Note

Distribution of Flavonols and Enzymes Participating in the Metabolism in Onion Bulbs: Mechanism of Accumulation of Quercetin and Its Glucosides in the Abaxial Epidermis

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Received May 20, 1999; Accepted August 25, 1999

Onion bulbs contain quercetin, quercetin 4'-glucoside (Q4'G) and quercetin 3,4'-diglucoside (Q3,4'G), almost all of which are localized in the abaxial epidermis of scales. There is a question of whether these compounds are synthesized in the abaxial epidermis or transported to the epidermis. To elucidate this, activities of enzymes that participate in the formation and degradation of quercetin glucosides were measured in the abaxial epidermis, adaxial epidermis and mesophyll. Activities of glucosyltransferases, which transform quercetin to Q4'G and Q4'G to Q3,4'G, were highest in the abaxial epidermis. Activity of phenylalanine ammonia-lyase was also higher in the abaxial epidermis than in other tissues. These results suggest that the flavonol glucosides are synthesized in the abaxial epidermis. Activities of glucosidases, which transform Q3,4'G to Q4'G and Q4'G to quercetin, were highest in the abaxial epidermis, suggesting that quercetin, which is present in the abaxial epidermis, is formed by deglucosidation of quercetin glucosides in the epidermal cells.

Keywords: metabolism of quercetin glucosides, onion bulbs, quercetin, quercetin glucosides

Flavonoids are a group of phenolic compounds that are normally present in vascular plants. Nutritionally, they have been termed vitamin P by Szent-Györgyi and their function is to reduce the increased permeability of capillaries. Recently, it has been found that compounds of this group have antioxidative functions (Bors *et al.*, 1997; Jovanovic *et al.*, 1997): they can scavenge active oxygen species like as superoxide anion radical (Sichel *et al.*, 1991; Takahama, 1983) and the radicals generated during lipid peroxidation (Jan *et al.*, 1991; Pratt, 1965; Takahama, 1985; Younes & Siegers, 1981). Flavonoids also have anti-carcinogenic functions (Carroll *et al.*, 1997) and can prevent cardiovascular disease (Hertog & Katan, 1997). Compounds of this group are contained in onion bulbs in a large quantity (Bilyk *et al.*, 1984; Hirota *et al.*, 1998; Patil *et al.*, 1995; Price *et al.*, 1997; Starke & Herrmann, 1976; Tsushida & Suzuki, 1995) and the major components are quercetin 4'-glucoside (Q4'G) and quercetin 3,4'-diglucoside (Q3,4'G) (Hirota *et al.*, 1998; Tsushida *et al.*, 1995). These glucosides are rather stable during boiling (Hirota *et al.*, 1998; Price *et al.*, 1997) and frying (Price *et al.*, 1997), suggesting that onion bulbs are a good source of flavonols for human.

In addition to quercetin glucosides, presence of an aglycone quercetin in onion bulbs has been reported (Bilyk *et al.*, 1984; Hirota *et al.*, 1998; Patil & Pike, 1995). It is mainly

contained in the brown dry skin, and in live scales, its amount increases from the inner to the outer scales. The amounts of quercetin glucosides also increase from the inner to the outer scales. Thus, it is deduced that the activities of enzymes, which synthesize and hydrolyze quercetin glucosides, change as a function of the position of the scale in a bulb. Such changes in activities of glucosyltransferase and glucosidase have been reported by Tsushida and Suzuki (1996). In the onion bulb scale, quercetin and its glucosides are mainly present in the abaxial epidermis (Hirota *et al.*, 1998). There are questions whether the glucosides are synthesized in the abaxial epidermis or are transported there and how quercetin is accumulated in the abaxial epidermis. The objective of this study was to determine the enzymatic activities that participated in the synthesis and deglucosidation of quercetin glucosides in each tissue of the scales of onion bulbs. Based on the results obtained, it is discussed that quercetin glucosides can be formed and deglucosidated in the abaxial epidermis.

Materials and Methods

Chemicals Quercetin and quercetin 3-glucoside (Q3G) were obtained from Wako Pure Chem. Ind. Co. (Osaka) and Extrasynthese (Genay, France), respectively. Q4'G and Q3,4'G were prepared from onion bulbs by thin layer chromatography as reported previously (Hirota *et al.*, 1998).

