广 西 植 物 Guihaia 28(3):395 — 401 2008 年 5 月

Existent states of anthocyanins in vacuole and their coloration effects in higher plants

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Abstract: This review sums up the reasons of anthocyanins being sequestered into vacuole, the existent states of anthocyanins in vacuole and the corresponding coloration effects of anthocyanins on plant cells. Transporting of anthocyanins from the biosynthesis site, namely the cytoplasm of plant cell, into vacuole is to detoxify the toxicity of anthocyanins on the functional molecules of the cell, such as proteins and DNAs. The vacuolar compartmentalization of anthocyanins is the prerequisite for anthocyanin function normally in plant cells. In a wide range of plant species and in most cases, anthocyanins dissolve completely in vacuole. However, in vacuoles, anthocyanins can also form granules which can be classified into two categories, namely anthocyanoplast (ACP) and anthocyaninic vacuolar inclusion (AVI). ACP is membrane-bounded, its formation is the result of the progressive coalescence of the smaller pigmented vesicles in vacuole and fully developed ACP is typically spherical and more deeply red-colored than the vacuole. In vacuole, ACP is high density and insoluble globule highly concentrated with anthocyanins. The emergence of ACP can provide intense coloration in the vacuole. AVI may be protein matrix and it possesses neither a membrane boundary nor an internal structure, its formation is the result of the anthocyanins transported into the vacuole bind with a protein matrix. In vacuole, AVI is irregular and jelly-like in shape. In AVIs, the attachment of anthocyanins to the matrix protein is likely to be via H-bonds to a sterically restricted site. AVI is suggested to act as vacuolar anthocyanin "trap", preferentially for anthocyanidin 3,5-diglycosides or acylated anthocyanins. The emergence of AVI can enhance color intensity and results in the "blueness" of color in the vacuole.

Key words: anthocyanin; existent state; coloration effect; anthocyanoplast; anthocyaninic vacuolar inclusion

CLC Number: Q946.83 Document Code: A Article ID: 1000-3142(2008)03-0395-07

Anthocyanins are the coloured end products of the flavonoid pathway and consist of anthocyanidins and saccharides(Holton & Cornish, 1995). They are typically found in flower and fruit tissues, and in the superficial cells of organs such as leaves and stems. The anthocyanin-pigmented cells are typically restricted to the epidermis and hypodermis(Pecket & Small, 1980).

The most apparent and important function of anthocyanins in plant life is that they act as water-soluble vacuolar pigments(Harborne, 1976; Gould *et al.*, 1995; Zhao *et al.*, 2004a). Generally, depending on constituent substitutions and complexes formed with metal ions and copigments, anthocyanins can appear red, blue or purple colors under the low pH, vacuolar conditions (Mueller *et al.*, 2000; Zhao *et al.*, 2004b). Almost all vascular plants possess basic anthocyanins such as pelargonidin 3-O-glucoside and cyaniding 3-O-glucoside responsible for the red to magenta coloration of flowers and fruits and delphinidin 3-O-glucoside introducing blue tones to the floral organs of plants(Harborne & Williams, 2000; Zhao *et al.*, 2004c, 2006). The coloration of anthocyanins is radically decided by their anthocyanidins. Three different classes of anthocyanidins are responsible for the primary shades of the flower and

Received date: 2006-12-10 **Accepted date**: 2007-05-05

Foundation item: Supported by the Provincial Department of Science and Technology of Yunnan(2006C0030Q); Startup Fund for Doctor of Yunnan Agricultural University(A2002096)

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fruit colors: pelargonidin(orange to brick red), cyanidin(pink to red) and delphinidin(purple to blue)(Harborne, 1976; Zhao *et al.*, 2004c, 2006).

However, so far, no comprehensive account concerning why anthocyanins accumulate in vacuole and how their coloration effects are realized has been published (Grotewold, 2004). Today, anthocyanins have been one of the targets of plant metabolic engineering with the aim of creating new, or altering the properties of existing colored compounds. Understanding of the cytological mechanisms of the coloration effects of anthocyanins in plants is of great and potential significance to plant breeders and molecular biologists who are interested in creating novel colored flowers or fruits or enhancing anthocyanin production.

