Betalains

Jean-Pierre Zrÿd* & Laurent Christinet
Laboratory of Plant Cell Genetics
Department of Plant Molecular Biology
Université de Lausanne, CH 1015 Lausanne, Switzerland
* jzryd@ie-pc.unil.ch & http://www.unil.ch/lpc/docs/bv.htm

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Betalain pigments

Betalain pigments are water-soluble vacuolar yellow (betaxanthins) and violet (betacyanins) pigments that replace anthocyanins in most plant families of the order Caryophyllales. They are also found in some species of the fungal genera *Amanita* and *Hygrocybe*. Betalains are conjugates of the chromophore betalamic acid that derives from dihydroxyphenylalanine by an oxidative 4,5- extradiol ring opening mechanism. The term betalain was introduced to describe these pigments as derivatives from betalamic acid (Wohlpart and Mabry, 1966). Betalains were erroneously named in the past «nitrogenous anthocyanins» and today often referred as «chromo-alkaloids» due to the presence of a nitrogen atom in the chromophore. It is still common to find erroneous references, even in contemporary textbooks, to anthocyanins in «lieu et place» of betalains (Fig. 1).

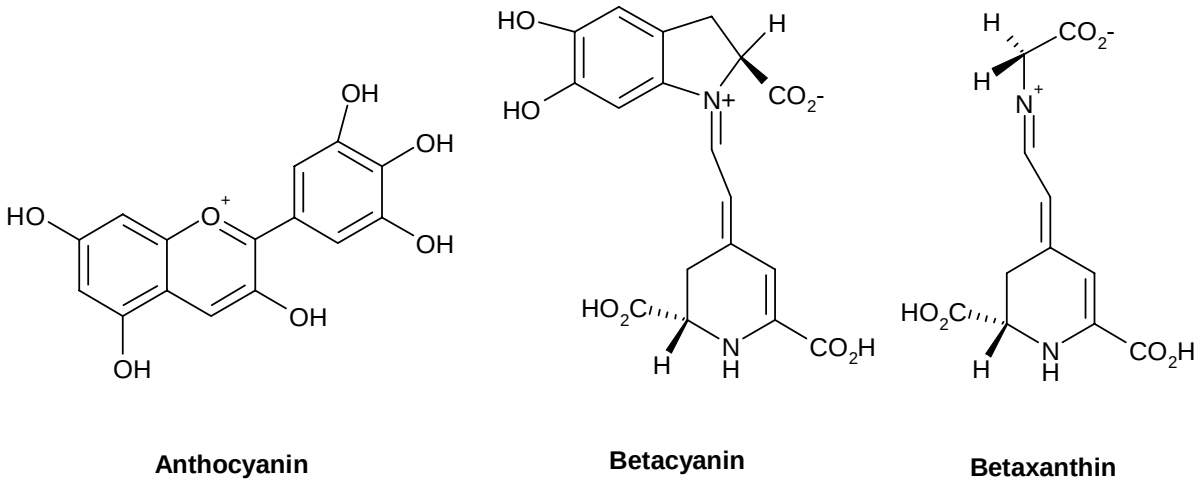


Fig 1 The structure of anthocyanins (delphinidin), betacyanins (betanidin) and betaxanthins. R = amino acid or amine

Chemistry

The main pigment of red beet, betanin (Schudel, 1919), has been used as a model for the determination of the structure and biosynthetic pathway of betalains. Our knowledge of the chemistry of betalain started in the late fifties with the pioneering work of Dreiding's laboratory at the University of Zürich (Wyler et al., 1963a). The short but fascinating «compte-rendu» by Mabry of its early years as a post-doc give an idea of the atmosphere surrounding this work and the progresses made by the group (Mabry, 2001). Since then works by Piatelli in Italy, Mabry in the USA and Wyler in Switzerland added to our knowledge of this unique family of pigments. The common characteristic of all betalains is the presence of the betalamic acid chromophore, a dihydropyridine moiety attached via a vinyl group to another nitrogenous group (Miller et al., 1968). Its lemon yellow color (lmax 424 nm) results from the resonance system induced by the presence of three conjugated double bonds (Fig. 2). Betaxanthins are formed by the condensation of an amino acid or an amine with the aldehyde group of betalamic acid, resulting in a Schiff-base (Fig.2). This structure is responsible for the strong yellow or yellow-orange colors of betaxanthins and the maximum of absorbance between 470-486 nm. Betacyanins are also formed of a betalamic acid unit linked to a molecule of cyclo-DOPA (Wyler and Dreiding, 1961) (Fig.2). The latter highly aromatic structure is responsible for the deep violet color this pigment; this aromatic structure also induces a strong batochrome shift of 60-70 nm (lmax 534-554 nm). The basic betacyanin and betaxanthin structures can be modified in numerous ways; conjugation reactions like glycosylation or acylation are common. A detailed view on new structures and the methods used for their characterization can be found in the recent review by Strack and co-workers (Strack et al., 2003).

