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Suppressive Effects of Coumarins on Pumpkin Seedling Growth and Glutathione *S*-Transferase Activity

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Abstract

The effects of some coumarins (coumarin, 7-hydroxycoumarin, scopoletin and esculetin) were investigated on pumpkin (*Cucurbita maxima* Duch.) seedlings and on pumpkin glutathione *S*-transferases (GSTs). Coumarin and esculetin suppressed the growth of seedlings, especially the elongation of roots as well as hypocotyls. Among the compounds tested, only esculetin inhibited the activity of a particular pumpkin GST by 50%, CmGSTU3 toward 1-chloro-2, 4- dinitrobenzene (CDNB) and at a concentration of 22 μM. Both ethylacetae (EtOAc) and water fractions in pumpkin seedlings and different organs of one-month-old pumpkin plants contained esculetin or similar hydrophobic fluorescent substances as well as hydrophilic substances, which showed different degrees of inhibitory effects on *Cm*GSTU3 activity.

Key words: esculetin, *Cucurbita maxima*, fluorescent substance, inhibitor, glutathione *S*-transferases.

Introduction

Plants and other organisms have developed mechanisms to defend themselves against harmful herbivores, insects, pathogens and chemical compounds. Glutathione S-transferases (GSTs, EC 2.5.1.18) are an important family of enzymes in plants, which catalyze the conjugation of toxic and mutagenic substances with reduced glutathione (Wilce and Parker 1994). They have additional important roles, such as metabolism of endogenous substances (Mueller et al. 2000), binding and subcellular transport of phytochemicals (Droog et al. 1995; Walbot et al. 2000) and protection of plants from adverse effects of stresses (Marrs and Walbot 1997).

Pumpkin plant contains high level of glutathione (Nakagawa et al. 1986) and the young seedlings as well as the culture cells induced from fruit express high GST activity, particularly under stressful conditions (Fujita et al. 1994, 1995; Fujita and Hossain 2003a). Previously, three tau-type GST species (*Cm*GSTU1, *Cm*GSTU2 and *Cm*GSTU3) have been isolated from pumpkin callus induced from sarcocarp tissues of mature fruit and the cDNAs of the GSTs have been cloned successfully (Fujita and Hossain 2003a). Among the GSTs, *Cm*GSTU1 tended to be expressed more in fully expanded mature organs, *Cm*GSTU2 expressed preferentially in leaves and petioles and *Cm*GSTU3 could be expressed in the roots of five-day-old pumpkin seedlings (Hossain and Fujita 2002).

The activity of plant GSTs can be controlled by multiple natural and synthetic compounds including plant phenols (Droog et al. 1995; Mueller et al. 2000). Among many phenolic substances, coumarins are able either to promote or to inhibit plant growth (Brown 1981). They also have effects on the action of plant growth regulators (Karanov 1972) on nitrogen uptake and metabolism (Abenavoli et al. 2001) and on respiration and photosynthesis (Macias et al. 1999). The most obvious effects of coumarins are inhibition of root growth and modification of root morphology and histology (Svensson 1971). Furthermore, certain coumarins are strong inhibitors of various enzymes (Chang and Chiang 1995; Masamoto et al. 2003) and possess free radical scavenging capacity (Kostova 2005). However, the effects of coumarins on the activity of pumpkin GSTs have not been reported yet. Therefore, in the present investigation, we tested the relative effects of various coumarin derivatives on the

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growth of pumpkin seedlings and on the activities of different tau-type pumpkin GSTs, particularly *Cm*GSTU3 (Fig. 1). We furthermore examined the presence of esculetin or esculetin-like hydrophobic fluorescent substances in pumpkin organs, which showed inhibitory effects on *Cm*GSTU3 activity