This review attempts to sum up the reasons of antocyanins being sequestered into vacuole, the existent states of antocyanins in vacuole and the corresponding coloration effects of anthocyanins on plant cells.

1 Anthocyanins accumulate in vacuole

1.1 Transporting of anthocyanins from cytoplasm into vacuole is to detoxify the toxicity of anthocyanins on the functional molecules of the plant cell

Anthocyanins, including their hydrophobic aglycone anthocyanidins, are potentially cytotoxic and genotoxic compounds that can oxidize proteins and intercalate into DNAs (Matile, 1984; Matile, 1987; Hrazdina, 1992; Ibrahim, 1992; Ahmed *et al.*, 1994; Klein *et al.*, 1996; Li *et al.*, 1997; Wink, 1997; Grotewold *et al.*, 1998; Klein *et al.*, 2000; Debeaujon *et al.*, 2001), and the corresponding detoxification process is accomplished by the transport of anthocyanins from the synthesis site, namely cytoplasm, into vacuole (Nozue *et al.*, 1993; Coleman *et al.*, 1997; Klein *et al.*, 2000; Bartholomew *et al.*, 2002). Generally, the vacuole has the potential to detoxify and store not only endogenous but also foreign, biotic, and abiotic glucosylated substances (Taiz, 1992). Compartmentalization of anthocyanins in the vacuole is required both to limit the mutagenic and oxidative effects of the anthocyanin biosynthetic pathway intermediates and to express the proper biological function of the anthocyanins (Ahmed *et al.*, 1994; Rueff *et al.*, 1995; Wink, 1997). So, anthocyanins, like a number of other secondary products of plant metabolism, can not uaually be in the cytoplasm (Xu *et al.*, 2001), and normally accumulate in the vacuole (Harborne, 1976; Saunders & Conn, 1978; Wagner, 1979; Stafford, 1990; Hrazdina & Jensen, 1992; Gould *et al.*, 1995; Mol *et al.*, 1998).

It is currently believed that the molecular modifications of anthocyanins are directly related to the transport of the anthocyanins from cytoplasm into vacuole. Glycosylation or acylation of anthocyanins appears to be the prerequisite for the vacuolar uptake of anthocyanins (Matern *et al.*, 1986; Hopp & Seitz, 1987; Wink, 1997; Bartholomew *et al.*, 2002; Springob *et al.*, 2003). It was further found that the glucose residue attached to the molecules is not sufficient to act as a signal of the vacuolar sequestration of anthicyanins (Frangne *et al.*, 2002).

1.2 The vacuolar compartmentalization of anthocyanins is the precondition of anthocyanin function normally in plant cells

In general, vacuoles offer a larger storage space, which is important for anthocyanins to reach concentrations great enough to function in the protection against predators and pathogens or as UV light sunscreens or attractants (Klein *et al.*, 2000; Debeaujon *et al.*, 2001). As a matter of fact, until vacuolar compartmentation of anthocyanins is completed, they are not stabilized, and are unable to function in plant cells, e. g. as pigments (Winkel-Shirly, 2001; Kitamura, 2006). The pigmentation of anthocyanins is finally realized in vacuole because it is the acidic environment of vacuole that causes the alteration of anthocyanins from colorless to colored pigments (Spelt *et al.*, 2002; Kitamura, 2006).

2 Existent states of anthocyanins in vacuole

2.1 Anthocyanins dissolve totally in vacuole

In a wide range of plant species and in most cases, the water-solubility of anthocyanins makes them usually dissolve uniformly in the vacuolar solution, resulting in

the relatively even color of plant cells(Pecket & Small, 1980; Markham *et al.*, 2000). Therefore, anthocyanin pigments become a good marker to reflect the vacuole size(Wagner *et al.*, 1978; Taiz & Zeuger, 1991).

