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OCCURRENCE, LOCATION AND DEVELOPMENT OF ANTHOCYANOPLASTS

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Key Word Index—*Brassica oleracea*; Cruciferae; red cabbage; anthocyanoplast; anthocyanin; photocontrol.

Abstract—Intensely pigmented organelles (anthocyanoplasts) have been found in anthocyanin-producing cells of more than 70 species representing at least 33 families of angiosperms. When fully developed these structures are typically spherical and normally only one is present in each pigmented cell. The development of the anthocyanoplast has been studied in both light and dark-grown red cabbage seedlings and the location of the mature organelle has been shown, by the use of isolated protoplasts and vacuoles, to be within the main cell vacuole. Evidence is presented which suggests that the anthocyanoplast is membrane-bounded and that it is the site of anthocyanin biosynthesis.

INTRODUCTION

With the exception of some of the later steps, the pathway of flavonoid biosynthesis is becoming reasonably well-established [1] and it is known that it is subject to phytochrome control [2]. Anthocyanin pigments, which are usually dissolved in the cell sap, are commonly found in angiosperms but are replaced by betacyanins in all families of the Centrospermae except the Caryophyllaceae [3]. Anthocyanins are typically found in flower and fruit tissues, and in the superficial cells of organs such as leaves and stems [4]. However, while a good deal is known about the distribution of these pigments, little information exists concerning the subcellular location of their synthesis.

The first significant anatomical observations appear to have been made by Politis [5], who recognized that in some anthocyanin-containing cells an intensely pigmented structure was present and he termed this a cyanoplast. Later, Lipmaa [6] observed a similar body and, apparently unaware of the earlier work, named it an anthocyanophore. Since that time such structures have been reported in a number of other species but although passing consideration has been occasionally accorded to them [7, 8], no comprehensive account of their distribution in the plant kingdom has been published and their role in anthocyanin biosynthesis is thereby difficult to assess. The purposes of the present communication are firstly to provide such a compilation for angiosperms, based upon a survey of the literature together with personal observations, and secondly to report upon a detailed investigation of the location and development of the anthocyanin-containing structures in red cabbage seedlings. Our findings indicate that these structures are likely to be the site of anthocyanin biosynthesis and it is proposed that they be known henceforth as anthocyanoplasts.

RESULTS AND DISCUSSION

Taxonomic and tissue distributions

The evidence concerning the taxonomic distribution of anthocyanoplasts (Table 1) demonstrates that they are much more widespread in occurrence than has been previously recognised. The survey reveals positive records for more than 70 species covering at least 33 families spanning both dicotyledons and monocotyledons. Of these, our personal observations include the first records for identified species in 5 families and 11 genera (Table 1). Four of the species listed have been employed in physiological studies on the photocontrol of anthocyanin biosynthesis, namely *Sinapis alba* [36], *Brassica oleracea* [38], *Raphanus sativus* [17] and *Phaseolus aureus* [37]. Mature anthocyanoplasts are spherical and more deeply red-coloured than the cell vacuole. Only one mature anthocyanoplast is normally found in each anthocyanin-containing cell (Fig. 1a) and similar structures have not been reported in unpigmented cells. The anthocyanin-pigmented cells are typically restricted in organs such as leaves and stems to the epidermis and hypodermis.

The above survey does not itself provide evidence as to whether anthocyanoplasts are the sites of biosynthesis or sites of accumulation of these pigments. Personal observations have so far failed to reveal the presence of these structures in the tissues of a number of anthocyanin-containing species, which at first sight might cast doubt upon them being sites of synthesis. However, we have also observed that anthocyanoplasts tend to degenerate and become less readily detectable as pigmented cells approach the completion of their development (see below). Furthermore, the positive records (Table 1) overwhelmingly relate to young tissues (e.g. hypocotyls or pedicels) or relatively short-lived ones (e.g. cotyledons or flower parts), as recognized by Politis [16], while by contrast anthocyanin pigmentation is retained by long-lived cells for substantial periods of time. It therefore seems desirable to examine tissues at a time when anthocyanin can be observed to be forming and to regard negative observations with caution. On the other hand, it is interesting to note that similar organelles have not so far been observed in betacyanin-containing species of the Centrospermae [4].

