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PRELIMINARY STUDIES ON ORGAN SPECIFICITIES OF 2,4-DICHLOROPHENOXYACETIC ACID-INDUCIBLE GLUTATHIONE *S*-TRANSFERASES AND NEW GLUTATHIONE *S*-TRANSFERASES PREFERENTIAL TO DEVELOPING ORGANS IN PUMPKIN (*CUCURBITA MAXIMA* DUCH.)

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The organ specificities of glutathione *S*-transferase (GST ; EC 2.5.1.18) species in pumpkin (*Cucurbita maxima* Duch.) plants were investigated. There were the apparent differences between the four GST proteins, Puga, Pugb, Pugc, and Pugd, which had been shown to be principal and 2,4-dichlorophenoxyacetic acid-inducible proteins in pumpkin callus (*Plant Cell Physiol.* (1994) 35 : 275－282). Puga was suggested to be a principal GST in the mature organs and seeds. Pugb preferentially distributed in leaves. Pugd distributed as a minor GST throughout the plant bodies. Pugc was not found in any organs of the normal plant. Two kinds of GSTs, designated GSTf1 and GSTf2, were newly found as principal ones in flowers, developing leaves, and developing fruits. These two GSTs seemed to decline as the organs matured. The relative existence ratio of pumpkin GSTs were conjectured to fluctuate by maturing of the organs as well as environmental changes.

Key Words : glutathione *S*-transferase, pumpkin (*Cucurbita maxima* Duch.), organ specificity, 2,4-dichlorophenoxyacetic acid.

Introduction

Glutathione *S*-transferases (GSTs ; EC 2.5.1.18) are thought to be enzymes which conjugate hydrophobic and electrophilic xenobiotics to the sulfide group of glutathione. Therefore, they are also thought to play an important role in resistance to herbicides in higher plants. On the other hand, recently, GSTs which possess auxin-binding activity have been reported in the soluble fractions from *Hyoscyamus*[1] and *Arabidopsis*[2]. GST which have GSH-peroxidase activity has also been reported in *Arabidopsis*[3]. Furthermore, in the molecular biological studies on expression of auxin activities, various species of auxin-responsive genes have been isolated and some of them have been elucidated to encode GSTs or the proteins similar to GST in primary structure[4-10]. These reports show that multiplicity in function and structure of plant GSTs, suggesting that various kinds of plant GSTs may take part in wide variety of physiological phenomena.

We have shown that there were at least four principal GST proteins, designated as Puga, Pugb, Pugc, and Pugd, in pumpkin cultured cells maintained with 2,4-dichlorophenoxyacetic acid as a plant growth regulator and that they had affinity with one another and formed homo- or heterodimers[11,12]. On the other

Abbreviation : GST, glutathione *S*-transferase

hand, it has appeared that there was little GST other than Puga in sarcocarp tissues of mature pumpkin fruit, which were parent tissues for cultured cells. In this study, in order to speculate the roles of these four GST species, the organ specificities of them were preliminaly investigated in normal pumpkin plant by two-dimensional gel electrophoresis and DEAE-cellulose column chromatography. As a result, it was found that these GST proteins each had different patterns of distribution with respect to the developing stages of organs as well as the kinds of organs. Furthermore, two new GST species were found in flowers and young developing organs.

Materials and Methods

Plant materials Pumpkin organs (*Cucurbita maxima* Duch.) harvested at the farm of Kagawa University were promptly rinsed with deionized water and used. Pumpkin seeds were planted ca. 2cm deep into trays filled with vermiculite watered to saturation. The trays were incubated at 25℃ for 4 days in the dark. After that, the trays were further incubated at 25℃ for 6 days in the dark or under continuous illumination of fluoresent. The seeds at day 0, the germs at day 2 and at day 5, and the seedlings at day 10 after sowing were harvested and promptly used.

Preparation of crude enzyme solution Each kind of organs or seedlings was homogenized in an equal volume of 25mM Tris-HCl buffer (pH8.0) containing 1mM EDTA, 1% (w/v) ascorbate, and 0.5mM phenylmethylsulfonyl fluoride. The homogenate was squeezed through two layers of nylon cloth. The filtrate was centrifuged at 12,000x*g* for 10min and the supernatant was used as a crude enzyme solution. All procedures were performed at 0 to 4℃.