Plant materials *Allium cepa* L. (cv. Satsuki) was obtained from a local market. To study tissue distribution of phenolics and enzymatic activities, the outermost scales of bulbs were divided into three tissues, namely, the abaxial epidermis, adaxial epidermis and mesophyll.

Abbreviations: FW, fresh weight; PAL, phenylalanine ammonia-lyase; PVP, polyvinylpyrrolidone; Q3G, quercetin 3-glucoside; Q4'G, quercetin 4'-glucoside; Q3,4'G, quercetin 3,4'-diglucoside.

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Determination of levels of phenolics Each tissue [1 g of fresh weight (FW)] described above was ground with a mortar and pestle in 10 ml of 80% methanol. After centrifugation at $20,000\times g$ for 5 min, the supernatants were used to determine the levels of quercetin glucosides and aromatic amino acids by high performance liquid chromatography (HPLC). To determine the levels of hydroxycinnamic acids, the supernatants were concentrated to about 1 ml with a rotary evaporator. Five milliliters of 2 M HCl was added to the concentrated solutions and incubated for 20 min in boiling water. After cooling, the acidic solutions were extracted with 3 ml of diethyl ether twice. The ether extracts were combined and ether was evaporated under a stream of N_2 gas. The residues were dissolved in methanol and levels of hydroxycinnamic acids were measured by HPLC.

HPLC to measure the amounts of the above phenolics was performed using a Shim-pack CLC-ODS column (6 mm i.d. $\times$ 15 cm, Shimadzu, Kyoto) and a spectrophotometric detector with a photodiode array (SPD M1A, Shimadzu). Quercetin and its glucosides were separated and identified as described previously using a mixture of methanol and 25 mM KH_2PO_4 (1 : 1, v/v) as a mobile phase (Hirota *et al.*, 1998). Q3G was used as a standard to quantify flavonol glucosides and all data are presented as amounts equivalent to Q3G. Tyrosine and tryptophan were separated from other components using a mixture of methanol and 25 mM KH_2PO_4 (1 : 6, v/v) as a mobile phase, and were identified by comparing retention times and absorption spectra with authentic compounds. Retention times of tyrosine and tryptophan were 4.0 and 12.2 min, respectively. Ferulic and sinapic acids were separated from other components using a mixture of methanol and 25 mM KH_2PO_4 (1 : 3, v/v) as a mobile phase and identified by comparing retention times and absorption spectra with authentic compounds. Retention times of ferulic and sinapic acids were 22.1 and 23.1 min, respectively. Flavonols, aromatic amino acids and hydroxycinnamic acids were quantified from areas under the peaks at 360, 260 and 320 nm, respectively. The flow rate of mobile phases was 1 ml min^{-1} .

Preparation of cell-free extracts To measure the activities of glucosidase and glucosyltransferase, the abaxial epidermis, adaxial epidermis and mesophyll (1 g of FW) were homogenized with a mortar and pestle in 1 ml of 0.1 M sodium phosphate (pH 6.0) in the presence of 0.1 g of polyvinylpyrrolidone (PVP) and 0.1 g of sea sand. The activity of phenylalanine ammonia-lyase (PAL) was measured by homogenizing each tissue (1 g of FW) in 20 ml of 0.1 M sodium borate-HCl (pH 8.8) in the presence of 0.1 g of PVP. The homogenates were centrifuged at $20,000\times g$ for 10 min at 4°C. The supernatants were used to measure the enzymatic activity.

Measurements of enzymatic activities Activities of glucosyltransferase were measured according to the method reported by Tsushida and Suzuki (1996) with a slight modification. The reaction mixture contained 2 μ l of 10 mM quercetin dissolved in methanol, 20 μ l of 40 mM UDPG, 20 μ l of water, 20 μ l of the above supernatant and 48 μ l of 0.1 M sodium phosphate (pH 6.0) (quercetin 3-glucosyltransferase) or 0.1 M Tricine-NaOH (pH 8.0) (quercetin 4'-gluco-