2.2 Anthocyanins form granules in vacuole

2.2.1 Discovery of the anthocyanin granules in vacuole and their diversification of denominations In certain species, anthocyanins are found to localize in discrete regions of the vacuole(Pecket & Small, 1980; Markham *et al.*, 2000), suggesting that anthocyanins can exist in the form of granules in vacuole. Early in 1911, it appeared to be Politis who made the first significant anatomical observations on anthocyanin-pigmented cells. He recognized that an intensely pigmented structure was usually present in some anthocyanin-containing cells, and he took the lead in terming it"a cyanoplast". In 1926, Lipmaa observed a similar body in anthocyanin-pigmented cells and named it"an anthocyanophore". Since that time, such subcellular structures have been found in anthocyanin-producing cells of more than 70 plant species representing at least 33 families of angiosperms spanning both dicotyledons and monocotyledons(Pecket & Small, 1980).

Historically, the anthocyanin granules in vacuole were denominated optionally and independently. They have been described as"blue spherules"in the epidermal cells of rose(*Rosa rugosa*) petal(Yasuda, 1974), "blue crystals" in larkspur, *Consolida ambigua* petals(Asen *et al.*, 1975), "ball-like structures" and "crystals" in stock, *Matthiola incana*, petals(Hemleben, 1981), and red "crystals" in mung bean(*Phaseolus radiatus*) hypocotyls(Nozzolillo & Ishikura, 1988), "intravacuolar spherical bodies" in *Polygonum cuspidatum* seedlings (Kubo *et al.*, 1995). The petals of a number of other flowers also contain similar intensely colored intravacuolar bodies(Markham *et al.*, 2000).

Despite the tremendous progresses involved in the worldwide exploition on the anthocyanin granules in vacuole, not much is known about the mechanisms by which anthocyanins form granules in vacuole and give birth to specific coloration effects on the plant cell. Nowadays, from the angle of structural and biochemical differences, it should be reasonable to classify the anthocyanin granules in vacuole into two categories: ACP and AVI.

2.2.2 Categories of the anthocyanin granules in vacuole and their coloration effects ACP appears to be the first term proposed by Pecket and Small (1980) to describe the membrane-bounded anthocyanin granules in vacuole. It is not the site of anthocyanin biosynthesis but one of the existent states of anthocyanins in vacuole after anthocyanins are transported into vacuole.

(1) Formation course, existent shape and dynamic changes: Formation of ACP is the result of the progressive coalescence of the smaller pigmented vesicles in vacuole. First, numerous unassociated small red vesicles form in vacuole, while only faint pigmentation is visible in the vacuole. Then, the red vesicles appear to be associating, resulting in the gradual decrease of the vesicle amount. Later, one vesicle becomes substantially larger than the others and the coloration of the vacuole becomes more obvious. As development proceeds, the larger red vesicle, namely the so-called ACP, increases further in size, and the small vesicles disappear completely. Normally, only one ACP is present within the central vacuole. It is thought above process is positively related with the biosynthesis of anthocyanins (Pecket & Small, 1980; Nakamura, 1993). In addition, it is observed that the occurrence of ACP appears to be inhibited by the presence of abscisic acid(ABA) and 2, 4-D(Nozue *et al.*, 1993; Kim *et al.*, 1997).

After formation, the existence of ACP in vacuole is not quiescent but dynamic. Fully developed ACP is typically spherical and more deeply red-colored than the vacuole, e. g. in the young and relatively short-lived tissues of red cabbage(*Brassica oleracea* var. *capitata*). When the pigmented cells approach the completion of the development, their ACPs tend to degenerate and become less easily detectable(Pecket & Small, 1980), which has something to do with the gradual depigmentation of the mature cells.