Analyses of GST isozymes and subunits Protein was precipitated with ammonium sulfate between 30% and 70% saturation from the crude enzyme solution extracted from various organs. The protein was dialyzed against 10mM Tris-HCl buffer (pH8.0) that contained 0.01% (w/v) β-mercaptoethanol (Buffer A) overnight. For analysis of GST isozymes, the dialyzate was put on a column (1.27cm i.d. x 55cm) of DEAE-cellulose (DE-52 ; Whatman, U.K.) that had been equilibrated with Buffer A. The column was washed with Buffer A and eluted with a linear gradient of 0 to 180mM KCl in 1,000mL of Buffer A. Fractions of 6.13mL were collected. GST activity in each fraction was determined. For analysis of GST subunits, the dialyzate prepared from organs of 20 to 30g was put on a column (1.5cm i.d. x 4 to 6cm) of DEAE-cellulose that had been equilibrated with Buffer A. The column was washed with Buffer A and eluted with Buffer A containing 0.15M KCl. Fractions of 2mL were collected. The fractions with GST activity were combined and immediately put on a column of (0.3cm i.d. x 0.5cm) of *S*-hexylglutathione-agarose (Sigma Chemical Co., U.S.A) that had been equilibrated with 20mM Tris-HCl (pH8.0). The column was washed with 20mM Tris-HCl (pH8.0) containing 0.2M KCl and eluted with 20mM Tris-HCl (pH8.0) containing 0.2M KCl and 1.81mM *S*-hexylglutathione. Fractions of 0.5mL were collected. The above procedures were performed at 0 to 4℃. The fraction with high concentration of GST was analyzed by two-dimensional gel electrophoresis as described previously[11]. Gels were stained with silver.

Assay of enzymatic activity GST activity was measured by the method of Booth *et al.*[13].

Measurement of protein Protein was measured by the method of Bradford[14].

SDS-PAGE SDS-PAGE was done in 12.5% gel containing 0.1% (w/v) SDS by the method of Laemmli[15]. Gels were stained with silver.

Immunoblotting The GST subunits separated by two-dimensional gel electrophoresis were tested for

the cross-reactivity with anti-Puga rabbit antiserum as described previously[12].

Results and Discussion

The specific activity of GST in various kinds of pumpkin organs were examined (Fig. 1). The specific activity was not much different between the organs, suggesting that GST uniformly distributes all over the plant body. Therefore, the GST may play a basic role in life conservation of normal pumpkin plant. However, the values of individual samples varied widely in the cases of stems and flowers, suggesting that GST content in the individual organs may change depending on environmental changes and the own developmental programs.

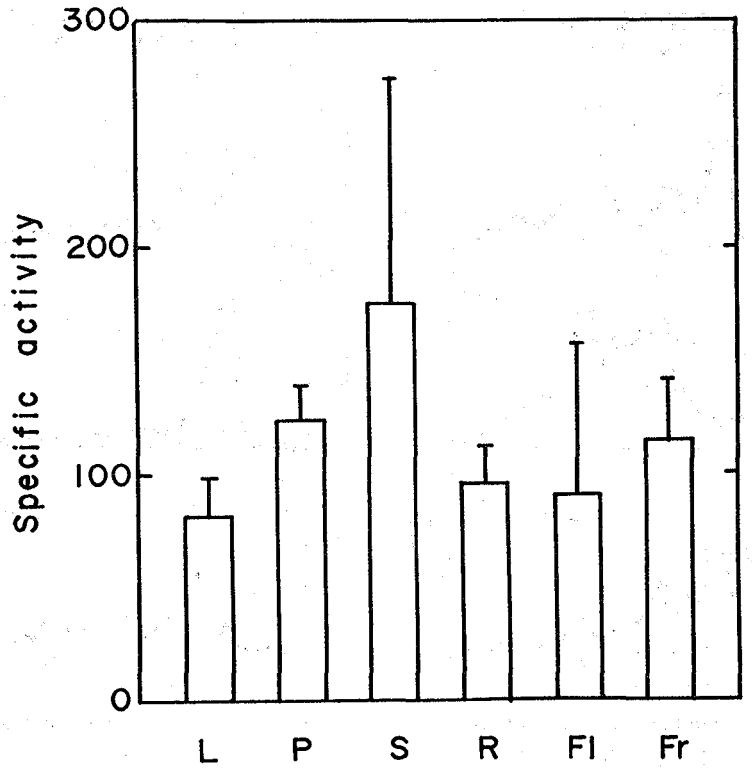

Fig. 1. Specific activity of glutathione *S*-transferase in the soluble protein fractions from various organs of pumpkin.
L, leaf ; P, petiol ; S, stem ; R, root ; Fl, flower ; Fr, fruit.
GST activity was determined with 1-chloro-2, 4-dinitrobenzene as substrate. The specific activity is expressed in nmol min^{-1} $(mg\ protein)^{-1}$. Each bar is the mean from 5 independent experiments and the SE are indicated.

