stressful temperature (4 or 42°C). For dehydration stress, seedlings were placed in a cup without water and kept at 25°C. Seedlings were placed in 20 ml of 50 mM NaCl solution for salt stress and kept at 25°C. Four seedlings were used in each treatment and were incubated for 24 h in the dark.

**Extraction of soluble protein.** The callus or organs were homogenized in an equal volume of 25 mM Tris-HCl buffer (pH 8.0) that contained 1 mM EDTA and 1% (w/v) potassium ascorbate. The homogenate was squeezed through two layers of nylon cloth. The filtrate was centrifuged at 12000 × g for 15 min, and the supernatant was used as a soluble protein solution for enzyme purification and western blotting analysis.

**Purification of Pugf.** Protein in the soluble protein solution was precipitated with ammonium sulfate at 30% to 70% saturation. The protein was dialyzed overnight against 5 mM Tris-HCl buffer (pH 8.0) that contained 0.01% (v/v) β-mercaptoethanol (buffer A). The dialysate was put on a column (1.6 cm i.d. × 34.6 cm) of DEAE-cellulose (DE-52; Whatman, Kent, UK) that had been equilibrated with buffer A. The column was washed with buffer A and eluted with a linear gradient of 0 to 0.18 M KCl in 1,000 ml of buffer A. Fractions of 5.8 ml were collected. The fractions corresponding to the highest GST active peak, around 0.035 M KCl, were combined as the Pugf pool. The pool was put on a column (0.76 cm i.d. × 5.5 cm) of S-hexylglutathione-agarose (Sigma, St. Louis, MO) that had been equilibrated with 20 mM Tris-HCl buffer (pH 8.0) containing 0.01% (v/v) β-mercaptoethanol (buffer B). The column was washed with buffer B containing 0.2 M KCl and eluted with buffer B containing 2.4 mM S-hexylglutathione. Fractions of 2.9 ml were collected. Protein fractions eluted with S-hexylglutathione were combined and dialyzed against buffer B. The dialysate was used as the purified Pugf solution.

**Production of polyclonal antibodies against Pugf.** A rabbit (weighing about 3 kg) received subcutaneous injections of a total of 1.26 mg of purified GST protein in Freund’s complete adjuvant at several sites. After two weeks, the rabbit was given a first booster injection of 1.26 mg of the purified GST protein in incomplete adjuvant, and then a second booster injection of 1.26 mg of the purified GST protein in incomplete adjuvant was given two weeks after the first booster injection. Blood was taken from the ear vein one week after the second booster injection.

**Pumpkin flower cDNA library.** Total RNA (5.58 mg) was obtained from 26.7 g of pumpkin flowers by the method of Vries et al. A pumpkin flower cDNA-Uni Zap library was constructed with 3 µg of purified poly(A)⁺ mRNA with a titer of 2.3 × 10⁶ plaque-forming units (pfu) for the library as recommended by the manufacturer (Stratagene, La Jolla, CA). The library was screened as described below.

First, 48,000 pfu of the pumpkin cDNA library was plated for primary screening. Plaques were allowed to form on NZ amine-yeast extract (NZY) top agarose at 42°C for 4 h. To lift plaques, a nitrocellulose filter (Hybond ECL, Amersham Pharmacia, Buckinghamshire, UK) treated with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was put on the surface of the agarose where plaques had formed, and the whole was incubated at 39°C for 3.5 h. After incubation, the filter was removed from the agarase and examined by immunodetection done by so-called enhanced chemiluminescence (ECL) with antiserum against Pugf. Plaques that reacted with anti-Pugf antiserum were further purified through two more rounds of screening under the same conditions.

The purified cDNA clones were rescued from the phage by the Stratagene in vivo excision protocol. The cDNAs rescued in pBluescript SK(-) were sequenced with an ABI Prism 310 genetic analyzer (PE Applied Biosystems Japan, Tokyo). Nucleotide sequences and deduced amino acid sequences were analyzed with a GENETYX program (Software Development Co. Tokyo).

**Genomic DNA for Pugf.** Pumpkin genomic DNA was extracted from pumpkin callus with a plant DNA extraction kit (Nucleon Phytopure, Amersham Pharmacia). PCR amplification of the Pugf genomic gene was done with genomic DNA extracted as a template, and 5'CGCTAATCCTGTTTTCGTG3' for the 5'flanking site and 5'GCACAGTCAGGCTCCATT3' for the 3' flanking site as primers. The amplified DNA fragment was subcloned in pT7Blue T-vector and used for sequence analysis.