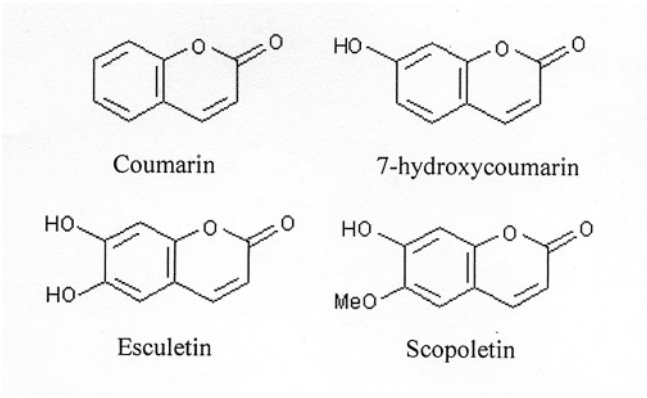

Fig. 1. Structures of compounds tested in the study.

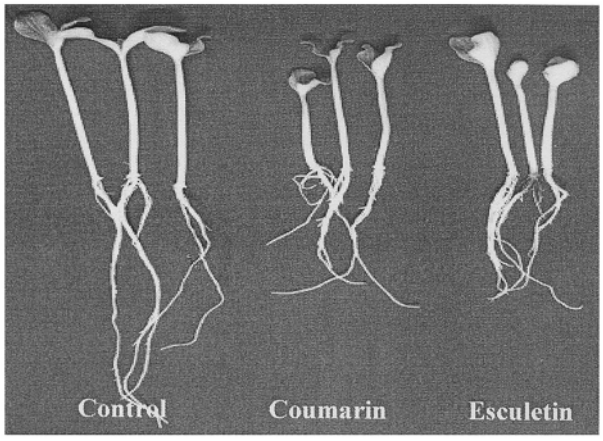

Fig. 2. Effects of coumarin and esculetin on the growth of pumpkin seedlings. Seedlings were treated with 50 µM of solutions and data were taken after six days of germination.

Materials and methods

Chemicals

Coumarin, 7-hydroxycoumarin, esculetin, scopoletin, cinnamic acid, caffeic acid, chlorogenic acid, pyrocatechol and dihydroxyascorbic acid were purchased from Wako Pure Chemical Industries, Osaka, Japan.

Raising of pumpkin seedlings and treatment with coumarin and esculetin

Mature pumpkin seeds were sown in three separate trays. The Vermiculite in two trays was saturated with 50 µM solution of coumarin or esculetin and vermiculite in the control tray was saturated with deionized water and incubated in the dark. After emergence of the seedlings, the vermiculite was further irrigated with the above-mentioned solutions/deionized water every day. To facilitate photosynthesis, the seedlings were transferred to white light (3.5 K. Lux) after two days of dark treatment. At six days after emergence, the final growth of roots and hypocotyls were observed after taking out seedlings from the incubator, removing from the vermiculite,and washing off with tap water.

Enzyme preparation and activity assay

Enzymes were extracted from *E. coli* cells, transformed with pBluescript [SK(-)] containing pumpkin GST cDNA genes in frame by a standard method (Hossain et al. 2007). GST activity was determined spectrophotometrically by the method of Fujita and Hossain (2003b). The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 1.5 mM reduced glutathione, 1 mM CDNB and enzyme solutions in a final volume of 0.7 ml. The enzyme reaction was initiated by the addition of CDNB and A_{340} was monitored at 25 ˚C for 1 min.

Preparation of plant extracts

Six-day-old pumpkin seedlings (excluding cotyledons) and roots, stems and leaves of one-month-old pumpkin plants were extracted as described by Masamoto et al. (2003) with slight modification. Ten g of each fresh material was extracted three times with 50% methanol. The combined extracts were evaporated to dryness and suspended in 15 ml of distilled water (DW). The oily components were removed twice by *n*-hexane. The remaining aqueous layer was extracted three times with an equal volume of ethyl acetate (EtOAc) and the combined extracts were evaporated to dryness and finally suspended in 1 ml of DW. The lower (water) phase was also evaporated and finally dissolved in 1.5 ml of DW. The comparative inhibitory effects of EtOAc and water fractions were examined on *Cm*GSTU3 activity toward CDNB.

