

Anatomical Observations of Anthocyanin Rich Cells in Apple Skins

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Abstract. Anatomical observations of anthocyanin rich cells in 'Fuji' apple skins were carried out by light microscopy and electron microscopy. Apple skins with fully developed red color had more layers of anthocyanin-containing epidermal cells than those of green skins. The density of anthocyanin was high in cells of the outer layer of the fruit skins and gradually decreased inward to the flesh. Anthocyanins were frequently found in clusters or in agglomerations that were round in the epidermal cells of the red skins. They accumulated in the inner side of developed vacuoles. Transmission and scanning electron microscopy revealed that the shapes of anthocyanins were cluster style, indeterminable forms, or complete spheres. Anthocyanin seemed to be synthesized around the tonoplast and condensed on the inward side of the vacuole. There was no distinct envelope membrane on the anthocyanin granule in the vacuoles of apple skin cells.

Anthocyanins, one of the major classes of flavonoids, are the plant pigments responsible for the various shades of red and blue color in fruit and flowers (Awad et al., 2000; Gross, 1987). They are ubiquitous in plants that display these colors and play an important role as a basis for identification and quality judgment of their fruit or flower products. With exposure to light, anthocyanin is observed to accumulate during ripening in epidermal and hypodermal cells of apple fruit skins when examined by light microscopy (Kim, 1990). However, little information is available on the ultrastructure of anthocyanin and the anatomical structural details of anthocyanin-containing cells in apple skins.

The biosynthesis and biochemistry of anthocyanins in various fruit and flowers have been studied extensively. It was found that anthocyanin-containing cell layers were more abundant in red apple skins than in immature apple skins with less developed color (Kim, 1990; Awad et al., 2000). The anthocyanin of apples is one of many flavonoid compounds, cyanidin-3-galactoside (Gross, 1987), which has two phenol rings with glycosylation (Awad et al., 2000). The glycosylation can make the anthocyanin structure stable or soluble. The anthocyanin often forms complexes with other flavonoid compounds such as hydroxycinnamic acid derivatives. It also forms complexes with other phenolic compounds, including tannin, for chemical stability (Bobbio et al., 1994; Clifford, 2000; Remy et al., 2000). The chemical structure of anthocyanin is intrinsic to phenolic compounds and has several functional groups

including hydro, methyl, hydroxyl or phenol groups which can be fixed with glutaraldehyde, formaldehyde, or osmium tetroxide (Awad et al., 2000; Hayat, 1989). In particular, formaldehyde reacts with phenolics promoting the formation of methylene bridges (Hayat, 1989). The phenol-containing regions within a cell are osmiophilic, thus, osmium tetroxide fixation can show high electron density in transmission electron micrographs (Hayat, 1989).

As for the cellular aspects of anthocyanin accumulation, it was suggested that an organelle anthocyanoplast, also referred to as cyanoplast, which is the site for anthocyanin biosynthesis, is present in vacuoles of sweet potato (Nozue et al., 1997) and red cabbage (Small and Peckett, 1982). The organelle was typically found to be spherical and occurring in anthocyanin-producing cells of angiosperms (Peckett and Small, 1980). Bound by a single membrane, the organelle was found to be separated from the hostile acidic environment of the vacuolar sap (Small and Peckett, 1982). Meanwhile, other works proposed that the globular inclusions involved in the biosynthesis of anthocyanin might be composed of protein matrices (Nozue et al., 1995, 1997), and that they might possess neither a membrane boundary nor an internal structure (Cormier, 1997; Nozzolillo, 1994). The objectives of this study were 1) to compare the profiles of anthocyanin accumulation in red skins with those in green skins, 2) to examine the morphological features of anthocyanin accumulation at the cellular level, and 3) to elucidate the membrane system of anthocyanin in apple skins.

Materials and Methods

Fruit. 'Fuji' apples at full maturity were

harvested in an apple orchard at Suwon, Korea. The fruit were washed with mild detergent to remove dirt, insect honeydew, and pesticide residues. They were then allowed to air dry at room temperature. Two types of fruit skins were prepared from the fruit as follows: 1) green skins of the fruit and 2) red skins of the fruit.

