Flavonoid Biosynthesis and Accumulation and Related Enzyme Activities in the Skin of 'Fuji' and 'Oorin' Apples during their Development

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Summary

To ascertain the mechanism of apple skin coloration, changes in the flavonoid biosynthesis and accumulations and related enzyme activities were investigated during fruit development in red-skinned 'Fuji' and green-skinned 'Oorin' apples. In 'Fuji' anthocyanin accumulated from 139 days after full bloom (DFB) concurrent with fruit maturation. Proanthocyanidins and flavonols showed highest concentrations in very young fruit; then decreased gradually afterwards and remained almost constant throughout fruit development in both cultivars. Changes in phenylalanine ammonia-lyase (PAL) activity corresponded with these patterns, but they slightly increased in 'Fuji' 139 DFB. The changing pattern of chalcone isomerase (CHI) activity was similar to that of PAL until maturation when a slight increase in CHI activity was observed. CHI activity in 'Fuji' apple skin was higher than that of 'Oorin'. In the 'Fuji' apple skin, UDP-3-*O*-glycosyltransferase (UFGaT) activity markedly increased from 139 DFB, parallel with anthocyanin accumulation. Our results suggest that anthocyanin synthesis mainly occurs during coloration in 'Fuji'. PAL, CHI, and UFGaT enzymes are involved with anthocyanin synthesis; UFGaT seems to be the most important enzyme in red pigmentation of 'Fuji'.

Key Words: apple skin, chalcone isomerase, flavonoid, phenylalanine ammonia-lyase, UDP-3-*O*-glycosyltransferase.

Introduction

Apple skin color is an important factor for consumer appeal. Poor red coloration has been a serious problem of 'Fuji', the predominant apple cultivar in Japan. Many attempts have been made to improve red coloration in 'Fuji' apple, among which are paper bagging (Arakawa, 1988) and covering the orchard floor with reflecting films (Ju et al., 1999). However, characteristics of coloring in 'Fuji' apple have not been well understood; thus, the problem of poor coloration remains unsolved.

The development of red coloration in apple is thought to be dependent on anthocyanin production during the maturation period (Arakawa, 1988). Anthocyanins are one kind of flavonoids, of which cyanidin-3-galactoside is the main anthocyanin in apple skin (Duncan and Dustman, 1936). Other flavonoids, such as proanthocyanidins and flavonols, are also involved in apple skin color; they are co-pigmented with anthocyanin to produce skin color in apple fruit (Lancaster et al., 1994). Flavonoid synthesis in apple skin is influenced by such environmental factors, as light and temperature (Arakawa et al., 1986; Tan, 1979). However, the mechanism regulating fruit coloring still remains unclear, but some enzyme activities involved in flavonoid synthesis are

known, e.g., Phenylalanine ammonia-lyase (PAL) acts at the first step of anthocyanin synthesis by converting phenylalanine to cinnamic acid. It is also the key enzyme that regulates anthocyanin synthesis in apples (Faragher and Chalmers, 1977; Arakawa et al., 1986). Chalcone isomerase (CHI) isomerizes chalcone to naringenin which provides the first flavonoid, the most important step in its biosynthesis (Stafford, 1990). UDP-3-*O*-glycosyltransferase (UFGaT), the last enzyme in anthocyanin biosynthesis, catalyses glycosylation of anthocyanidin to produce anthocyanin. This activity of the UFGaT enzyme for anthocyanin synthesis has been reported in apple fruits and grape berries (Ju, et al., 1995; Kobayashi et al., 2001). The UFGaT activity and flavonoid concentration are intimately related in 'Splendour' and 'Granny Smith' apples (Lister and Lancaster, 1996). Anthocyanin synthesis was stimulated by increased light intensity through accelerating UFGaT gene expression (Ju et al., 1999).

In this study, we compared the changes of flavonoids and the enzymes involved in their synthesis during fruit development for 'Fuji' and 'Oorin' apple. This was done in order to elucidate the mechanism of anthocyanin synthesis and pigmentation of 'Fuji' apple.

Materials and Methods

Fruits were obtained from 10-year-old 'Fuji' and 12-year-old 'Oorin' apple trees, both grafted on M26, and

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grown in an orchard of the Agricultural and Forestry Research Center, University of Tsukuba. Fruits were collected at 2-week intervals from 46 days after full bloom (DFB) until harvest, 196 and 190 DFB in 'Fuji' and 'Oorin', respectively. Skin color was measured with a portable chromometer (Minolta CR-100) and the fruit chromaticity in L^* , a^* , b^* space coordinates were recorded. Each sample was then peeled and the skin was immediately frozen in liquid nitrogen and stored at -80°C prior to analysis.