Thin layer chromatography

The EtOAc and water fractions of plant extracts were applied to thin layer chromatography (TLC) plates (Si 70, F_{254}, Wako Pure Chemical Industries, Osaka, Japan) and developed with butanol-acetic acid-water (80:10:10, v/v/v). The fluorescent spots were identified under UV radiation at a wavelength of 254 nm. To check the inhibitory activity, the solvent moving zone on the plate was fractionated into equal divisions. The silica gel in each fraction was scratched out and the substances were extracted with 5.0 ml of 50% methanol. The solvent of each of the fractions was removed by evaporation and dried substances were dissolved in 0.5 ml of DW. The 30 µl from each of the concentrated fractions was used to assay the inhibitory activity toward *Cm*GSTU3.

Results and Discussion

Effects of coumarins on pumpkin seedlings

Plant physiologists have shown a great interest in coumarins

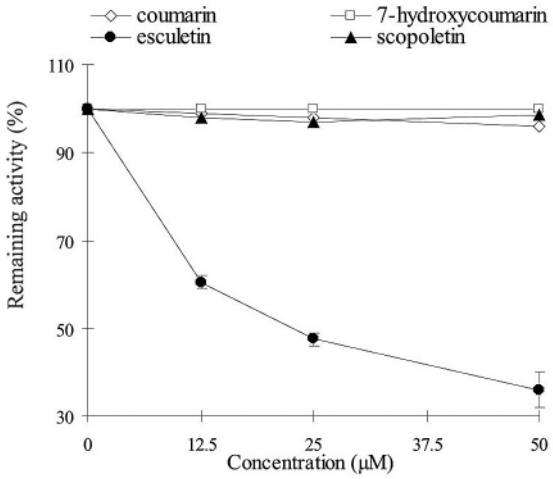

Fig. 3. Inhibition of *Cm*GSTU3 activity toward CDNB by different coumarins. Results were obtained from three independent experiments. Vertical bar represents standard error.

because of their inhibitory effects on plant growth (Alexieva et al. 1995). To investigate the effects on pumpkin plants, we treated pumpkin seedlings with coumarin and esculetin (as described in Materials and Methods) and found that both of the compounds reduced the growth (elongation) of roots as well as hypocotyls (Fig. 2). This result is in good agreement with a rich number of previously reports. Some derivatives of coumarin inhibited the stem growth of intact pea plants, stem and root growth of wheat and cucumber seedlings and elongation of excised wheat coleoptile segments (Alexieva et al. 1992, 1995). The growth inhibitory effects of various coumarins (coumarin, 7-HC, esculetin and scopoletin) have also been reported in *Avena* roots (Goodwin and Avers 1950) and *Plenum pratense* plants (Avers and Goodwin 1956). The inhibition of root growth might caused by slowing down of the metabolism and blocking of mitosis in root tissue (Goodwin and Avers 1950; Podbielkowska et al. 1995). But, the exact mechanism of growth inhibition has not been understood clearly.

Effects of coumarins on pumpkin GSTs

Coumarins are widely distributed in plants (Zobel and Brown 1995), which have multiple biological properties and various effects on different cellular systems (Kostova 2005). Therefore, to investigate whether they regulate GST enzymes or not, we inverstigated the effects of various coumarins (coumarin, 7-hydroxycoumarin, esculetin and scopoletin) on the GSH-CDNB-conjugating activity of a pumpkin GST, *Cm*GSTU3. Among the tested compounds, only esculetin (6, 7-dihydroxycoumarin) showed an interaction with the enzyme and at a concentration of 22 μM, inhibiting the activity of *Cm*GSTU3 by 50% (Fig. 3). The other two pumpkin GSTs (*Cm*GSTU1 and *Cm*GSTU2) were insensitive to the tested compounds except that the activity of *Cm*GSTU1 was slightly inhibited by esculetin (data not shown). Interaction of esculetin with plant GSTs has not been reported previously, but it has shown to be a strong inhibitor of lipoxygenase (Neichi et al. 1983), xanthine oxidase (Chang and Chiang 1995) and cyclooxygenase (Sekiya et al. 1982). The other forms of coumarins showed weak inhibitory effects (Chang and Chiang 1995). In this study, we did not observe any inhibitory effect of coumarin, 7-hydroxicumarin or scopoletin on pumpkin GSTs, suggesting that the enzymes are not responsive to those compounds.