Anthocyanoplast development in red cabbage

The ability to synthesize anthocyanin is restricted in young seedlings of red cabbage to the hypodermis and, to a

Table 1. Species of angiosperms which possess anthocyanoplasts

Order and family	Species	Tissue or organ	Reference
DICOTYLEDONS			
Centrospermae			
*Caryophyllaceae	*Dianthus caryophyllus* L.	flowers	[9]
	Silene dioica (L.) Clairv.	calyx, pedicel	
Ranales			
*Ranunculaceae	*Aquilegia glandulosa* Fisch.	petals	[10]
Lardizabalaceae	*Decaisnea fargesii* Franch.	fruit	[11]
Rhoeadales			
*Papaveraceae	*Papaver rhoeas* L.	anthers	[12]
*Cruciferae	*Aethionema grandiflorum* Boiss & Hohen.	seedlings	[13]
	Alyssum moellendorfianum Ascherson ex G. Beck		[13]
	Brassica oleracea L.	hypocotyl, cotyledons	[14 16]
	Cheiranthus cheiri L.	calyx	
	Chorispora tenella (Pallas) DC.	seedlings	[13]
	Cochlearia glastifolia L.		[13]
	Eruca vesicaria (L) Cav.		[13]
	Lepidium sativum L.		[13]
	Lobularia maritima (L) Desv.		[4]
	Malcolmia africana (L) R.Br.		[13]
	Raphanus sativus L.	hypocotyl, cotyledons	[13, 17]
	Sinapis alba L.		
	Sisymbrium strictissimum L.	seedlings	[13]
Resedaceae	*Reseda odorata* L.	radicle	[18]
Rosales			
*Crassulaceae	*Sedum spurium* Bieb.	leaves	
*Rosaceae	*Prunus virginiana* L.	fruit	[19]
	Pyrus communis L.	anthers	[12]
	Rubus fruticosus L.	fruit	[20]
*Leguminosae	*Phaseolus aureus* Roxb.	hypocotyl	[4]
	Wisteria sinensis (Sims) Sweet.	flowers	[20]
Geraniales			
*Geraniaceae	*Pelargonium zonale* Ait.	petals	[21]
Linaceae	*Linum bienne* Miller	anthers	[12]
	L. usitatissimum L.		[12]
Malvales			
*Malvaceae	*Hibiscus syriacus* L.	flower-buds	[20]
Myrtales			
*Haloragaceae	*Gunnera tinctoria* (Molina) Mirbel.	fruit	[22]
Umbelliflorae			
Umbelliferae	*Orlaya kochii* Heywood	anther	[12]
Ericales			
*Ericaceae	*Erica herbacea* L.	petals	[10]
Gentianales			
*Gentianaceae	*Centaurium littorale* Gilmour	flowers	[6]
	C. pulchellum (Savartz) Druce.		[6]
*Rubiaceae	*Sherardia arvensis* L.	anthers	[12]
Tubiflorae			
*Polemoniaceae	*Polemonium caeruleum* L.	penduncle	
*Convolvulaceae	*Convolvulus althaeoides* L.	anthers	[12]
	Ipomoea purpurea Roth.	hypocotyl	[12]
*Boraginaceae	*Pulmonaria officinalis* L.	flower-buds	[23, 24]
	P. rubra Schott.		[23, 24]
*Verbenaceae	*Clerodendrum thompsonianae* Balf.	petals	[10]
*Labiatae	*Ajuga reptans* L.	flowers	
	Glechoma hederacea L.	petals	[10]
	Lamium purpureum L.	anthers	
	Ocimum basilicum L.	seedlings	[4]
*Solanaceae	*Atropa belladonna* L.	fruit	[25]
	Datura inermis Facq.	hypocotyl, cotyledons	[16]
	D. meteloides Dunal.		[16]
	D. stramonium L.		[16]

Table 1.—*Continued*

Order and family	Species	Tissue or organ	Reference
	Hyoscyamus niger L.	flowers	[16]
	Lycopersicum esculentum Mill.	hypocotyl, cotyledons	[16]
	Solanum melongena L.	fruit	[20]
	S. racemosum Facq.	hypocotyl, cotyledons	[16]
	S. sisymbrifolium Lam.		[16]
Scrophulariaceae	*Nemesia strumosa* Benth.	petals	[26]
	Scrophularia peregrina L.	anthers	[12]
Dipsacales			
*Caprifoliaceae	*Sambucus nigra* L.	fruit	[20]
	Weigela florida A.DC.	petals	[10]
	W. japonica Thunb.		
Campanulales			
*Compositae	*Bellis perennis* L	pedicel, florets	
MONOCOTYLEDONS			
Liliflorae			
*Liliaceae	*Hyacinthus orientalis* L.	bulb	[27]
		petals	
	Lilium speciosum Thunb.	petals	[20]
	Ophiopogon japonicus Ker.	fruit	[10]
Tecophilaceae	*Cyanastrum cordifolium* Oliv.	petioles	[28]
*Iridaceae	*Iris germanica* L.	petals	[29]
	I. reichenbachii Heuff.		[30]
	I. tectorum Maxim.		[10]
Bromeliales			
Bromeliaceae	*Billbergia nutans* Wendl.		[5]
*Commelinaceae	*Tradescantia blossfeldiana* Mildbr.	sepals	
Glumiflorae			
*Gramineae	*Agrostis stolonifera* L.	leaf-sheath	
	Hordeum vulgare L.		
Orchidales			
*Orchidaceae	*Laelia anceps* Lindl.	petals	[10]