Determination of total anthocyanin content in apple fruit skins. Anthocyanin was extracted from apples by homogenizing 10 g of skin from each type of fruit skins with 10 mL of 0.1% HCl in methanol. The mixture was kept at low temperature (4 °C) overnight and filtered through Whatman No. 2 filter paper. Optical density was measured using a spectrophotometer (Bionate5; ThermoSpectronic) at 535 nm. The anthocyanin content in the two types of apple skins was calculated by Beer-Lambert's law (Gross, 1987). Four fruit of each type of apple were randomly selected, and data were pooled by skin type to calculate and compare the mean content of anthocyanin per fruit by Fisher's protected least significant difference (FLSD) test using SAS PROC GLM (SAS Institute, Cary, N.C.).

Light microscopy of fruit skins. Fruit skins (each 5 × 5 mm with about 2 mm of underlying tissues) were free-hand sectioned from the two types of fruit skins using a razor blade. They were then mounted on slide glasses with a drop of distilled water and examined in bright field using a light microscope (Axiophot; Carl Zeiss Inc., Oberkochen, Germany).

Scanning electron microscopy. To examine the three-dimensional, intracellular structures of apple fruit skins at ambient temperature, the osmium-dimethyl sulfoxide (DMSO)-osmium (O-D-O) method (Tanaka, 1987) was employed in this study. Two types of fruit skins (each 5 × 5 mm with about 2 mm of underlying tissues) were excised using a razor blade. The specimens were fixed with 1% (w/v) osmium tetroxide in phosphate buffer (pH 7.4) for 2 h. After rinsing with the same buffer, they were immersed in 25 and 50% (v/v) DMSO each for 30 min. The specimens were frozen on a metal plate that had been precooled with liquid nitrogen, and fractured using a razor blade or a hammer. The fractured pieces were immediately placed in 50% DMSO for thawing at room temperature. They were rinsed in the same buffer until the DMSO had been completely removed. The specimens were then immersed again in 1% osmium tetroxide in the same buffer for 1 h and 0.1% osmium tetroxide at 4 °C for 3 d. The specimens were treated sequentially with 1% osmium tetroxide for 1 h, 2% tannic acid overnight, and 1% osmium tetroxide for 1 h. They were dehydrated in a graded ethanol series: 30%, 50%, 70%, 80%, 95%, and three times in 100% ethanol, each for 10 min. The specimens were further treated with isoamyl acetate two times each for 10 min, and dried in a critical point drier (CPD 030; BAL-TEC Inc., Balzers, Liechtenstein) with liquid carbon dioxide as a transitional fluid. The specimens were mounted on metal stubs and sputter-coated with gold (about 30 nm in thickness). They were examined with a scanning electron microscope (JSM-5410LV; JEOL Ltd., Tokyo, Japan) at an accelerating voltage of 20 kV.

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Transmission electron microscopy. Ultrastructural aspects of anthocyanin accumulation were investigated in red apple fruit. Fruit skins (each 2×2 mm with about 2 mm of underlying tissues) were excised from red apples using a razor blade. The specimens were fixed with 2.5% (v/v) glutaraldehyde and 2.5% (v/v) paraformaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) at room temperature for 4 h, and washed with the same buffer three times each for 10 min. The specimens were post-fixed with 1% osmium tetroxide in the same buffer at 4°C for 2 h, and washed briefly with distilled water twice. They were dehydrated in a graded ethanol series: 30%, 50%, 70%, 80%, 95%, and three times in 100% ethanol each for 10 min. The specimens were further treated with propylene oxide two times each for 30 min as a transitional fluid, and embedded in Spurr's resin. Ultrathin sections (about 50 nm in thickness) were cut with a diamond knife using an ultramicrotome (MT-X; RMC Inc., Tucson, AZ). The ultrathin sections were mounted on copper grids and stained with 2% (w/v) alcoholic uranyl acetate for 20 min, and 0.2% (w/v) lead citrate for 2 min (Small and Peckett, 1982). They were examined with a transmission electron microscope (JEM-1010; JEOL Ltd., Tokyo, Japan) operated at an accelerating voltage of 80 kV. In addition, thin sections (about $1\ \mu\text{m}$ in thickness) were made with a glass knife using an ultramicrotome. The thin sections were mounted on slide glasses and stained with 0.05% (w/v) toluidine blue for 5 min. They were then examined with a light microscope (Axiophot; Carl Zeiss Inc., Oberkochen, Germany).

Results and Discussion

Determination of total anthocyanin content in apple skins. The two types of fruit skins showed different profiles of anthocyanin accumulation depending on the skin color. The total anthocyanin content was approximately 6 to 7 times higher in red skins than in green skins (Fig. 1). Significant differences were found in anthocyanin content from the two types of fruit skins ($P=0.01$). This result was consistent with those of other studies (Kim, 1990).