In our previous study[11], it has been shown that the various GST isozymes from pumpkin callus were easily separated on DEAE-cellulose column with a linear gradient of KCl concentration. Therefore, in

order to examine the distribution of GST isozymes in pumpkin plant, DEAE-cellulose column chromatography was carried out with respect to soluble protein fractions prepared from various organs and the elution of GST activity was determined (Fig. 2). For both mature and immature leaves, the abundant activity was found at the KCl concentrations corresponding to ones with which PugaPugb and PugbPugb each were eluted. This was very characteristic to leaves. For mature leaves and mature fruits, the abundant activity was found at the fractions corresponding to PugaPuga. On the other hand, for flowers, stems, developing leaves, and developing fruits, the abundant activity was found at the KCl concentration corresponding to one with which PugaPugd was eluted. However, as described below, it was not PugaPugd but probably new GSTs. The above results suggest that the kinds of GST isozymes which predominatly existed were different according to growth levels of organs as well as kinds of organs.

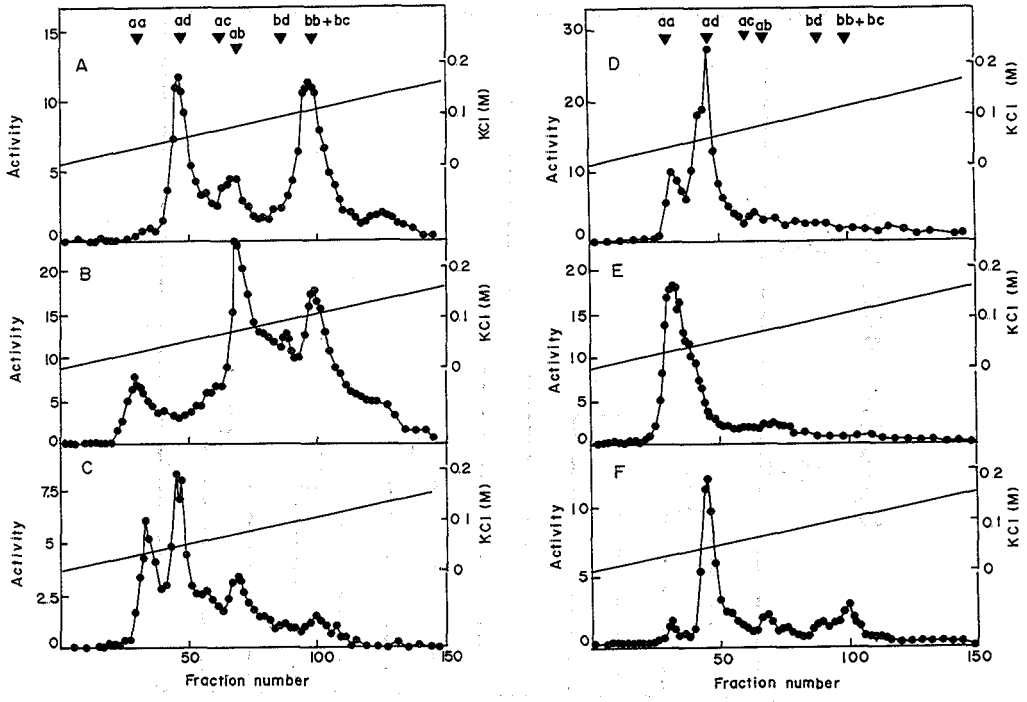

Fig. 2. Glutathione *S*-transferase isozymes of various pumpkin organs separated on DEAE-cellulose column.

The 30−70% ammonium sulfate fractions prepared from soluble protein fractions of various organs were applied to a DEAE-cellulose column and eluted as described in Materials and Methods. A, the sample was prepared from 114g of developing leaves ; B, from 220g of mature leaves ; C, from 269g of stems ; D, from 115g of developing fruits ; E, from 500g of mature fruits ; F, from 112g of flowers. GST activity was determined with 1-chloro-2, 4-dinitrobenzene as substrate. Activities of A, C, D, and F are expressed in nmol min $^{-1}$(260 μL) $^{-1}$ and B and E in nmol min $^{-1}$ (50 μL) $^{-1}$. The elution sites of known pumpkin GST isozymes are indicated by arrowheads on the top of the graph.