**Inducible expression of Pugf.** The open reading frame of the clone encoding Pugf was cloned to be in frame with the β-galactosidase gene α-complementation particle in pBluescript SK(-). For expression of GST as a fusion protein, XL1-Blue cells were transformed with phagemids and cultivated at 37°C in Luria-Bertani medium with 50 µg ml⁻¹ ampicillin and 1 mM IPTG for 19 h. The cells were collected and lysed in 25 mM Tris-HCl (pH 8.0) that contained 1 mM EDTA and 1% (w/v) potassium ascorbate. Cellular debris was pelleted through centrifugation (17,500 × g at 4°C for 10 min). After centrifugation, the supernatant for each clone was collected as en-
Table 1. Purification of Pugf, a Glutathione S-transferase, from Pumpkin Flowers
A total of 1,130 g of flowers was used. GST activity was measured with 1-chloro-2,4-dinitrobenzene as the substrate.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Total protein (mg)</th>
<th>Total activity (μmol min⁻¹)</th>
<th>Specific activity (μmol min⁻¹ mg⁻¹ protein)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>6,240</td>
<td>133</td>
<td>0.0213</td>
<td>100</td>
</tr>
<tr>
<td>30–70% saturated (NH₄)₂SO₄ fraction</td>
<td>4,670</td>
<td>104</td>
<td>0.0222</td>
<td>77.9</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>329</td>
<td>44.3</td>
<td>0.135</td>
<td>33.3</td>
</tr>
<tr>
<td>S-Hexylglutathione-agarose</td>
<td>4.8</td>
<td>11.6</td>
<td>2.4</td>
<td>8.7</td>
</tr>
</tbody>
</table>

Fig. 1. Column Chromatography on DEAE-Cellulose of 30–70% Ammonium Sulfate Fraction from a Soluble Protein Fraction Extracted from Pumpkin Flowers (1,130 g).

GST activity toward 1-chloro-2,4-dinitrobenzene is expressed in nmol min⁻¹ 50 ml⁻¹. The upper curve shows the KCl gradient.

Results and Discussion

Puga, Pugb, Pugc, and Pugd are major GSTs in pumpkin callus maintained on a medium containing 2,4-D. The results of preliminary experiments (based on DEAE-cellulose chromatography and two-dimensional gel electrophoresis) done previously suggested that a different species was the major GST in pumpkin flowers. Therefore, in this study, we first tried to purify Pugf, one species of GST, to identify its properties. A soluble protein fraction prepared from pumpkin flowers was put on a DEAE-cellulose column and eluted with a linear gradient of KCl (Fig. 1). Results of previous studies suggested that peaks of activity at 0.025, 0.060, and 0.090 M KCl arise from PugaPuga (a homodimer), PugaPugb (a heterodimer), and PugbPugb (a homodimer), respectively. Therefore, GST consisting of Pugf (the highest peak of GST activity, at or near 0.035 M KCl) seemed to be adequately separated from the other GST species consisting of Puga and Pugb. The Pugf fractions combined were finally put on a column of S-hexylglutathione-agarose (an affinity resin for GST) to complete the purification. Typical results of Pugf purification from pumpkin flowers are shown in Table 1. In this purification, the final product was purified 113 fold in purity with a yield of 8.7%. The crude homogenate contained the other species, Puga and Pugb, so the actual purity and yield could be higher than the ones in Table 1. The specific activity of purified Pugf was 2.4 μmol min⁻¹ mg⁻¹ protein for 1-chloro-2,4-dinitrobenzene. This value is one to two orders of magnitude lower than that of other pumpkin GSTs (86 μmol min⁻¹ mg⁻¹ protein for Puga, 23 μmol min⁻¹ mg⁻¹ protein for Pugb, and 124 μmol min⁻¹ mg⁻¹ protein for Pugc) that have been purified. A two-dimensional electrophoreogram of the purified product showed two adjacent protein spots (Fig. 2). The left spot was denser than the right one. The N-terminal amino acid residues of the protein in the denser spot was sequenced. First, the protein was blotted onto a polyvinylidene fluoride membrane after two-dimensional gel electrophoresis and treated by Edman degradation. A sequence AGIKVHGIPTS was obtained.

Next, we produced rabbit antiserum using the purified product. The antiserum produced did not cross-react with Puga, Pugb, Pugc, or Pugd (Fig. 3). Therefore, the antiserum was used as anti-Pugf antiserum. The organ specificity of Pugf in the pumpkin plant was examined by western blotting (Fig. 4). Pugf protein was at high concentrations in petioles, stems, roots, and young fruits as well as in flowers. The highest one was in young fruits. Comparison of expanding young leaves and fully expanded mature
Fig. 2. Two-dimensional Gel Electrophoresis of Pugf Purified from Pumpkin Flowers.
Two micrograms of the purified GST protein was used. The gel was stained with silver.

Fig. 3. Cross-reactivity of Anti-pugf Antiserum Produced against the Purified Product with Other Pumpkin Glutathione S-Transferases.
Soluble protein fraction from pumpkin callus was put through two-dimensional gel electrophoresis. The gel was further used for western blotting where the antiserum produced was used as the first antibody.

leaves and comparison of expanding young fruits and fully expanded mature fruits suggested that Pugf protein is more abundant in expanding young organs than in fully expanded mature organs. Puga protein is most abundant in fully expanded mature organs, Pugb protein is at high levels in leaves, and Pugc protein is below the detectable level in all organ. The expression pattern of Pugf was different from the patterns of Puga, Pugb, and Pugc.