Light microscopy of fresh and fixed apples. The examination of fresh anthocyanin-containing tissues revealed different anatomical features in the two types of fruit skins. The red pigment (mainly anthocyanin) released from freshly sectioned fruit skins was more intense in red skins than in green skins (Fig. 2). The density of red pigment was high in cells of the outer layer of the fruit skin, and gradually decreased inward to the flesh (Fig. 2A). No significant red pigment was observed in the wax or the epidermis, which might be due to pigment release from the squash-mounted fresh fruit skins. The individual epidermal cells in red skins were usually square-like with three to five anthocyanin-containing cells clustered within approximately five cell layers from the top (Fig. 2A and B). The epidermal cells were arranged in more regular patterns and were smaller than the flesh cells. Some cells in the hypodermis contained uniformly

red-colored vacuoles. Anthocyanin-containing cells were found sporadically in the flesh. Compared with the red skins, features of the green skins were different in the intensity and distribution of red pigment (Fig. 2C and D). Only a few cells showed red color and they were located sporadically in the hypodermis, resulting in the low intensity of red pigment.

Awad et al. (2000), Kim (1990), and Markham et al. (2000) reported similar tissue features in cross-sectioned apple fruit skins to those observed in this study.

In fixed and embedded red fruit skins, lignified cell walls and phenolic compound-like materials in vacuoles were densely stained by toluidine blue (Fig. 3) as described previ-

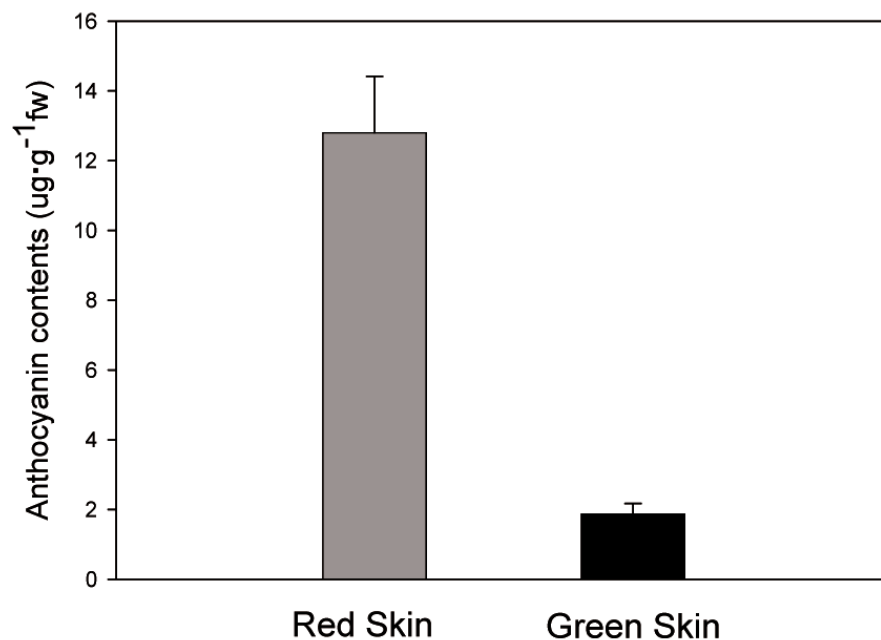


Fig. 1. Anthocyanin content of green and red apple skins. Bars in the columns indicate the standard deviations. The different letter denotes a significant difference at $P = 0.01$ according to the FLSD test.