syltransferase) containing 5 mM mercaptoethanol. Formation of Q3,4'G from Q4'G was measured at pH 6.0. The mixture was incubated for 10 min at 37°C and the reaction was terminated by adding 100 μ l of methanol. After centrifugation at $4000\times g$ for 5 min, quercetin glucosides formed were quantified by HPLC. For the measurements of glucosidase activity, 100 μ l of 2 mM Q3G, Q4'G or Q3,4'G and 10 μ l of 0.1 M ascorbic acid were added to 100 μ l of the above supernatants and incubated for 1 h at 37°C. The reactions were terminated by adding 200 μ l of methanol and centrifuged at $4000\times g$ for 5 min. Ten microliters of the samples was applied to an HPLC column, and HPLC was performed using a Shim-pack CLC-ODS column and a mixture of methanol and 25 mM KH_2PO_4 (1 : 1, v/v) as a mobile phase. Activity of PAL was measured in a reaction mixture that contained 10 mM phenylalanine in 20 ml of the above cell-free extract. The mixture was incubated at 38°C for 2 h, and the reaction was terminated by adding 1 ml of 5 M HCl. *trans*-Cinnamic acid formed from phenylalanine was extracted with 10 ml of diethyl ether twice. The ether extracts were combined and ether was evaporated under a stream of N_2 gas. The residues were dissolved in 100 μ l of a mixture of methanol and 25 mM KH_2PO_4 (2 : 3, v/v) and analyzed by HPLC using the column described above. The mixture of methanol and 25 mM KH_2PO_4 was used as a mobile phase to measure the levels of *trans*-cinnamic acid.

Results and Discussion

Table 1 shows distribution of flavonols and hydroxycinnamic acids in tissues of scales. As has been reported (Hirota *et al.*, 1998), two quercetin glucosides were localized primarily in the abaxial epidermis and a flavonol aglycone, quercetin, was detected only in this tissue. Hydroxycinnamic acids were present in all tissues although in amounts much less than the amounts of flavonols, and there was also a difference in the amounts among tissues. To ascertain whether flavonols and hydroxycinnamic acids were synthesized in each tissue, the activity of PAL, which is a key enzyme for synthesis of flavonols and hydroxycinnamic acids, was measured (Table 2). PAL activity was detected in all tissues and the activity was about 6 and 18 times higher in the abaxial epidermis than the adaxial epidermis and mesophyll, respectively. This indicates

Table 1. Levels of flavonols, hydroxycinnamic acids and aromatic amino acids in different tissues.

	Abaxial epidermis	Adaxial epidermis	Mesophyll
Flavonol		(μ mol g^{-1} FW)	
Quercetin	2.10 $\pm$ 0.55	BD	BD
Q4'G	38.44 $\pm$ 14.42	0.63 $\pm$ 0.37	0.10 $\pm$ 0.05
Q3,4'G	25.08 $\pm$ 7.91	0.28 $\pm$ 0.19	0.06 $\pm$ 0.04
Hydroxycinnamic acid		(nmol g^{-1} FW)	
Ferulic acid	1.50 $\pm$ 0.09	1.58 $\pm$ 0.32	0.85 $\pm$ 0.21
Sinapic acid	15.00 $\pm$ 1.27	6.70 $\pm$ 1.41	1.89 $\pm$ 0.55
Amino acid		(nmol g^{-1} FW)	
Tyrosine	BD	ND	946 $\pm$ 62
Tryptophan	348 $\pm$ 68	83 $\pm$ 32	696 $\pm$ 89

Values are means $\pm$ SDs ($n=4$). BD, below detection. ND, not determined. A peak of which retention time was the same as tyrosine was detected, but the absorption spectrum was not the same as that of tyrosine.

Table 2. Activity of PAL in different tissues.

Abaxial epidermis	Adaxial epidermis (nmol g ⁻¹ FW h ⁻¹)	Mesophyll
0.57±0.06	0.09±0.01	0.03±0.02

Values are means±SDs (n=4).

Table 3. Activities of glucosyltransferase in different tissues.

		Abaxial epidermis	Adaxial epidermis	Mesophyll
		(nmol g ⁻¹ FW min ⁻¹)		
Substrate	Product			
Quercetin	Q3G	0.43±0.04	0.15±0.06	0.23±0.05
	Q4'G	12.29±3.12	2.30±0.50	1.40±0.60
Q4'G	Q3,4'G	0.62±0.17	0.17±0.02	0.16±0.02

Values are means±SDs (n=4).

that *trans*-cinnamic acid, which is a material to synthesize flavonols and hydroxycinnamic acids, is formed in each tissue. The amounts of aromatic amino acids, tyrosine and tryptophan, in different tissues are also shown in Table 1; they were largest in mesophyll. From this result, it is deduced that tissue distribution of enzymes that participate in the synthesis of aromatic amino acids is different from that of enzymes that participate in the synthesis of phenolics of secondary metabolites. At present, we do not understand why aromatic amino acids are accumulated in plant cells. Phenylalanine, which is the substrate of PAL, was not detected in any tissue.