To discover the physiological properties of Pugf, we examined its quantitative changes in hypocotyls and roots of seedlings under various stresses by western blotting (Fig. 5). Dehydration caused a slight increase in the protein level, but high and low temperatures and salty stress had no effect. The responses of Pugf to physiological stresses were weaker than those of Puga, Pugb, and Pugc.

Using pumpkin cells maintained on 2,4-D-free medium, we did an experiment to find whether 2,4-D increases the amount of Pugf protein in the cells (Fig. 6). Neither low nor high concentrations of 2,4-D in the medium increased the protein level. In contrast, Puga, Pugb, and Pugc increase in the presence of 2,4-D. The results suggest that Pugf is a GST species that has physiological functions different from those of Puga, Pugb, and Pugc.

We constructed a expression cDNA library using mRNA prepared from pumpkin flowers and immunoscreened it with the anti-Pugf antiserum. Four clones encoding a putative GST were obtained by three rounds of screening. Two of them were polyadenylated at the same site, but the others were polyadenylated at different sites. Figure 7A shows the nucleotide sequence and the corresponding amino acid sequence of the longest cDNA clone. The cDNA consisted of 1,064 bp encoding a polypeptide of 214 amino acid residues having a predicted molecular mass of 23,483 Da and a predicted isoelectric point of 6.5. The deduced N-terminal amino acid residues were consistent with those of the dense-spot protein mentioned above. When compared with a corresponding PCR-amplified fragment of genomic DNA, the cDNA was found to be produced through splicing at two sites (200–201 and 248–249 bp in Fig. 7A). The two introns are shown in Fig. 7B. To find whether...
this cDNA encodes GST, we expressed the protein that the cDNA encoded as a fusion protein of β-galactosidase in XL1-Blue cells in the presence of IPTG (Table 2). GST activity was induced by IPTG in the cells transformed with pBluescript that had Pugf cDNA inserted, showing that the cDNA codes GST. In western blotting, the fusion protein expressed in the cells was bound with anti-Pugf antiserum (data not shown), indicating again that the cDNA was that of Pugf.

Pugf had little homology (23–24% identity in amino acid sequence) with Puga, Pugb and Pugc. Of plant GSTs that have been sequenced, Pugf belonged to the phi-type group (Fig. 8). As described above, the Pugf genomic gene contained two introns. The phi-type GSTs of known gene structure contain three exons and two introns, though tau GST contain two exons and one intron, and zeta GST contains ten exons and nine introns. Therefore, Pugf can be named CmGSTF1 by the nomenclature system proposed by Edwards et al.17) Pugf is likely to be classified into subclass cluster A, which contains GSTs from Dicotyledonae. The GSTs that belong to cluster A are thought to have differentiated from one gene that existed before the plants separated into families, because there are no GSTs of the same family that have GST of another family between themselves in cluster A of the dendogram. The range of sequence homology between Pugf and other GSTs of cluster A is narrow (58–61% in identity, 70–72% in similarity). Therefore, GSTs that belong to cluster A might be useful in monitoring the establishment of families in the Dicotyledonae. HM GST and AT PM24, which both have auxin-binding activity, belong to cluster A. In this study, Pugf protein was most abundant in expanding young organs. Perhaps Pugf is involved in cell expansion controlled by auxin. On the other hand, most GSTs in cluster A are induced by stresses and seem to help mitigate damage arising from stress. For example, ERD11 is induced by dehydration; AT PM2428) is induced by ethylene; GST29) 93% identical with PARB, is induced by aluminum; SoC GST (Genbank accession number AF002692) is induced by low temperature. Am GST2 and PM239X14 have glutathione peroxidase activity in the phi-type group, although no GST in cluster A has yet been identified. We did an experiment to find whether Pugf has activity toward cumene and tert-butyl hydroperoxides using bacterial expression systems as reported by Roxas et al.,30) but found no meaningful activity.
Fig. 7. Nucleotide Sequence of Pugf cDNA and Deduced Primary Structure of Protein (A) and Two Intron Sequences (B).

Note that the pumpkin GST coding sequence is in frame with the α-complementation particle of the β-galactosidase gene encoded by pBluescript. Potential polyadenylation signal sequences are underlined. Two splicing sites are marked by arrows.
**Fig. 7.** Dendrogram Showing Similarities between Plant Glutathione S-Transferases Classified as Phi-type.

The dendrogram was constructed with the following protein sequences (Genbank/SWISSProt accession numbers in parentheses): *Nicotiana plumbaginifolia* GST (NP GST; Z71749), tobacco GST (PARB; P30109), *Hyoscyamus muticus* GST (HM GST; P46423), *Solanum commersonii* GST (SoC GST; AF002692), *Euphorbia esula* GST (EE GST; AF242309), *Arabidopsis thaliana* GSTs (AT PM24; P46422, ERD11; P42760, ERD13; P42761, PM239X14; P42769), *Silene cucubalus* GST (SC GST; Q04522), *Petunia hybrida* GST (PH GST; Y07721), and maize GSTs (ZM GST1; P12653, ZM GST3; Patent Publication No. WO 96/23072 A1).
References