The above sequence of families is arranged following the Engler system as employed in *Flora Europaea* [31]. Species are quoted according to the latter work or following Bailey [32] and cases in the literature where the identity of the material was in doubt, or where the pigmented organelles did not conform to the general pattern, have been omitted. Where no reference is cited the observations are those of the authors. Unspecified materials of the following genera also appear to contain anthocyanoplasts—*Cosmos* [4], *Delphinium* [33, 34], *Fuchsia* [11] and *Vitis* [20, 35].

*Families reported by Harborne [3] to contain reliably identified anthocyanins.

lesser extent, the epidermis of the hypocotyl and cotyledons [38]. After 2 days' growth the seedlings begin to enter the phase of most rapid synthesis of anthocyanin [38] and at this stage numerous unassociated small red vesicles are present in the superficially located cells (Table 2), while only faint pigmentation is visible in each main cell vacuole. By the following day, the red vesicles appear to be associating and are fewer in number. Later, one vesicle becomes substantially larger than the others and the pigmentation of the cell vacuole becomes more prominent. As development proceeds, the larger red body increases further in size, reaching over 10 μm in diameter in light-grown material when the seedlings are 4 days old (Table 2). The smaller bodies have disappeared by then and the single structure is clearly identifiable as an anthocyanoplast. The above observations lead to the conclusion that the anthocyanoplast is formed as a result of the progressive coalescence of the smaller pigmented vesicles. As the anthocyanoplast enlarges, some anthocyanin presumably leaks out into the cell vacuole causing it to become progressively more pigmented. Anthocyanoplasts of light-grown seedlings were consistently larger than those of dark-grown seedlings (Table 2), which accords with the greater ability of the former to synthesize pigment [38]. Similar structures were found in the cells of the superficial layers in leaves of mature red cabbage plants (Fig. 1a). Treatment of intact cells with either 1% *n*-propanol or a dilute detergent (0.1% Triton X-100) led to the loss of anthocyanin but a colourless spherical entity corresponding to the anthocyanoplast remained in each cell (Fig. 1b). This suggests that these structures are membrane-bounded rather than simply being hydrophobic droplets. Cells of unpigmented layers were devoid of these structures.

The observations on seedlings were made on transverse sections cut from close to the top of the hypocotyl, since in this region the cells undergo only limited extension growth and the chances of finding anthocyanoplasts were good. Longitudinal sections revealed that in the substantially elongated cells lower down in the hypocotyl the anthocyanoplasts had become disorganised and had largely lost their ability to retain pigment at a higher concentration than that of the cell vacuole.

Table 2. Influence of seedling age and illumination on anthocyanoplast development in red cabbage

Age (days)	Diameter of pigmented vesicles (μ)* Light	Dark	Description
2	3.2	2.4	numerous
3	7.2	5.0	fewer
4	11.3	6.4	few
5	10.6	8.1	one

*Measurements were made on samples of 55 anthocyanin-pigmented cells in transverse sections cut from close to the top of the hypocotyl.

Sub-cellular location of anthocyanoplasts

The location of anthocyanoplasts within cells has not previously been determined. However, from the size and position of these organelles in cells observed in sections of red cabbage seedlings it seemed possible, though somewhat surprising, that they lay in the cell vacuoles. In order to provide incontrovertible evidence regarding the location of the mature anthocyanoplasts, work was carried out to isolate protoplasts and thence cell vacuoles from hypocotyl tissue. The protoplasts were isolated and then collected on a sucrose-sorbitol gradient using a modification of the method of Edwards *et al.* [39]. The protoplasts were examined microscopically (Fig. 1c) and cell vacuoles were released from them by addition of 0.2 M K_2HPO_4 at pH 8.0 [40]. Anthocyanoplasts were clearly retained within the isolated vacuoles (Fig. 1d) and were normal in appearance. Observations of cells at earlier