Flavonoid analysis

Flavonoids were determined as described by Lister et al. (1994) by grinding 1 g of apple peel with mortar and pestle in liquid nitrogen. Thirty milliliters of 15% acetic acid in methanol was then added to the homogenate and incubated overnight at 4°C . The extract was concentrated to 1 ml in vacuo and centrifuged at $10\,000 \times g$ for 5 min. The supernatant was analyzed using high-performance liquid chromatography (HPLC; JASCO MD-910) equipped with photodiode arrays and a Crestpak C18S column (JASCO, 4.6×150 mm). The eluting solvents were 10% acetic acid in water (A) and acetonitrile (B) for the gradient program which ascended from 0 to 20% B in A for 20 min and then kept for 10 min at the final concentration and a flow rate of $1.0\text{ ml} \cdot \text{min}^{-1}$. Proanthocyanidins, flavonols, and anthocyanin were monitored at 280 nm, 350 nm, and 530 nm, respectively; proanthocyanidins consisted of catechin, epicatechin, chlorogenic acid, and phloridzin, while the flavonols were: quercetin-3-galactoside, quercetin-3-rhamnogalactoside, quercetin-3-glucoside, quercetin-3-rhamnoside and so forth.

Enzyme extraction and assay

The extraction was carried out according to Dong et al. (1995) with slight modifications. Ten grams of apple skin were ground with mortar and pestle in liquid nitrogen until a fine powder was obtained. Polyvinylpyrrolidone (PVP-40, Sigma) was then added at a rate of 10% of the fresh weight and mixed.

Thirty milliliters of 100 mM sodium phosphate buffer (pH 8.0) containing 14 mM 2-mercaptoethanol were added and the mixture was gently stirred until completely thawed. After the homogenate was centrifuged at $12\,000 \times g$ for 15 min and 1 g of Dowex 1-X 4 was added, the supernatant was incubated for 20 min on ice and centrifuged again at $12\,000 \times g$ for 10 min. Ammonium sulfate was added to the supernatant to give 30% saturation and the mixture centrifuged at $12\,000 \times g$ for 10 min. Protein was precipitated from the supernatant by adding ammonium sulfate to 80% of final saturation, and sample centrifuged at $12\,000 \times g$ for 10 min. The protein pellet, resulting from the second precipitation at 80% ammonium sulfate, was resuspended in 5 ml of 100 mM sodium phosphate buffer, pH 8.0, containing 14 mM 2-mercaptoethanol. The extract was assayed for

enzymes at 4°C .

PAL activity was measured as the rate of formation of trans-cinnamic acid by recording an increase in absorbance at 290 nm (Zucker, 1965). The assay mixture (2 ml) consisted of 0.1 M phosphate buffer (pH 8.0), $20\text{ }\mu\text{M}$ L-phenylalanine, and 1 ml enzyme solution. The mixture was incubated for 60 min at 40°C . A unit of PAL is defined as the production of $1\text{ }\mu\text{mol}$ of cinnamate per min, and PAL activity are expressed as $\text{U} \cdot \text{mg}^{-1}$ protein.

CHI activity was measured according to Lister and Lancaster (1996), in which chalcone was added to the reaction mixture (2 ml) consisting of 50 mM Tris buffer (pH 7.6), 50 mM KCN, $18.5\text{ }\mu\text{M}$ 2',4,4',6'-tetrahydroxy-chalcone, and 100 μl enzyme solution, initiate the reaction. Activity was recorded as a change in absorption at 370 nm. A unit of CHI is defined as the disappearance of $1\text{ }\mu\text{mole}$ of chalcone per min, and the results are expressed as $\text{U} \cdot \text{mg}^{-1}$ protein.

UFGalT activity was assayed according to Lister and Lancaster (1996). The reaction mixture, containing 100 μl of enzyme extract, 100 μl of 50 mM bicine buffer, 1 mM quercetin, and 2.5 mM UDP-galactose as substrate, was incubated at 30°C for 30 min. The reaction was terminated by adding 75 μl of 20% trichloroacetic acid. The product, quercetin-3-galactoside, detected at 350 nm using HPLC, was quantified by comparing its peak area with that of the standard quercetin-3-galactoside (provided by Funakoshi) solution. A unit of UFGalT is defined as the production of quercetin-3-galactoside per min and is expressed as $\text{U} \cdot \text{mg}^{-1}$ protein.