Preliminarily, in order to examine which kinds of GST subunits preferentially exist in which organ, soluble protein fractions from individual organs each were applied on an affinity column of *S*-hexylglutathione-agarose and the adsorptives were analyzed by SDS-PAGE. For all the organs examined, a predominant protein band was found at the site corresponding to mobility of Puga (Mr 22,000). However, for the organs other than leaves, thick protein bands comparable with the protein band corresponding to Mr 22,000 were not observed at any sites, including sites corresponding to mobilities of Pugb (Mr 23,000), Pugc (Mr 24,000), and Pugd (Mr 24,500). From the results, the abundant activity found at the elution site of PugaPugd in the DEAE-cellulose column chromatography of flowers and developing organs was suspected to depend on a pI-isomer of Puga or a novel type of GST with a molecular weight similar to Puga, but not on Pugd. For leaves, a thick Pugb band comparable with Puga band was detected, supporting the results obtained from DEAE-cellulose column chromatography.

In order to examine the kinds and the composition of GST subunits in individual organs of pumpkin plant in detail, the soluble protein fractions prepared from various organs were applied to a DEAE-cellulose column, followed by a *S*-hexylglutathione-agarose column and the adsorptives were analyzed by two-dimensional gel electrophoresis (Fig. 3). In flower, Puga, Pugb, and Pugd were present as minor GST components, while two kinds of GSTs which have molecular weights similar to one of Puga and possess isoelectric points higher than Puga (pI 5.45) were present as main subunits. They each are hereinafter refered to as Pugf 1 (pI 6.10) and Pugf 2 (pI 5.80). These pI values were the most highest ones among the GSTs found in pumpkin cells. From the immunoblot analysis of GST subunits separated by two dimensional gel electrophoresis, it was shown that GSTf 1 and GSTf 2 did not cross react with anti-Puga rabbit antiserum (data not shown), suggesting that GSTf 1 and GSTf 2

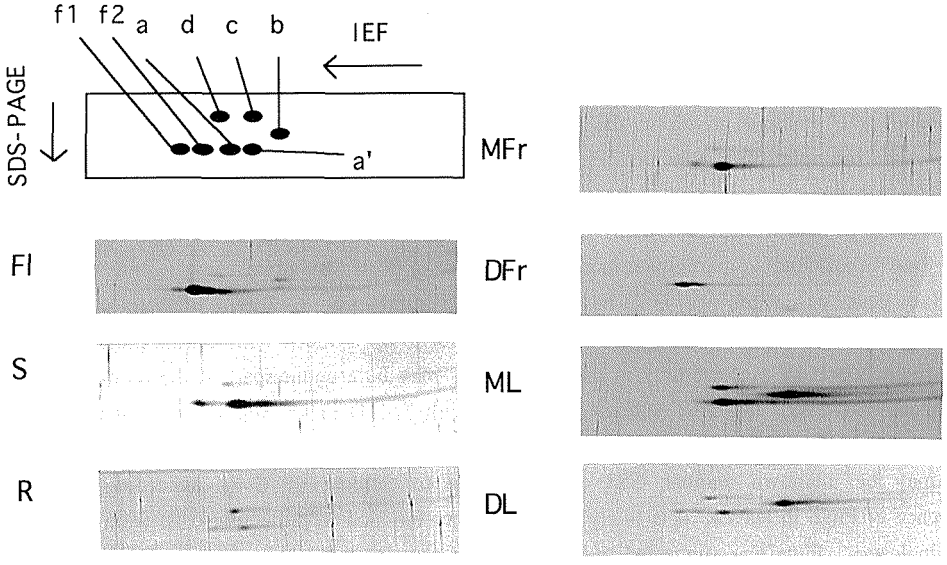

Fig. 3. Glutathione *S*-transferase subunits in various pumpkin organs.
The GST protein fractions were prepared from various organs (20−30g in weight) and analyzed by two-dimensional gel electrophoresis as described in Materials and Methods. Fl, flower ; S, stem ; R, root ; MFr, mature fruit ; DFr, developing fruit ; ML, mature leaf ; DL, developing leaf.