If flavonol glucosides are formed in the tissues where they are present, activities of the enzymes that participate in the synthesis of the glucosides would be correlated with the amounts of flavonol glucosides. Table 3 shows activities of glucosyltransferases in different tissues. Activities of the transferases that transformed quercetin to Q3G and Q4'G were higher in the abaxial epidermis than in the adaxial epidermis and mesophyll, and activity of the transferase that transformed Q4'G to Q3,4'G was about 4 times higher in the abaxial epidermis than the other two tissues. Since Tsushida and Suzuki (1996) reported that Q3G is not glucosylated to Q3,4'G in onion bulbs, we did not measure the activity of this enzyme.

The presence of PAL and glucosyltransferase in not only mesophyll but also adaxial epidermis and abaxial epidermis was reported in leaves of *Pisum sativum* by Hrazdina (1992). Since the activities of PAL and glucosyltransferase correlated with the amounts of quercetin glucosides (Tables 1–3), it is deduced that quercetin glucosides are formed from phenylalanine in the tissues where the glucosides are present. The activity of chalcone syntase, which catalyzes formation of chalcone from *trans*-cinnamic acid and/or hydroxycinnamic acids, is detected in epidermis but not in mesophyll of leaves of *P. sativum* (Hrazdina, 1992). In parsley leaves, chalcone syntase is present in epidermis (Schmelzer *et al.*, 1988). According to these reports, it is expected that the activity of chalcone syntase might be higher in the abaxial epidermis of onion scales. The difference in amounts of ferulic and sinapic acids among tissues was not as large as that in the amounts of flavonols, suggesting that the difference in activity of cin-

Table 4. Activities of glucosidases in different tissues.

		Abaxial epidermis	Adaxial epidermis	Mesophyll
		(nmol g ⁻¹ FW min ⁻¹)		
Substrate	Product			
Q3G	Quercetin	0.14±0.08	0.32±0.16	0.17±0.12
Q4'G	Quercetin	2.18±0.48	0.07±0.02	0.15±0.05
Q3,4'G	Quercetin	0.10±0.03	BD	BD
	Q3G	0.05±0.02	BD	BD
	Q4'G	0.21±0.02	0.05±0.02	0.03±0.01

Values are means±SDs (n=4).

namate 4-hydroxylase among tissues is not as large as that in the activity of chalcone syntase.

The amount of quercetin was much larger in the abaxial epidermis than in other tissues. There are two possible explanations for this. One is that the rate of synthesis of quercetin from chalcone is faster than the rate of the glucosidation of quercetin, and the other is that deglycosidation of quercetin glucosides to quercetin is faster than formation of quercetin glucosides from quercetin. To evaluate the potential of each possibility, the activities of glucosidases in each tissue were measured (Table 4). The glucosidase activity which transformed Q4'G to quercetin was higher than other glucosidase activities. When the activity was compared among tissues, it was 15–30 times higher in the abaxial epidermis than in other tissues. Since Q4'G is one of the major flavonols in onion bulbs, there is a possibility that quercetin is formed by deglycosidation of quercetin glucosides; this is supported by the results that in the dry skin of onion bulbs, where *de novo* synthesis of flavonol glucosides does not seem to proceed, there are high levels of quercetin (Bilyk *et al.*, 1984; Patil & Pike, 1995). Tsushida and Suzuki (1996) discussed that the formation of quercetin is due to deglycosidation, based on their result that the activity of glucosyltransferase is lower in the outermost scales than in other scales and that the reverse is true for glucosidase.

Quercetin formed in the abaxial epidermis of outer scales may be transformed to brown components which are present in dry skin by the action of peroxidase (Hirota *et al.*, 1998). Ferulic and sinapic acids were detected not only in 80% methanol extracts but also in the apoplast of scales (data not shown). Since peroxidase that was present in cell walls of onion scales oxidized ferulic and sinapic acids (data not shown) and these compounds bind to polysaccharides in cell walls (Fry, 1986; Iiyama *et al.*, 1994), ferulic and sinapic acid residues in cell walls might be utilized to form cross-links making the cell walls rigid during storage.

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