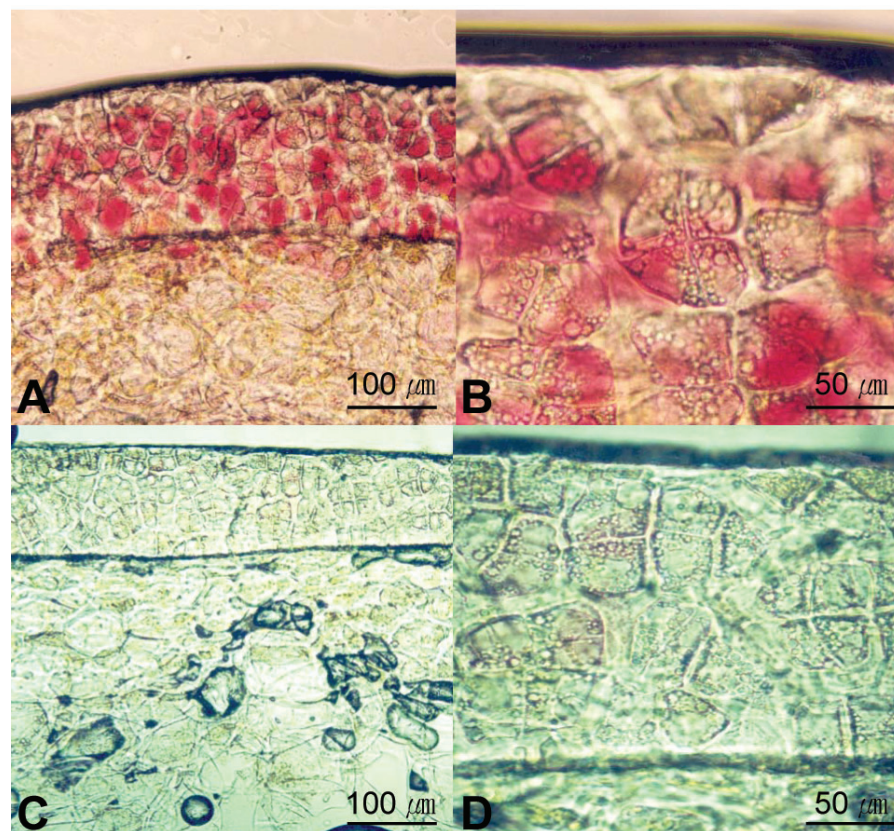


Fig. 2. Light microscopy of fresh apple ('Fuji') skins. (A and B) red skins; (C and D) green skins. The density of red pigment was higher in the outer cell layer of the red skins than in the green skins.

ously (Berlyn and Miksche, 1976; O'Brien and McCully, 1981). Given that the vacuole is the site where soluble phenolic compounds accumulate, it is likely that many of the densely stained materials observed in the vacuoles were anthocyanins. These materials were frequently found to have round shapes in the epidermal cells of the red skins. Such cells occurred more commonly beneath the cuticle than the flesh. Epoxy embedding tissues of cranberry also revealed the frequent occurrence of two pigment-bearing cell layers of granule or clustered objects beneath the cuticle (Sapers et al., 1983).

Scanning electron microscopy. The freeze-fractured tissues provided three-dimensional information on anthocyanin accumulation in the two types of fruit skins (Fig. 4). The most striking differences between green and red skins were found in the cytoplasmic content of

epidermal and subepidermal cells. Characteristic globular or ovoid structures that are assumed to be anthocyanins were frequently found in red skins (Fig. 4A), but rarely found in green skins (Fig. 4B). The cells in green fruit were highly vacuolated and some parts of those appeared to be nearly empty. Some anthocyanins formed clumps together in vacuoles, showing a rather indefinite form (Fig. 4C). Higher magnifications of anthocyanins revealed that they were positioned closely appressed to the vacuole membrane (Fig. 4D). The O-D-O method used in this study had been considered more effective for the stability of anthocyanin than other methods (Bobbio et al., 1994). The intracellular structures of apple fruit skins were well preserved, allowing their accumulated anthocyanin to be clearly viewed. This study showed that the O-D-O method could be an alternative to cryo-scanning electron microscopy, which employs a cooling system to maintain a cold temperature in the specimen chamber.

Transmission electron microscopy. Cross sections of red skins revealed typical components including nuclei, mitochondria, plastids, and vacuoles in cytoplasm enclosed by a cell wall (Fig. 5). Electron-dense materials were accumulated inside central vacuoles and several vesicles were developed along the tonoplast (Fig. 5A). Based on their texture and electron density, these materials likely correspond to phenolic compounds, particularly phenols containing *O*-dihydroxy groups, which readily react with osmium tetroxide and appear electron-dense (Rey et al., 1998). Furthermore, their presence

in the vacuoles and their close association with tonoplast strongly suggest that the phenolic compounds include anthocyanins responsible for the red pigmentation in red skins. Anthocyanin is very labile in neutral or basic solutions but stable in the vacuole, because the vacuole solution is acidic in most plant tissues (Jurd, 1972; Nakamura et al., 1980). The average pH of the vacuole in grape berries was reported to be about 2.78 due to the accumulation of malic and other organic acids (Moskowitz and Hrazdina, 1981). Future work remains to be done to cytochemically characterize the biochemical nature of these anthocyanins from apple fruit that may have different structural and conformational isoforms or subunits. Nearly round, electron-dense materials were deposited along the inner tonoplast and condensed inward in the vacuole (Fig. 5B–D). Hrazdina et al. (1978) found that the activation of enzymes or chemical reactions involved in anthocyanin biosynthesis took place in the cytosol and vacuoles. The overall conversion of phenylalanine to anthocyanin takes place near the endoplasmic reticuli occurring in close association with the tonoplast (Hrazdina et al., 1978).