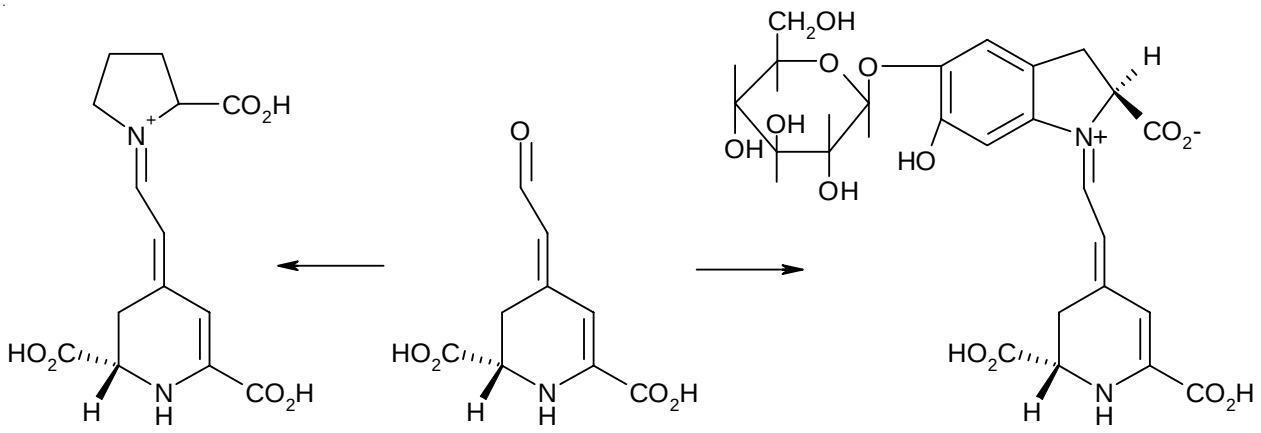


Figure 2. Chemical structure of betalamic acid, the main chromophore of betalains. This structure is present in all betaxanthins associated to an amino acid or an imino compound (ex: indicaxanthin from *Opuntia ficus-indica*) and in all betacyanins associated to cyclo-DOPA (ex: betanin from *Beta vulgaris*).

Betacyanins

All betacyanins need two molecules of tyrosine as precursors. The simplest natural betacyanins are the non-glycosylated betanidin or isobetanidin chromophores obtained by the condensation of cyclo-DOPA with betalamic acid (Wyler et al., 1963b). Both molecules are differing only by the absolute configuration of their C-15 chiral center (Wilcox et al., 1965) (Fig. 3). In beet hypocotyls the ratio between the two compounds concentration is about 4 to 1.

