BIOLOGIA PLANTARUM 53 (3): 565-568, 2009

BRIEF COMMUNICATION

Effect of esculetin on activities of pumpkin glutathione *S*-transferases and growth of pumpkin seedlings

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Abstract

The effect of esculetin and some related coumarins (coumarin, 7-hydroxycoumarin and scopoletin) on growth of pumpkin (*Cucurbita maxima* Duch.) seedlings and the activities of pumpkin glutathione *S*-transferases (GSTs) were investigated. Coumarin and esculetin affected the growth of seedlings. The hypocotyls of affected seedlings became weak and bent at the mid region, roots became very soft with brownish discoloration, and finally seedlings died. Among the compounds tested, only esculetin inhibited *Cm*GSTU3 and *Cm*GSTU2 activities measured with 1-chloro-2,4-dinitrobenzene (CDNB) and at a concentration of 22 μM, it inhibited the activity of *Cm*GSTU3 by 50 %.

Additional key words: coumarins, *Cucurbita maxima*, detoxification, fluorescent substance, inhibitor.

In all living organisms, glutathione *S*-transferases (GSTs, EC 2.5.1.18) defense against harmful compounds by catalyzing the conjugation with reduced glutathione (GSH; Wilce and Parker 1994). Besides detoxification of endogenous and xenobiotic compounds, plant GSTs have additional important roles in acting as career proteins (Walbot *et al*. 2000), in cellular response to auxins (Bilang and Sturm 1995), in production of secondary metabolites (Mueller *et al*. 2000) and in protection against oxidative stress (Marrs 1996).

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 seemed to be expressed preferentially in leaves and petioles, and *Cm*GSTU3 could be expressed in the root of 5-d-old pumpkin seedling (Hossain and Fujita 2002). It is also reported that these GSTs are induced by different stresses (Hossain *et al.* 2006).

The activity of pumpkin GSTs can be inhibited by α,β-unsaturated carbonyl compounds and related aldehydes/alcohols (Fujita and Hossain 2003b). Inhibitory effects of various natural and synthetic compounds including plant phenols against certain plant GSTs have also been reported (Mueller *et al*. 2000). Among phenolic substances, coumarins are able to inhibit root growth and modify root morphology and histology (Svensson 1971). They are strong inhibitors of various enzymes (Chang and Chiang 1995) and possess free radical scavenging capacity (Kostova 2005). However, the effects of coumarins on the activity of plant GSTs have not been reported yet. Therefore, we investigated the effects of different coumarin derivatives on growth of pumpkin seedlings and on the activities of tau-type pumpkin GSTs.

To raise seedlings, mature pumpkin (*Cucurbita maxima* L.) seeds were sown in *Vermiculite* saturated with deionized water and incubated in the dark at 25 ºC. Five-day-old seedlings were removed from *Vermiculite* and all traces of *Vermiculite* were washed off carefully with tap water. After preparation, one seedling for each treatment was placed in a glass cup that contained 20 cm^3 of 2, 10 or 50 μM solution of coumarin or esculetin. Each cup was covered with a polyethylene tube to avoid undesirable mixing of the vapour of different chemicals. The cups containing seedlings were then incubated at 25 °C under white light (55 μmol m^{-2} s^{-1}). Seedlings incubated with 20 cm^3 of distilled water were used as controls. Each experiment was replicated twice. The solution of each treatment was changed every day and at

Received 26 July 2007, *accepted* 25 April 2008.
Abbreviations: CDNB - 1-chloro-2,4- dinitrobenzene; GSH- glutathione; GST - glutathione *S*-transferase.
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6 d after treatments, the differences in growth of seedlings were observed.

Enzymes were extracted from *E. coli* cells transformed with *pBluescript* [SK(-)] containing pumpkin GST cDNA (Hossain *et al.* 2007). GST activity was determined spectrophotometrically by the method of Fujita and Hossain (2003a). For control treatment, each reaction mixture (0.7 cm^3) consisted of either 0.01 cm^3 *Cm*GSTU1 with specific activity 3708 nmol mg^{-1}(protein) min^{-1}, 0.05 cm^3 *Cm*GSTU2 with specific activity 352 nmol mg^{-1}(protein) min^{-1} or 0.005 cm^3 *Cm*GSTU3 with specific activity 5501 nmol mg^{-1}(protein) min^{-1}, and the mixed solution of 100 mM potassium phosphate buffer (pH 6.5), 1.5 mM reduced glutathione, and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB). For inhibition study, different concentration of coumarin, 7-hydroxycoumarin, esculetin (6,7-dihydroxycoumarin) or scopoletin (6-methoxy-7-hydroxycoumarin) were added to the reaction mixture. The enzyme reaction was initiated by the addition of CDNB, and absorbance (A_{340}) was monitored at 25 °C for 1 min.

The effect of esculetin was found to be stronger than that of coumarin (Fig. 1). Coumarin damaged the seedlings only at a high concentration, whereas the effect of esculetin appeared even at a low concentration (2.0 μM). In the case of affected seedlings, the hypocotyls became weak and bent at the mid region, roots became very soft with brownish discoloration, and finally seedlings died.