may not be what Puga sustained some posttranslational modifications. These two new kinds of GST proteins may form homo- or heterodimer each other as main GST subunits in flowers. In stems, Puga and Puga' (pI 5.30), which may be a pI isomer of Puga, were present as main subunits, while Pugb and Pugd were present as minor subunits. A relatively thick protein spot was also found at the site corresponding to Pugf 1, but not spot was detected at the site of Pugf 2. In mature fruits, Puga was present as a main subunit, the other GSTs were scarcely found, while in developing fruits, Pugf 1 was present as a main subunit. In roots, Puga and Pugd were present as main subunits, while Pugb was present as a minor subunit. A minor spot was also found at the site corresponding to Pugf 1. In mature leaves, Puga, Pugb, and Pugd were present as main subunits. Though a minor spot was found at the site corresponding to Pugf 1, no protein spot was observed at the site of Pugf 2. In contrast with the mature leaves, the developing leaves contained more Pugf 1. Pugc was not observed in any organs. The above results suggest that the pumpkin GST subunits distribute in different patterns with respect to growth levels of organ as well as kinds of organ.

In order to find the relation between GST and growth of organs, changes of GST activity and the ebb and flow of GST subunits were examined during the germination of pumpkin seeds (Fig. 4). For 5 days after sowing (for the period which the seeds and germs exist in vermiculite), the total activity increased as the weight of one individual increased. Until 10 days after sowing, the total activity furthermore continued to increase for the etiolated seedlings (9 to 12 cm in shoot length at day 10) grown in the dark, while for the greened seedlings (3 to 6 cm in shoot length at day 10) grown in the light, the total activity was same or lower than one of the germs (about 2 cm in shoot length) of day 5. The seedlings grown in the dark also had higher specific activity than those grown in the light. The GST subunits in the seeds and the seedlings grown for 10 days after sowing were examined by two-dimensional gel electrophoresis. Puga was present as a main subunit and the others were none or little in seeds like mature fruits. However, Pugf 1 and Pugf 2 were newly expressed as principal GST subunits, and Pugd and Pugd were also produced as minor GST subunits in the seedlings grown in the dark. On the other hand, the seedling grown in the light showed a middle character between both. These things suggest that multiple GST subunits are induced, particularly, Pugf 1 and Pugf 2 are induced well during germination of seeds and elongation of seedlings. Bilang *et al.*[1] have reported that a soluble auxin-binding protein from *Hyoscyamus muticus* was a GST. Hence, it may be important to elucidate if or not Pugf 1 and Pugf 2 participate in elongation of cells caused by auxins.

Various genes associated with GST have been cloned in tabacco. *C-7* gene of them has been reported to be expressed preferentially in mature leaves and not to be induced with auxins including 2,4-D[8]. Pugb which is present preferentially in pumpkin leaves may be a counterpart of the product of *C-7* gene in tabacco. However, this GST subunit is induced with 2,4-D in sarcocarp tissues of pumpkin fruit[16] and the cultured cells[12,17]. This difference may be caused by genetic difference between their species, or there may be unknown another GST corresponding to product of *C-7*. According to preferential occurrence of Pugb in leaves, it is likely that Pugb may function in protecting cells from photo-oxidative stress in leaves. Puga was suggested to be present as a major subunit in the organs which had stopped growing. Hence, it should be elucidate if this tendency merely depends on its proteinous stability or means its physiological importance in mature organs. Pugd seemed to be present in relatively high amount in roots and leaves over the plant body. Pugd seemed to take a middle character between Puga and Pugb. Pugc was almost absent throughout the plant body. It may be absent in normal plant but induced when the plant suffers environmental stimulations and invading by xenobiotics and so forth.

Though these four kinds of GST subunits form various heterodimers as well as homodimers in callus cultured cells, they each may actually function at different organs one another in plant bodies. Pugf 1 and Pugf 2 which were suggested to be present preferentially in flowers and developing organs have never been observed as principal GSTs in the callus cells cultured with 2,4-D. This may suggest that they are not induced with 2,4-D. If it is ture, they may be classified into a group alien from Puga, Pugb, Pugc, and Pugd. Droog *et al.*[18] have genetically classified plant GSTs into the three classes. The GSTs of class Ⅲ have a characterisitics to be induced with 2,4-D effectively. Class Ⅱ has an ethylene-inductive carnation flower GST[19]. Class Ⅰ has pathogene-inductive wheat GSTA[20] and auxin-inductive tobacco *parB*[7] and so forth. It may give a clue for the speculation about the roles of Pugf 1 and Pugf 2 to clear to which class they belong on inducition.