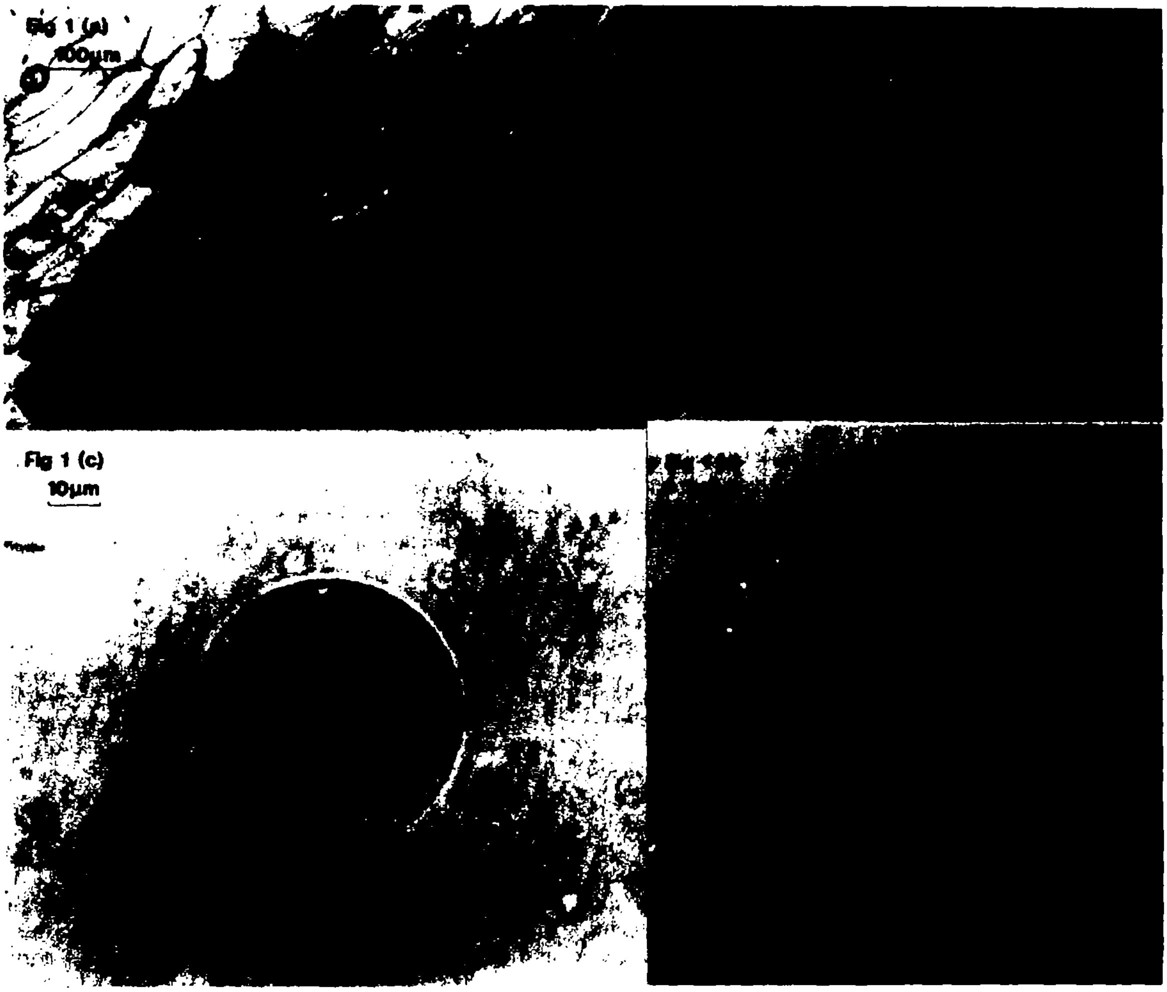

Fig. 1. Anthocyanoplasts of red cabbage: (a) *in situ* in surface section of leaf from the heart, showing one anthocyanoplast per pigmented cell. (The cells on the left-hand side have lost their pigment as a result of being damaged in sectioning but the anthocyanoplasts are still visible.) (b) As in (a) but after treatment with dilute Triton X-100, which leads to loss of pigment but the colourless anthocyanoplast is still visible; (c) an isolated pigmented protoplast containing a densely coloured anthocyanoplast and (d) an isolated vacuole with the anthocyanoplast retained within it.

stages of development suggested that the vesicles, which later form the anthocyanoplast, are also in the cell vacuole at the time at which they can be first recognized by their colour.