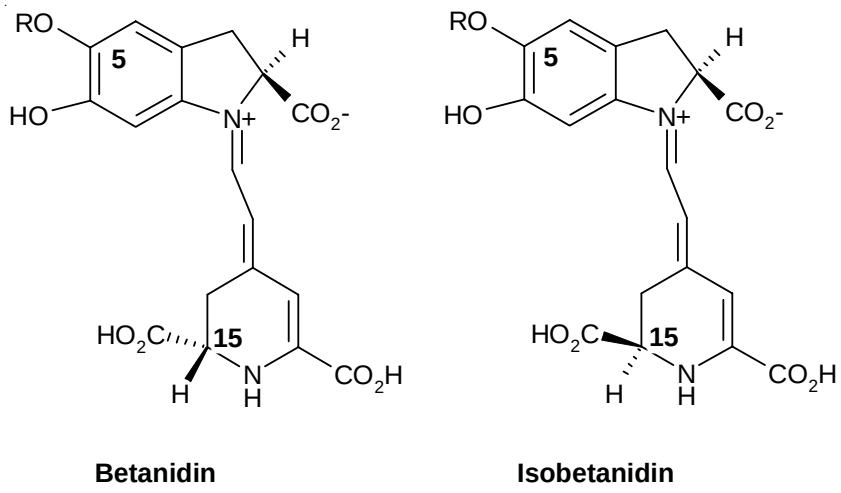
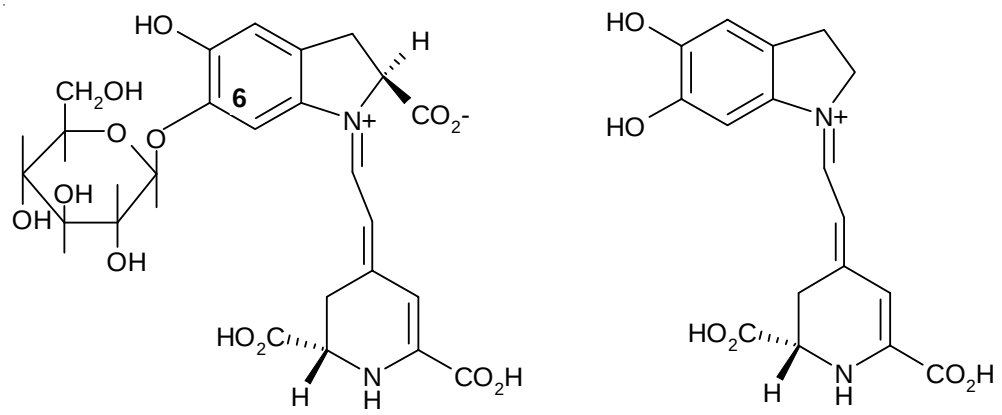


Figure 3. Betacyanins non-glycosylated chromophores differ only by the C-15 chiral center (R=H).

Other betacyanins derive from these two isomers by *O*-glycosylation on one of the two free hydroxyl groups of cyclo-DOPA (Table 2). Glucosylation in position 5 is called betanin and is the major red beet pigment (Fig. 4). Some rare pigments, like gomphrenins have been identified as 6-*O*-glucosides (Minale, 1967). Until recently, the only known non-glycosylated betacyanin was 2-descarboxy-betanidin (Fig. 4), a minor pigment from *Carpobrotus acinaciformis* flowers (Aizoaceae) containing a decarboxylated cyclo-DOPA moiety (Piattelli and Imperato, 1970). Lately, small amount of this pigment was also identified in yellow beet (Schliemann et al., 1999)

accompanied by two new similar pigments, the 2-descarboxy-betanin and the 6'-*O*-malonyl-2-descarboxy-betanin (Kobayashi et al., 2001).

The majority of betacyanins are acylated by ferulic acid or less frequently by cinnamic acid on their glycoside part via an ester linkage. Malonylation is also present in betacyanin structure; it is known for stabilizing pigments in flowers, preventing anthocyanins from beta-glycosidase attacks (Suzuki et al., 2002). A new type of acylated betacyanin containing both an aliphatic and a hydroxycinnamoyl aromatic acyl residue has been detected in *Phytolacca americana* (Schliemann et al., 1996). This kind of acylation is also observed in complex anthocyanins.



Gomphrenin I **2-Descarboxy-betanidin**

Figure 4. Different types of glycosylation of betacyanins: betanidin 6-*O*-glucoside (Gomphrenin I) from *Gomphrena globosa* and the non-glycosylated 2-descarboxy-betanidin from *Carpobrotus acinaciformis* .