The glycosyltransferase involved in the glycosylation of anthocyanidin has been thought to be located in the vacuole or at the inner side of the tonoplast (Fritsch and Grisebach, 1975). It was proposed that dihydroflavonol is converted to anthocyanidin at the membrane and that its glycosylation then takes place on the tonoplast or in the vacuole (Fritsch and Grisebach, 1975). Thus, several steps of anthocyanin biosynthesis are thought to be accomplished in close proximity to cytosol, and the intermediates transported to the tonoplast where the processes are completed and the products accumulated in the vacuole (Hrazdina et al., 1978; Nakamura et al., 1980).

Various morphological types of anthocyanin were observed in vacuoles in our study: an indeterminate type (Fig. 5A), a hemispherical type (Fig. 5B and C), and a spherical type (Fig. 5D–F). Self association is most likely involved in increasing the stability of the chemical and physical structure of anthocyanin. Its high density functions to scatter light so that the underlying pericarp tissue is not penetrated by ultraviolet rays (Moskowitz and Hrazdina, 1981). Higher magnifications of anthocyanins revealed that they did not possess a distinct envelope membrane to keep the spherical type anthocyanins bound (Fig. 5F). In this study, neither the organelles proposed by Peckett and Small (1980) and Small and Peckett (1982), nor anthocyanoplast or cyanoplast (Nozue et al., 1997) were found in 'Fuji' apple fruit skins. Peckett and Small (1980) reported that only single membrane-bound anthocyanoplasts were present within the vacuole, which was the site of anthocyanin biosynthesis. In some species, it was reported that anthocyanin was localized at a compartmented space in the vacuole (Markham et al., 2000).

In conclusion, this study increased our knowledge of the cytology of anthocyanin accumulation in apple fruit. Its main location in outer cell layers of fruit skins and several



Fig. 3. Light microscopy of fixed and resin-embedded red apple skins. Anthocyanin granules (arrows) in vacuoles were densely stained by toluidine blue.

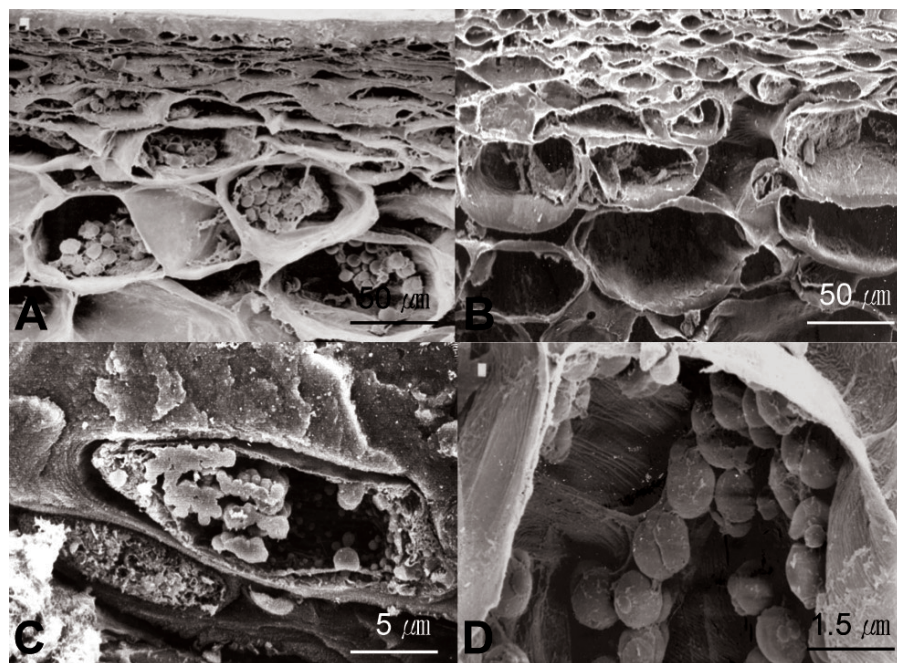


Fig. 4. Scanning electron microscopy of freeze-fractured apple skin. (A) red skins, (B) green skin. (C) Epidermal cells of a red skin. Many strands of cytoplasm with remains of cell components formed a network stretching out to a cell membrane are shown. (D) Red skin. Anthocyanin granules were found in the vacuole.