Many research groups have reported growth inhibitory

Fig. 1. Effects of coumarin and esculetin on the growth of pumpkin seedlings. Seedlings were treated with 2, 10 or 50 μM of coumarin or esculetin after being separated from *Vermiculite*. Growth of the seedlings was observed frequently and data were taken after 6 d of treatment.

Table 1. Inhibition of pumpkin GSTs activities toward CDNB by different coumarins. Each value for remaining GST activity (expressed as a percentage of control value) is the mean of three independent experiments ± SE.

Coumarins	Conc. [μM]	Remaining activity [%] *Cm*GSTU1	*Cm*GSTU2	*Cm*GSTU3
Coumarin	25	100 ± 1.0	102 ± 1.0	98 ± 2.0
	50	103 ± 0.5	102 ± 1.5	96 ± 2.5
7-hydroxycoumarin	25	102 ± 3.0	103 ± 2.0	100 ± 0.5
	50	109 ± 1.5	109 ± 3.5	100 ± 0.5
Esculetin	25	87 ± 2.0	103 ± 1.0	48 ± 1.5
	50	80 ± 2.0	99 ± 2.0	36 ± 4.0
Scopoletin	25	103 ± 2.0	102 ± 0.5	97 ± 2.0
	50	105 ± 3.5	102 ± 0.5	99 ± 1.5

effects of coumarins. Alexieva *et al*. (1992, 1995) found that 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. The inhibitory effects of coumarin, 7-hydroxycoumarin, esculetin and scopoletin have also been reported in *Avena* roots (Goodwin and Avers 1950) and *Phleum pratense* plants (Avers and Goodwin 1956). However, the exact mechanism of growth inhibition caused by coumarins has not been understood clearly.

A possible explanation of this inhibitory action is that esculetin inhibits certain vital enzymes, restricts cell division and proliferation, decreases metabolism of toxic substances, disables defense mechanisms and finally blocks the cell cycle, leading to cell death. Since, live is associated with many key factors and esculetin has diverse known and unknown effects on plant physiology, there might be causes of plant death other than those mentioned above.

To investigate whether the growth inhibition is associated with regulation of GST, we investigated the effects of coumarin, 7-hydroxycoumarin, esculetin and scopoletin on the GSH-CDNB-conjugating activities of tau-type pumpkin GSTs (*Cm*GSTU1, *Cm*GSTU2 and *Cm*GSTU3). Among the tested compounds, only esculetin showed an interaction with the GSTs. It showed the

strongest inhibitory effect on *Cm*GSTU3 activity (I_{50} = 22 μM) and also showed a small inhibitory effect on *Cm*GSTU1 activity (Table 1). Many studies have suggested that some coumarins act as strong inhibitors of various enzymes (Chang and Chiang 1995, Masamoto *et al*. 2003). In this study, we did not observe any effect of coumarin, 7-hydroxycumarin, or scopoletin on activities on pumpkin GSTs, suggesting that the enzymes are not responsive to those compounds.

Interaction of esculetin with plant GSTs has not been reported previously, but it is a strong inhibitor of soybean lipoxygenase (Lee and Lillard 1997) and mushroom tyrosinase (Masamoto *et al*. 2003). 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). Although the expression of *Cm*GSTU3 in different organs of the mature pumpkin plant has been reported to be under the detectable level (Fujita and Hossian 2003a), the GST was expressed in the roots of 5-d-old pumpkin seedlings (Hossain and Fujita 2002). Similarly, root tissues of the pumpkin plant might contain esculetin since it has been reported to be abundant in the bark, leaves and roots of *Umbelifereae, Rutaceae* and *Euphorbiacae* (Masamoto *et al*. 2004). Therefore, *in vivo* interaction of *Cm*GSTU3 and esculetin in the pumpkin plant is possible.

Due to its polyphenolic structure, esculetin might be toxic also 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). However, we did not observe any interaction between esculin and pumpkin GSTs (data not shown). Additionally, we found that the inhibition of *Cm*GSTU3 caused by esculetin was competitive toward CDNB (data not shown). Therefore, there remains the possibility that esculetin is a substrate of the GST and that the enzyme plays an important role in the detoxification of this compound. Therefore, at least three mechanisms regarding the metabolism of toxic esculetin in plant cells can be suggested: *1*) through glycosylation into esculin or other forms thereby transported into the vacuole by H^+-antiporter, *2*) through formation of a conjugate with GSH catalyzed by GST and thereafter transported into the vacuole by ABC transporters, or *3*) through binding by GST (ligandin) and remaining in the cytosol as a non-toxic protein-esculetin complex.

The result of the present investigation, however, thrusts further research for elucidating the cause of detrimental effect on pumpkin seedlings, the mechanism of interaction between esculetin and plant GSTs and finally for establishing the concept of metabolism of toxic esculetin in plant cells.

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