Betaxanthins

The yellow betaxanthins are immonium conjugates of betalamic acid with an amine or an amino acid. All protein amino acids and any of the 220 known non-protein amino acids found in plants can participate to the betaxanthins molecules (Trezzini and Zryd, 1991b), therefore numerous betaxanthins can be found in plants, but only some have been fully characterized, most of them being present only in trace amounts. The classification of betaxanthins distinguishes the amino acid-derived compounds from the amine-derived conjugates (Table 2). The first isolated structure was indicaxanthin (Fig. 2) from the fruit of Cactus pear (*Opuntia ficus-indica*) (Piattelli et al., 1964). Numerous new structures have been identified during the last fifteen years (Fig. 5). In *Portulaca grandiflora*, two pigments, one containing tyrosine (portulacaxanthin II) and the other glycine (portulacaxanthin III) were characterized (Trezzini and Zryd, 1991a). In Amaranthaceae, tryptophan-betaxanthin and the first methylated betaxanthin, 3-methoxytyramine-betaxanthin have been isolated from *Celosia argentea* (Schliemann et al., 2001). The latter compound seems to be methylated already at the catecholic stage rather than at the betaxanthins stage.

Table 1. Some betacyanins arranged according to their structures.

AGLYCONES	
Betanidin	(Wyler et al., 1963b)
Isobetanidin	(Wilcox et al., 1965)
2-Descarboxy-betanidin group	
2-Descarboxy-betanidin	(Piattelli and Imperato, 1970)
2-Descarboxy-betanin	(Kobayashi et al., 2000)
6'-O-Malonyl-2-descarboxy-betanin	(Kobayashi et al., 2001)
GLYCOSIDES	
5-O-glycosylation	
Betanin group	
Betanin: betanidin 5-O-glucoside	(Wyler and Dreiding, 1961)
Phyllocactin: 6'-O-malonyl-betanin	(Piattelli et al., 1969)
2'-Apiosyl-phyllocactin	(Kobayashi et al., 2000)
2'-(5''-O-e-Feruloylapiosyl)-betanin	(Schliemann et al., 1996)
2'-(5''-O-e-Feruloylapiosyl)-phyllocactin	(Schliemann et al., 1996)
Hylocerenin:6'-O-(3''-hydroxy-3''-methyl)-betanin	(Wybraniec et al., 2001)
Amaranthin group	
Betanidin 5-O-(glucuronide)-glucoside-amaranthin	(Sciuto et al., 1974)
Iresinin I : hydroxymethylglutaryl-amaranthin	(Cai et al., 2001)
Celosianin I, II : coumaroyl and feruloyl-amaranthin	(Cai et al., 2001)
6-O-glycosylation	
Gomphrenin group	
Gomphrenin I: betanidin 6-O-glucoside	(Piattelli and Minale, 1964)
Gomphrenin II: coumaroyl derivative of gomphrenin I	(Heuer et al., 1992)
Gomphrenin III: feruloyl derivative of gomphrenin I	(Heuer et al., 1992)
Betanidin 6-O-(hydroxycinnamoyl)-b-sophoroside derivatives	(Heuer et al., 1994)

Recently, seven new pigments from cactus pear were characterized, six of which were new in plants (Stintzing et al., 2002). That brings the question of the choice of the methodology to identify betaxanthins often present in trace amount. According to Stintzing, enrichment of the plant extract in betaxanthins, after separation from sugars and pectins, gives a more detailed profile close to the «in vivo» chemical diversity (Stintzing et al., 2002).