As we found esculetin as a strong inhibitor of *Cm*GSTU3, its nature of inhibition is of great significance to understand its role in the enzymatic reaction. Therefore, we investigated the type of inhibition of *Cm*GSTU3 caused by esculetin in respect of CDNB. The apparent K_i value for the inhibitor was determined using varying concentrations of esculetin (from 5 to 40 μM). In the assay system, we used different concentrations of CDNB (0.25 to 1 mM) and a fixed concentration (1.5 mM) of GSH. Fig. 4 indicated that the inhibition occurred in a competitive manner. The apparent K_i value calculated from the plotted data was 11.54±2.06 μM. This suggested that esculetin formed conjugate with GSH by the

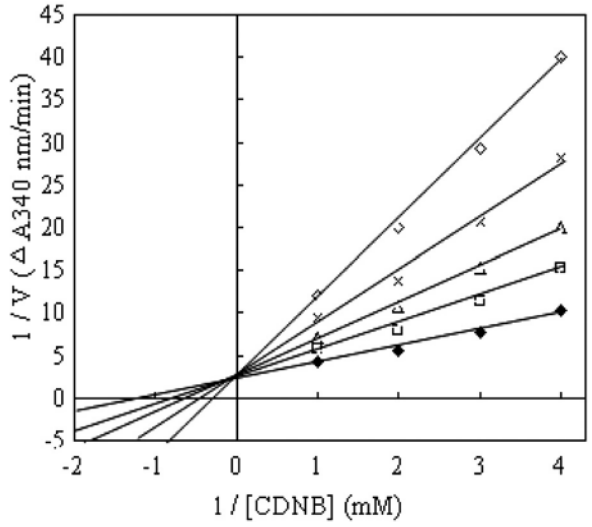

Fig. 4. Inhibitory kinetics of *Cm*GSTU3 by esculetin (◆ : 0 μM, □ : 5 μM, △ : 10 μM, × : 20 μM and ◇: 40 μM) with varying concentrations of CDNB and 1.5 mM of GSH. Plotted values represent the means of three independent experiments.

catalytic activity of *Cm*GSTU3, although we could not obtain any evidence of enzymatic product formation by TLC analysis.

It has been reported that *Cm*GSTU3 exhibited strong identity and similarity with auxin-induced tobacco GST Nt 103 (Fujita and Hossain 2003a), which is expressed in tobacco root tips (van der Zaal et al. 1991). Similarly, *Cm*GSTU3 has reported to been expressed in the roots of five-day-old pumpkin seedlings (Hossain and Fujita 2002). Since esculetin is present in the bark, leaves and roots of *Umbelifereae, Rutaceae* and *Euphorbiacae* (Masamoto et al. 2003), root tissues of pumpkin plant might contain it. Therefore, *in vivo* interaction of *Cm*GSTU3 and esculetin in the pumpkin plant is possible.

Esculetin has two hydroxyl groups at the C6 and C7 positions of the coumarin skeleton that serve as targets for *O*-methylation or *O*-glycosylation (Kim et al. 2006) and also play an important role in the expression of tyrosinase inhibitiory activity (Masamoto et al. 2003). To determine whether these OH groups

correlate with GST inhibition, we examined the effects of some compounds possessing two adjacent OH groups, namely, caffeic acid, chlorogenic acid, pyrocatechol and dihydroxyascorbic acid along with cinnamic acid. None of the tested compounds showed any effect on the activities of *Cm*GSTU3 (data not shown). It revealed that the inhibition of the GST caused by esculetin was mainly structure-specific, but did not depend only on the general properties of the adjacent OH groups.