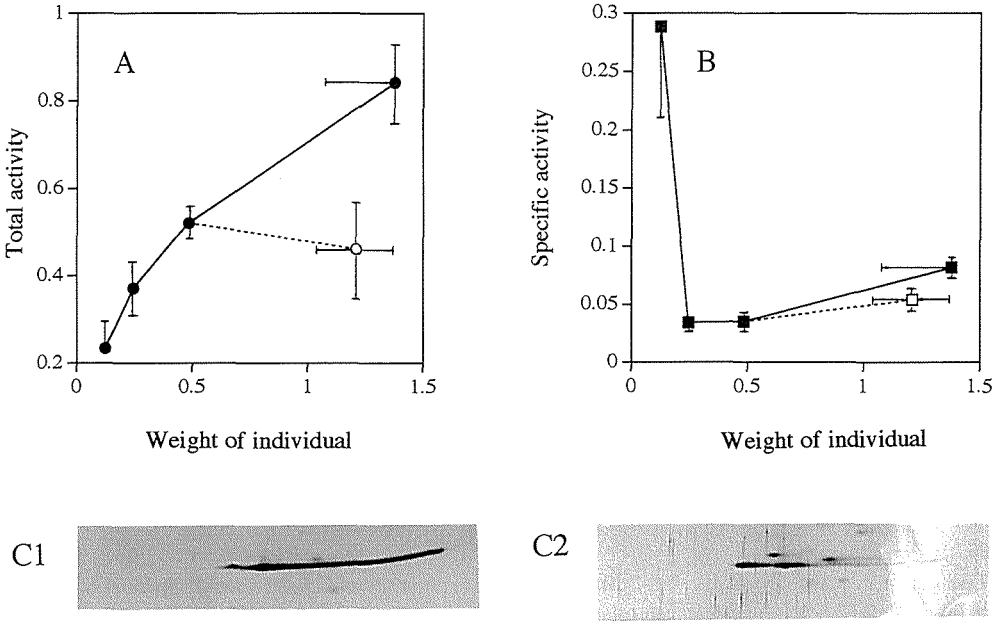

Fig. 4. Change of activity and induction of subunits of glutathione *S*-transferase during germination of pumpkin seeds.
The seeds at day 0, the germs at day 2 and day 5, and the seedlings at day 10 after sowing were harvested. Closed symbols are expressed samples grown in the dark, open symbols under continuous illumination of fluoresent. Total activity, specific activity, weight of one individual are expressed in μmol min^{-1}, μmol min^{-1} (mg protein)$^{-1}$, and g, respectively. GST activity was determined with 1-chloro-2,4-dinitrobenzene as substrate. Each symbol is the mean from 5 independent experiments and the SE are indicated. C1 and C2 show the two-dimensional gel electrophoretograms of subunits from seeds at day 0 and etiolated seedlings at day 10 after sowing, respectively.

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正常カボチャ，*Cucurbita maxima* Duch.，植物体における2,4-ジクロロフェノキシ酢酸誘導性グルタチオン*S*-トランスフェラーゼの器官分布および発育途上の器官に特異的な新規グルタチオン*S*-トランスフェラーゼ

藤田政之・花田勇樹・富田裕伸・高尾憲晶・谷口直樹

要　　約

正常カボチャ，*Cucurbita maxima* Duch.，植物体におけるGST分子種の器官分布を調べた．カボチャ培養細胞において明らかとなった2,4-D誘導性の4種の主要GSTタンパク質，Puga，Pugb，Pugc，Pugd，の間には分布に顕著な違いが見られた．Pugaは成熟した器官および種子において主要GSTとして存在していることが示唆された．Pugbは葉に優先的に分布する分子種であることが示唆された．Pugdはマイナー GSTとして植物体全体に見られた．Pugcは正常な植物体においてはほとんど存在しないことが示唆された．新たなGSTタンパク質，Pugf1およびPugf2，が花および生育途上にある葉および果実において主要GSTとして見い出された．これらのGSTは器官の成熟にともない減少するようであった．しかし、カボチャGST分子種の存在量は器官の発育段階だけでなく外部環境の変化等でも変動する可能性が示唆された．