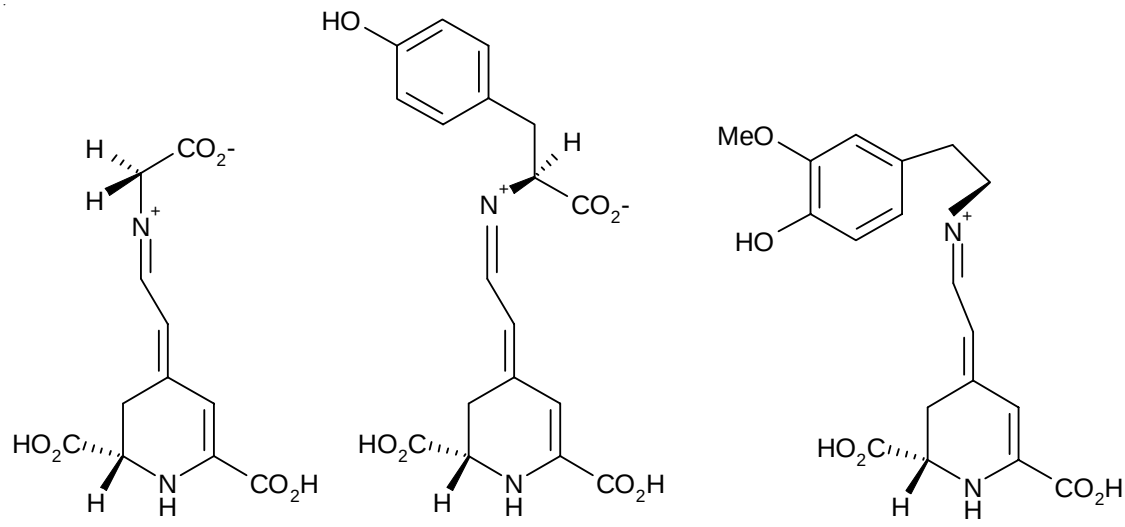


Figure 5. Chemical structure of some betaxanthins: Portulacaxanthin III (Glycin-betaxanthin), Portulacaxanthin II ((S)-tyrosine-betaxanthin) from *Portulaca grandiflora* and 3-methoxytyramine-betaxanthin (derived from dopamine-betaxanthin) from *Celosia sp.*

Several betaxanthins have been identified in the fungus *Amanita muscaria*, including seven orange musca-aurins (lmax 480 nm) (Döpp and Musso, 1973b, Döpp and Musso, 1973a, Trezzini and Zryd, 1991b). *A. muscaria* does not contain betacyanins but red and yellow betalamic acid derived compounds, called respectively musca-purpurin (lmax 540 nm) and musca-flavin (lmax 420 nm) (Terradas and Wyler, 1991a, Mueller et al., 1997b).

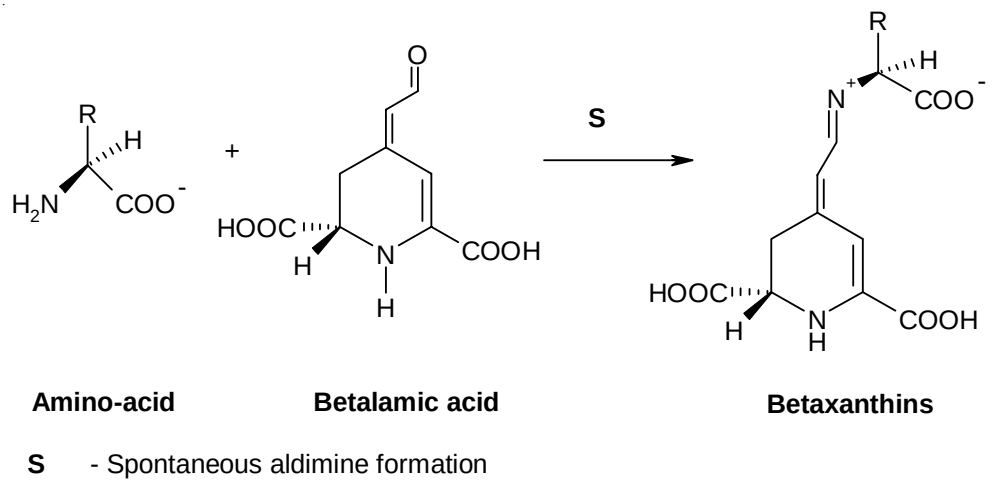


Figure 6 – Betaxanthins are formed by a spontaneous, non-enzymatic reaction at neutral and slightly acidic pH

Betaxanthins can be easily synthesized non-enzymatically «in vitro» by mixing betalamic acid and the desired amine at neutral or slightly acidic pH (Trezzini, 1990, Trezzini and Zryd, 1991b); this occurs spontaneously in the acidic plant vacuole (Schliemann et al., 1999) (Fig. 6).