Isolation of esculetin and related fluorescent substances from pumpkin plants

Many plant species contain esculetin (Chang et al. 1996; Liu et al. 2005; Masamoto et al. 2003), which can be isolated by different methods (Masamoto et al. 2003; Wu et al. 2007). Therefore, we extracted the similar substances from 6-day-old pumpkin seedlings and from roots, stems and leaves of 1-month-old pumpkin plants and examined their inhibitory effects on *Cm*GSTU3 activity. Both EtOAc and water fractions inhibited the activity of the enzyme (Fig. 5).

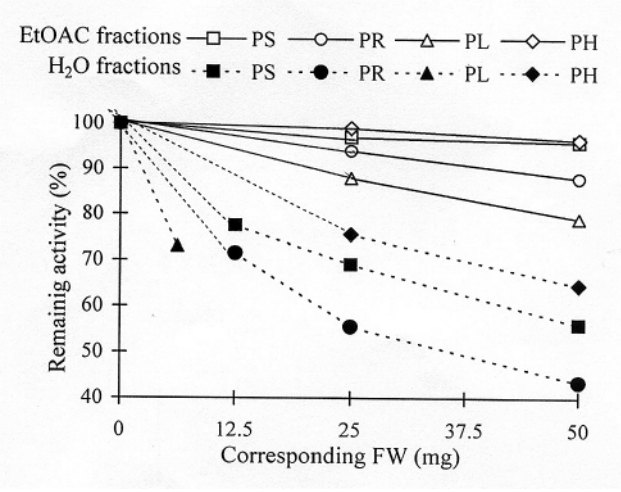

Fig. 5. Inhibitory effects of different extracts prepared from pumpkin seedlings (PS) and from roots (PR), stems (PSt) and leaves (PL) of 1-month-old pumpkin plants on *Cm*GSTU3 activity toward CDNB. The concentration as well as UV absorption of PL extract was too high to assay activity with a higher volume. Results were obtained from two independent experiments.

Although the water fractions contained a large amount of substances with high inhibitory potentials, the EtOAc fractions contained esculetin or its related substances (Wu et al. 2007). The highest inhibitory potencies of the water fractions were mainly due to some hydrophilic substances (Fig. 6B), whereas the inhibitory potencies of EtOAc fractions were the cumulative effects of hydrophilic and hydrophobic fluorescent substances (Fig. 6A). Among different plant parts, leaf extract demonstrated the strongest inhibitory effects followed by root extract. The extracts of seedlings and stem showed rather small inhibitory effects.

The EtOAc and water fractions of different extracts were analyzed by TLC method. The fluorescent substances, which migrated to the position similar to that of esculetin, demonstrated greater inhibition on *Cm*GSTU3 activity (Fig. 6A). The concentration of

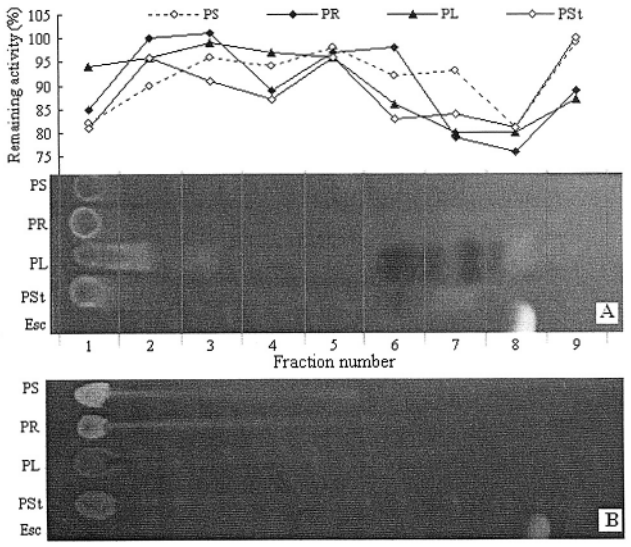

Fig. 6. Thin layer chromatography of EtOAc (A) and water (B) fractions of pumpkin seedlings (PS) and roots (PR), leaves (PL) and stems (PSt) of one-month-old pumpkin plants along with esculetin (Esc). In the case of EtOAc (A) fractions, 320 µl (corresponding to 3.2g fresh tissue components) of each of the extracts except for PL was applied to a TLC plates (Si 70, F_{254}, Wako 5×20cm). For PL, 80 µl of extract (corresponding to 0.8 g fresh tissue components) were applied. In case of water fractions (B), 50 µl (corresponding to 0.33 g fresh tissue components) of each of the extracts were applied to the same type of TLC plate. The chromatographic condition is described in detail in 'Materials and Methods'. The upper line graph shows the relative inhibitory activities of individual fractions toward *Cm*GSTU3.