(2) Strcutrue and chemical property: ACP is found to be membrane-bounded, e. g., in the seedling hypocotyl and leaves of red cabbage(*Brassica oleracea* var. *capitata*), ACP is bounded by a single tripartite membrane approximately 10 nm in thickness(Pecket & Small, 1980). So, ACP is thought to be one kind of or-

ganelle rather than simply being hydrophobic droplet (Pecket and Small,1980;Nozzolillo *et al.*,1988;Nakamura *et al.*,1993;Grotewold *et al.*,1998;Xu *et al.*,2001). In vacuole, ACP is strongly osmiophilic, high density and insoluble globule highly concentrated with anthocyanin(Nozue *et al.*,1993).

(3) Coloration effects: The emergence of ACP can provide intense coloration in the vacuole. It seems that, just in the enlarging course of ACP, some anthocyanin leaks into the vacuole causing it to become progressively more pigmented (Pecket & Small, 1980; Nakamura, 1993). The formation and enlargement of ACP resulted in the intense red coloration of the grape berry(Nakamura,1989,1994). At the same time, it is demonstrated that the presence of ACP can not prolong the pigmentation time of the cell(Pecket & Small,1980).

AVI appears to be the first term proposed by Markham *et al.* (2000) to describe the non-membrane-bounded anthocyanin granules in vacuole. AVI is not the site of anthocyanin biosynthesis either. Just like ACP,it is also one of the existent states of anthocyanins in vacuole after anthocyanins are sequestered into vacuole.

(1) Formation course,existent shape and dynamic changes: Formation of AVI is the result of the combination of anthocyanins with proteins in vacuole. After anthocyanins are transported into the vacuole, they bind with a protein matrix and form AVI(Conn *et al.*, 2003), suggesting that the proteins may exist in the vacuole in advance or they are transported into the vacuole just at the time when anthocyanins are being sequestered. In AVIs,the attachment of anthocyanins to the matrix protein is likely to be via H-bonds to a sterically restricted site. At the pH of the vacuole, the bonding is strong enough to effectively integrate the anthocyanins and the protein, and the protein acts, in fact,as an efficacious vacuolar "trap" for anthocyanins (Markham *et al.*,2000).

After formation in vacuole, AVI is irregular and jelly-like in shape(Markham *et al.*,2000). In addition, independently of variations in the levels of anthocyanins, light appears to induce the coalescence of AVIs,resulting in the spread of anthocyanins from the inclusions into the vacuolar sap(Irani & Grotewold,2005).

(2) Strcutrue and chemical property: AVI may be protein matrix(Nozue *et al.*,1995;Nozue *et al.*,1997; Markham *et al.*,2000) and it possesses neither a membrane boundary nor an internal structure(Nozzolillo, 1994;Cormier,1997;Nozue *et al.*,1997). Therefore,it appears not to be organelles(Markham *et al.*,2000). Moreover, AVI may be composed of several protein species(Zhang *et al.*,2004). In the AVIs of lisianthus (*Eustoma grandiflorum*) cells, three proteins were found to have approximate molecular weights of 50000,35000 and 34000 Daltons and pIs in the range 4—5(Markham *et al.*,2000).

AVI is found to be insoluble in most aqueous buffers and is not solubilized with common detergent such as SDS, Triton X-100 and Nonidet P40. It is also only partially soluble in concentrated denaturing solutions such as 6 mol/L urea and 6 mol/L guanidine hydrochloride. Complete solubilization is only achieved with high concentration of both denaturant and reductant(Markham *et al.*,2000).

AVI exhibits a high degree of specificity to the molecular structures of anthocyanins. AVI is suggested to act as vacuolar anthocyanin "traps", preferentially for anthocyanidin 3,5-diglycosides(Markham *et al.*,2000) or acylated anthocyanins(Conn *et al.*,2003;Zhang *et al.*,2004). In lisianthus(*E. grandiflorum*) cells,bound to the AVIs' matrix are four cyanidin and delphinidin acylated 3,5-diglycosides,and the "trapped" anthocyanins are shown to differ from solution anthocyanins only in that they lack a terminal rhamnose on the 3-linked galactose(Markham *et al.*,2000).