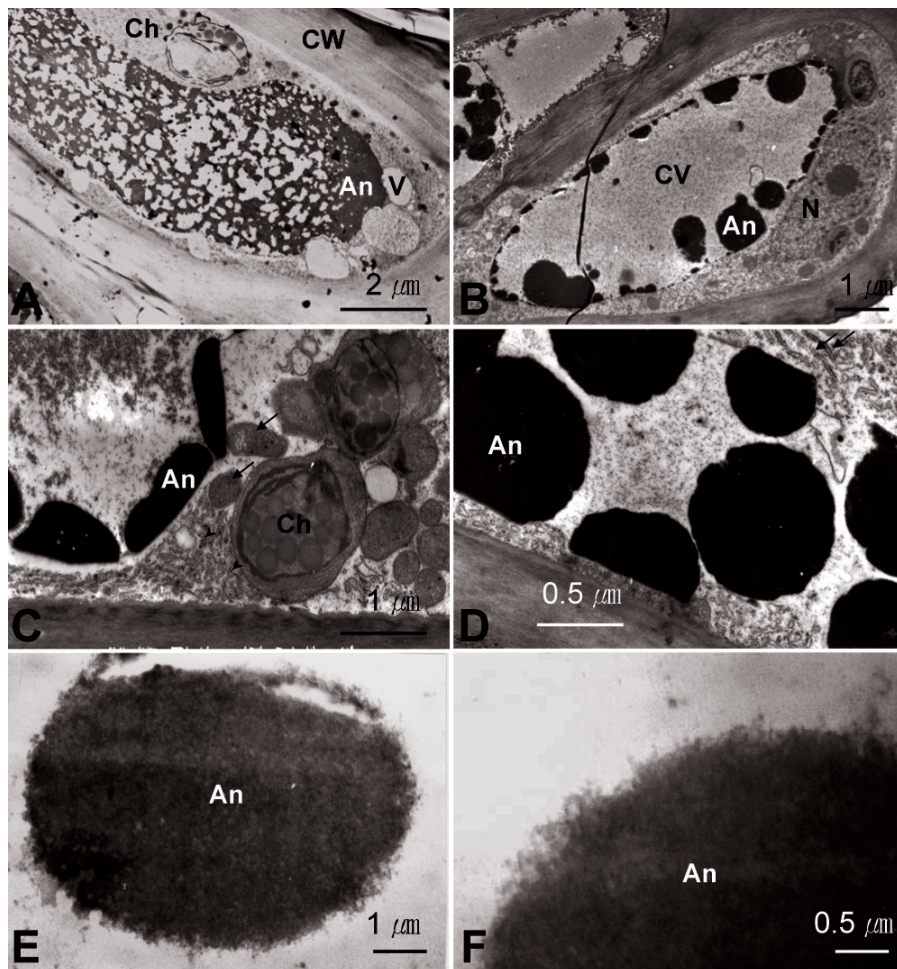


Fig. 5. Transmission electron microscopy of red apple skins. (A) Indeterminate form of anthocyanin. (B) Clustered irregular type. CV = central vacuole. (C) Clustered irregular type. Arrowheads indicate endoplasmic reticuli. Arrows indicate mitochondria. (D) Large spherical type. Arrows indicate endoplasmic reticuli. (E and F) Magnified anthocyanin granule. An = anthocyanin, Ch = chloroplast, CW = cell wall, N = nucleus, V = vacuole. There was no distinct envelope membrane on the anthocyanin granule.

morphological types were presented at the cellular level in this study. The implication of vacuoles and tonoplast in anthocyanin accumulation suggests that the organellar enzymes would perhaps present site-specific targets for the regulation of pigment expression of ornamental plants. The knowledge of the developmental transition and regulation of enzyme activities and the occurrence of individual enzyme isoforms involved in anthocyanin biosynthesis will enhance our understanding of the nature of anthocyanin and other pigments in plants.

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