Fritsch and Grisebach [41] have suggested that some of the enzymes involved in flavonoid biosynthesis may occur in the tonoplast or in the vacuolar sap, while Ginsberg [42] has shown in root tissue of *Reamuria* that tannins are present in cisternae which coalesce to form the central vacuole. Steinitz and Bergfeld [43] have taken the view that the formation of a central vacuole is a prerequisite for anthocyanin biosynthesis in mustard seedlings. However, observations on red cabbage reveal that the central vacuole appears well before the time at which the pigmented vesicles are first evident. It would therefore seem more likely that the crucial event in preparation for anthocyanin biosynthesis is the production of the small vesicles themselves. Unfortunately no evidence is available about their origin but by analogy with current views concerning the origin of vacuoles in general, it seems possible that they arise from the endoplasmic reticulum (ER) [44]. Other evidence suggesting the involvement of the ER in phenolic biosynthesis comes from the association of a part of the phenylalanine ammonia lyase activity, and nearly all that of cinnamate 4-hydroxylase, with a microsomal preparation from potato tissue [45]. The association of droplets of condensed tannins with smooth ER has been observed in the shoot apex of *Oenothera* [46].

From the facts presented above it is concluded that anthocyanoplasts are likely to prove to be of general occurrence in anthocyanin-containing cells at the time when pigment synthesis is taking place. Evidence in favour of the view that the coloured vesicles and the anthocyanoplasts themselves are the sites of anthocyanin biosynthesis is that (a) these structures occur predominantly in young and often short-lived tissues, (b) pigmentation appears in the vesicles before it is apparent in the cell vacuole and (c) light exposure leads to an increase in both anthocyanin production and the size of the anthocyanoplasts. While this view requires further substantiation, the fact that the anthocyanoplast appears to be membrane-bounded is also consistent with it being the site of anthocyanin production. This appears to be the first report of a functional synthesizing organelle within the cell vacuole. It seems realistic to regard the anthocyanoplast as a compartment in which biosynthesis of anthocyanin can be 'buffered' from the presumably hostile environment of the main cell vacuole. Once the pigment has been formed, it leaks into the cell vacuole.

There is clearly a need to investigate the enzyme complements of anthocyanoplasts, the properties of their membranes, and the ability of the isolated organelles to synthesize pigment. Progress towards these ends will depend upon the establishment of a method for isolating anthocyanoplasts in quantity from pigmented tissue. A further understanding of the photocontrol of anthocyanin biosynthesis will depend upon the determination of the origin of the vesicles from which the anthocyanoplasts are formed.

EXPERIMENTAL

Plant materials and growth conditions. Anthocyanin-containing tissue from a range of families of angiosperms was examined for the presence of anthocyanoplasts by optical microscopy. In the study of anthocyanoplast development in red cabbage (*Brassica oleracea* L. cv Niggerhead obtained from Hurst, Gunson, Cooper and Taber Ltd.), seeds were grown at 25 ± 0.5 either in continuous darkness or in continuous illumination in a Fisons PG 94 plant growth chamber. Measurements of anthocyanoplast size were made daily using transverse sections taken from the top of the hypotocotyl.

Protoplasts and vacuole isolation. Protoplasts were prepared from the hypocotyl of 5-day-old light and dark-grown red cabbage seedlings. The upper region of the hypocotyl was chopped into segments which were floated on a soln of 2% cellulysin, 1% macerase, 1% K dextran SO_4 (all Calbiochem), 0.55 M sorbitol, 1 mM $MgCl_2$ and 25 mM KH_2PO_4 adjusted to pH 5.6. After vacuum infiltration for 30 min, the preparations were incubated at 35° for 4 hr. The protoplasts were released by teasing the segments apart and then expressing the suspension from a Pasteur pipette. Release of protoplasts was easier from young leaves of the heart of red cabbage than from seedling tissue. Protoplasts were centrifuged at 200 g for 2 min and resuspended in 2 cm^3 0.55 M sucrose, 1 mM $MgCl_2$ and 25 mM KH_2PO_4 at pH 5.6. On top of this was layered 1 cm^3 0.45 M sucrose, 0.1 M sorbitol, 1 mM $MgCl_2$ and 25 mM KH_2PO_4 at pH 5.6, followed by 1 cm^3 0.55 M sorbitol, 1 mM $MgCl_2$ and 25 mM KH_2PO_4 at the same pH. After centrifuging at 200 g for 4 min the pigmented protoplasts collected between the layers containing 0.55 M sorbitol and 0.45 M sucrose/0.1 M sorbitol. Vacuoles were isolated by placing a small drop of suspension on a well-slide and adding a drop of 0.2 M K_2HPO_4 at pH 8.0. Intactness of protoplasts and vacuoles was confirmed by their exclusion of 1% Evans Blue [47].

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