Protein concentration was determined by the method of Bradford (1976), using bovine serum albumin as a standard.

Results and Discussion

Changes of apple skin color

Skin color was expressed by a^* value which rises as the red coloration increases (McGuire, 1992). 'Fuji' and 'Oorin' showed red skin coloration 46 DFB, then gradually changed to green. Red coloration in 'Fuji' apple reappeared 153 DFB and increased greatly during maturation, whereas 'Oorin' remained green throughout its maturation period (Fig. 1A).

Changes of flavonoids compound in apple skin

The main anthocyanin in apple skin is cyanidin-3-galactoside (Duncan and Dustman, 1936) which are confirmed by HPLC tracing as the prominent anthocyanin in 'Fuji' and 'Oorin' apple skin and quantified by using ideain as authentic standard. Some anthocyanin biosynthesis occurred during the early period of fruit development in both cultivars. Accumulation of anthocyanin was almost nil throughout fruit development in 'Oorin', whereas that in 'Fuji' was started at 153 DFB and increased rapidly from 165 DFB during maturation

Fig. 1. Changes in color and flavonoids compounds of 'Fuji' and 'Oorin' apples during fruit development.

Vertical bars represent SE of the means.

A: skin color; B: anthocyanins; C: proanthocyanidins D: flavonols.

(Fig. 1B). Both proanthocyanidins and flavonols occurred at markedly high concentrations during the early stage of fruit development and steadily decreased thereafter (Fig. 1C, D). The patterns of change in both proanthocyanidin and flavonol concentrations were similar for both cultivars but concentration in 'Fuji' was higher than that in 'Oorin' throughout fruit development (Fig. 2C, D). The rise in concentration of proanthocyanidins on the skin of immature apples was reported by Lister et al. (1994) and Burda et al. (1990). Lister et al. (1994) reported an increase in proanthocyanidins concentration in 'Granny Smith' and 'Splendour' towards the end of the growing season. In this study, only a slight increase was observed in 'Fuji'. While flavonols increased slightly at 153 DFB coinciding with an increase in anthocyanin.

Changes in enzyme activity

PAL activity was highest in immature fruits then decreased rapidly during fruit development in both cultivars. A second increase, although very small, was observed during the maturation stage (Fig. 2A). These results are similar to those previously reported (Blankenship and Unrath, 1988; Lister et al., 1994). Aoki et al. (1970) observed PAL activity only in the red sections of apple skin and he suggested that a close relationship existed between PAL and anthocyanin synthesis. However, Blankenship and Unrath (1988) observed red color development irrespective of PAL activity in 'Red Delicious' apple, whereas Ju, Z. G. et al. (1995) found

that PAL activity was more closely related with a simple phenol than with anthocyanin in the mature apple fruit. Furthermore, the latter suggested that PAL is necessary in fruit coloration to supply precursors, but when the amount of precursor is sufficient in mature fruit, PAL activity has no effect on anthocyanin accumulation. Our results showed that the PAL activity in 'Fuji' was much higher than that in 'Oorin' during fruit maturation, and that activity coincided with an increase in anthocyanin concentration. These results suggest that PAL activity may regulate red coloration in 'Fuji' apple by supplying flavonoids in general, but PAL may not be the crucial enzyme in regulating anthocyanin biosynthesis.

CHI activity was highest in fruits sample 46 DFB. The rate, however, steadily decreased during fruit development until 139 DFB, then increased after 165 days, and reached a peak at 190 DFB, particularly in 'Fuji' (Fig. 2). Dong et al. (1995) reported that PAL and CHI activities rapidly increased upon exposure of fruit to UV light and white light. The exposure significantly enhanced red coloration by increasing the rate of anthocyanin biosynthesis, which signifies that CHI may be one of the critical enzymes involved in red coloration in 'Fuji'.