fluorescent substances in root extract seemed to be much lower than that in leaf extract, but the inhibitory effects of root extract were greater than that of leaf extract. This indicated that root tissue contains strong hydrophobic inhibitors, which are active at very low concentrations. In TLC, Fraction-1 of most of the extracts showed high inhibitory effects, presumably due to the presence of hydrophilic fluorescent compounds. In the case of water fractions, the substances did not separated clearly in TLC plate (Fig. 6B), but all of the extracts showed large amounts of fluorescent substances at the origin of application (Fraction-1). Logically, most of the hydrophilic fluorescent substances in tissues of pumpkin plant were extracted with water.

Due to its polyphenolic structure, esculetin might be toxic even to the plant cells that produce it. Therefore, it is crucial to reduce its toxicity by some mechanisms. In barley leaf mesophyll cells, esculetin is thought to be glucosylated in the cytoplasm, resulting in the formation of esculin, which is thereafter transported into the vacuole by the H^+-antiporter (Werner and Matile 1985). Although we could not obtain clear evidence of enzymatic product formation, we found that the inhibition of pumpkin GST caused by esculetin was competitive toward CDNB. So, it is possible that esculetin is a substrate of the GST and the enzyme plays an important role in the detoxification of the toxic compound. The pathways of coumarin metabolism proposed by Born et al. (2002) have suggested the formation of a conjugate between GSH and coumarin 3, 4-epoxide. Therefore, at least three mechanisms regarding the metabolism of toxic esculetin in plant cells can be suggested: i) through glycosylation into esculin or other forms thereby transport-

ed into the vacuole by H^+-antiporter, ii) through formation of a conjugate with GSH catalyzed by GST and thereafter transported into the vacuole by ABC transporters, or iii) through binding by

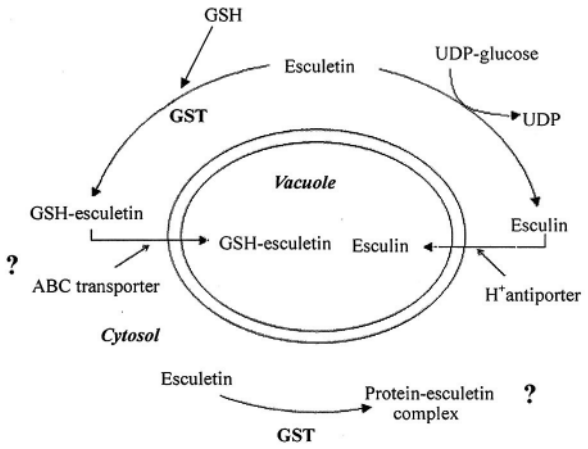

Fig. 7. Proposed mechanisms of the detoxification of esculetin in plant cells.

GST (ligandin) and remaining in the cytosol as a non-toxic protein-esculetin complex (Fig. 7). However, the result of the present investigation thrusts further research to established the concept.

Conclusion

It can be concluded that coumarin and esculetin have growth inhibitory effects on pumpkin seedlings and on a tau-type pumpkin GST, *Cm*GSTU3, which is highly responsive to esculetin. Different organs of pumpkin plant contain esculetin or similar compounds as well as more hydrophilic fluorescent substances that cause strong inhibition on the CDNB-conjugating activity of *Cm*GSTU3. However, further research is needed for elucidating the cause of growth inhibition, clarifying the mechanism of interaction between esculetin and plant GSTs and finally establishing the concept of metabolism of toxic esculetin in plant cells.

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