(3) Coloration effects:AVI is considered to be the storage site of anthocyanins(Zhang *et al.*,2004). The presence of AVI results in intensification in colour and a significant shift in the absorbance spectra of anthocyanins in the cells.

On one hand, AVI can enhance color intensity. In lisianthus(*E. grandiflorum*) flowers, the packaging of anthocyanins into AVI produces marked colour intensification by concentrating anthocyanins above levels that would be impossible in vacuolar solution(Markham *et al.*,2000).

On the other hand, AVI can result in the "blue-

ness" of color. A distinct bluing of color in the AVIs-rich petal zone of carnation (*Dianthus caryophyllus*) is observed, and the normally pink pelargonidin pigments produce a blue-grey colouration, which is evidenced by the enhanced absorptance in the longer wavelength bands at about 625 nm (Markham *et al.*, 2000). Flowers of the "Rhapsody in Blue" rose (*Rosa rugosa*) cultivar show a change in color induced by age, namely from red-purple to bluish-purple. This variation is associated with a progressive accumulation of anthocyanins into AVI-like structures, and cyanin is probably "trapped" into AVI at higher concentrations than would be possible in a vacuolar solution and in quinonoidal form, appearing purple-blue because of additional absorption in the range of 580—630 nm (Gonnet, 2003). Theoretically, absorption in the 580—630 nm region is attributed to the presence of anthocyanin quinonoidal bases in either neutral or ionized forms (Hoshino & Goto, 1990; Lin *et al.*, 1992; Brouillard and Dangles, 1994; Baranac *et al.* 1996). The "bluing" effect could be the result of a concomitant equilibrium shift favouring the quinonoidal base forms of the anthocyanin due either to the bonding or to self association (Hoshino & Goto, 1990).

As a result, nowadays research advances indicate that, in vacuole, anthocyanins can dissolve completely or form granules, affecting the color of plant cells directly and obviously. However, nothing is known about how anthocyanins choose different existent modes in the cells of different plant organs or species. Further studies are clearly needed to elucidate the mechanisms by which anthocyanins exist in vacuole in different states. Today, the storage of anthocyanins in vacuole and the corresponding pigmentation effects are the important aspect of anthocyanin-related studies because they are the cytological mechanism of the coloration of all anthocyanin-pigmented cells. It has been becoming an issue of central importance in understanding how anthocyanin coloration is controlled *in vivo* and may offer the opportunity to introduce new plant traits by genetic engineering.

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高等植物花色苷在液泡中的存在状态及其着色效应

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摘 要：综述了花色苷被摄入液泡的原因、花色苷在液泡中的存在状态及其对植物细胞的着色效应。花色苷在植物细胞质中合成后转运到液泡里是为了解除其对蛋白质和DNA等细胞功能分子的毒性。花色苷的液泡区隔化是花色苷在植物细胞中发挥正常功能的前提。在大多数植物中，花色苷在绝大多数情况下完全溶解在液泡里。但是，花色苷也能在液泡里形成颗粒，这些颗粒可以划分为花色苷体和花色苷液泡包涵体两类。花色苷体由膜包裹，其形成是液泡中小的有色囊泡逐渐合并的结果，发育完全的花色苷体为典型的球状、具比液泡更深的红色；液泡里的花色苷体具高密度，呈现为含高浓度花色苷的不溶性小球；花色苷体的存在可导致液泡的强烈色彩。花色苷液泡包涵体可能具备蛋白质基质，既无膜包裹又无内部结构，其形成是转运进液泡的花色苷与蛋白质基质结合的结果；液泡里的花色苷液泡包涵体形状不规则，象果冻；在花色苷液泡包涵体中，花色苷可能通过氢键连接于蛋白质基质的一个有限空间位点；花色苷液泡包涵体被认为是液泡中花色苷的"陷阱"，优先摄取花色素3,5－二糖苷或酰化的花色苷；花色苷液泡包涵体的存在可增加液泡色彩的强度并导致"蓝化"。

关键词：花色苷；存在状态；着色效应；花色苷体；花色苷液泡包涵体

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