While UFGaT in 'Fuji' had low activity 46 DFB, a surge occurred at 165 DFB and increased rapidly thereafter. However, UFGaT activity in 'Oorin' was almost nil throughout fruit development (Fig. 2). UFGaT catalyzes unstable cyanidin to stable anthocyanin in apple skin (Ju, Z. G. et al., 1995). Ju et al. (1999) reported

Fig. 2. Changes in enzyme activities of 'Fuji' and 'Oorin' apple skin during fruit development. Vertical bars represent SE of the means.
A: PAL, B: CHI, C: UFGaT.

that increasing light intensity by spreading both foil and metalized film under the tree canopy significantly increased UFGaT activity, stimulating anthocyanin synthesis. Our results showed that an increase in UFGaT activity in 'Fuji' apple closely paralleled the increase in anthocyanin content (Fig. 2C and Fig. 1A). UFGaT also catalyzes quercetin glycosylation as we observed that quercetin glycosides increased slightly 139 DFB in 'Fuji' apple skin only (data not shown); the increase coincided with the enhancement of UFGaT activity. The slight increase in flavonols 153 DFB may be due to the increase in UFGaT activity because a significant rise in UFGaT activity coincided with the onset of anthocyanin accumulation. These results clearly demonstrate that UFGaT is mainly responsible for anthocyanin synthesis during fruit maturation in 'Fuji'. Other reports showed high UFGaT activity even in the green apple

cultivar (Lister and Lancaster, 1996; Ju et al., 1999). Lister et al. (1994) reported that quercetin glycosides slightly increased during fruit maturation in 'Granny Smith', but our result with 'Oorin' did not show it. Thus different apple cultivars may have different coloration mechanisms even if they are the green type. 'Oorin' showed neither UFGaT activity nor any anthocyanin.

Thus, different trends in flavonoid accumulation were observed as well as the activity of the enzymes involved in flavonoid biosynthesis between red-skinned 'Fuji' and green-skinned 'Oorin'. The development of red coloration on the skin of 'Fuji' and anthocyanin synthesis occurred simultaneously. The increase in anthocyanin accumulation came just before the red coloration commenced. In 'Fuji' CHI and UFGaT activities increased rapidly while PAL activity increased only slightly when anthocyanin accumulation started. These results demonstrate that although PAL is the key enzyme involved in anthocyanin synthesis for supplying of anthocyanin precursors, other enzymes such as CHI and UFGaT are equally essential for anthocyanin biosynthesis once ample precursors are present. The increase of CHI activity occurred later, compared with UFGaT, their activities were synchronized. Flavonoid biosynthesis starts from naringenin, which is produced from chalcone catalysed by CHI (Stafford, 1990), and serves as a precursor of anthocyanin in 'Fuji'. Kobayashi et al. (2001) investigated the expression of seven anthocyanin biosynthetic genes in white and red grapes and observed that UFGaT gene was expressed in the red skin sport only; other genes were also expressed but at a lower level in the white sport. Their results suggest that the mutation from white to red grapes was the expression of the UFGaT gene. To establish the exact step-by-step mechanism of anthocyanin biosynthesis, investigation on the changes in enzymes and genes involved in is needed.

PAL, CHI, and UFGaT activities are light-inducible (Arakawa et al., 1986; Dong et al., 1995; Ju et al., 1999). These results, together with our data may offer some means of improving red coloration in apple skin and provide a key to the relationship between anthocyanin biosynthesis and regulating factors such as light, temperature, and fruit maturation.

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リンゴ 'ふじ' と '王林' の発育段階におけるフラボノイドの蓄積とフラボノイド生合成に関与する酵素活性の変化

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摘 要

リンゴ果実の着色機構を明らかにするために、鮮紅色に着色するリンゴ 'ふじ' と着色しない '王林' を用いて、果実発育段階におけるフラボノイドの蓄積とその生合成に関与する酵素活性の変化について比較検討した。'ふじ' では満開後139日からアントシアニンが蓄積し始め、果皮の着色を伴って著しく増加した。一方 '王林' では全発育段階を通じてアントシアニンの蓄積は認められなかった。プロアントシアニンとフラボノールは両品種とも幼果期に多く、果実発育と成熟に伴って減少した。PAL酵素活性は両品種とも幼果期に高く、発育に伴って減少したが、満開後139日に 'ふじ' のみで

やや活性が上昇した。CHI活性の変化は両品種とも成熟前はPALと同じ傾向であったが、成熟期になると両品種とも増加した。このCHI活性の増加は 'ふじ' が '王林' に勝っていた。'ふじ' のUGalT活性は満開後139日から著しく増加し、これはアントシアニン蓄積と一致した。一方 '王林' では全発育段階を通じてUGalT活性がみられなかった。以上の結果から、'ふじ' の着色期には主にアントシアニン生合成が行われ、またそのアントシアニン生合成にはPAL, CHI, UGalTが関係しているが、特にUGalT酵素が最も重要であると考えられた。