Amino acid-derived conjugates	Amino acids	Identification:
Dopaxanthin	DOPA	(Impellizzeri et al., 1973)
Indicaxanthin	proline	(Piattelli et al., 1964)
Miraxanthin I	methionine sulfoxyde	(Piattelli et al., 1965a)
Miraxanthin II	aspartic acid	(Piattelli et al., 1965a)
Portulacaxanthin I	hydroxyproline	(Piattelli et al., 1965b)
Portulacaxanthin II	tyrosine	(Trezzini and Zryd, 1991a)
Portulacaxanthin III	glycine	(Trezzini and Zryd, 1991a)
Vulgaxanthin I	glutamine	(Piattelli et al., 1965c)
Vulgaxanthin II	glutamic acid	(Piattelli et al., 1965c)
Tryptophan-betaxanthin	tryptophan	(Schliemann et al., 2001)
Amine-derived conjugates	**Amines**	
Miraxanthin III	tyramine	(Piattelli et al., 1965a)
Miraxanthin V	dopamine	(Piattelli et al., 1965a)
3-Methoxytyramine-betaxanthin	methoxy-tyramine	(Schliemann et al., 2001)
Humilixanthin	hydroxy-norvaline	(Strack et al., 1987)
Miraxanthin I	methionine sulfoxyde	(Schliemann et al., 2001)

Table 2. A selection of betaxanthin representative structures distributed in two groups according to the type of molecule conjugated with betalamic acid (amines or amino acids).

Physiology of betalains

Light

Synthesis of betacyanins or betaxanthins does not require light in most species examined till now, although light is one major factor controlling the quantities of pigments produced (Wohlpart and Mabry, 1968). Phytochrome is involved in the photo-control of amaranthin synthesis in *Amaranthus paniculatus*, one of the few species that have an absolute light requirement, (Rast et al., 1972); the red stimulation of pigment accumulation is made partially reversible by a far-red treatment. In *Amaranthus tricolor* seedlings, it has been shown that the photo-control occurs at the level of the formation of the dihydropyridine moiety of the molecule (Giudici De Nicola et al., 1975). This photo-control involves both blue light and red light and is thought to be mediated both by phytochrome and cryptochrome (Kochhar et al., 1981). It was shown recently that UV-A alone could induce the production of betacyanins and flavonol glycosides in bladder cells of *Mesembryanthemum crystallinum* leaves (Vogt et al., 1999b); this induction is maximum at wavelength between 305-320 nm (Ibdah et al., 2002).
(Girod and Zryd, 1987) observed the appearance of pigmented cells on green calli from *Beta vulgaris* when they were transferred from dim-light to high-light intensity; this trans-differentiation process is dependent of light intensity. Blue light induces pigmentation in *P. grandiflora* callus (Kishima et al., 1995).

Light is known to affect the competition between the dopamine pathway leading to catecholamines (adrenaline) and the betalain pathway; in *Portulaca* callus catecholamine are only synthesized from dopamine in the dark (Endress et al., 1984).

Cytokinins and other hormones

In *Amaranthus caudatus*, betacyanins are synthesized in the dark in the presence of adenine-derived compounds (Bigot, 1968). A bio-assay for the determination of cytokinins (6-benzyl aminopurine) in plant extracts based on the cytokinin-induced formation of betacyanins in the dark-grown *A. caudatus* seedlings in the presence of tyrosine has been developed (Biddington and Thomas, 1973). This bio-assay is one of the most sensitive method still available for the quantification of natural cytokinins in plants (Kubota et al., 1999). Cytokinins can mimic light treatment in the control of tyrosine utilization (Stobart and Kinsman, 1977). Studies on the molecular mode of action of cytokinin in the *Amaranthus* betacyanin assay with actinomycin D, alpha-amanitin and cycloheximide showed that rapid transcript induction and their translation were needed for amaranthin formation (Romanov et al., 2000). The mean intervals between transcription, translation and pigment formation were estimated to be approximately two hours. In *P. grandiflora*, auxin (2,4-D) is needed for the appearance of a stable pigmentation, in the absence of auxin pigmentation disappears almost entirely and become unstable (Trezzini, 1990). In cultured cells from *B. vulgaris*, the ratio of auxin (2,4-D) to cytokinin (6-BAP) is an important factor in the regulation of betalain biosynthesis (Girod and Zryd, 1991b).

Cell and hairy root cultures

Cell and hairy root cultures of betalain producing plants have been established long ago either for basic research on the biosynthetic pathways or with the purpose of producing high-quality violet and yellow pigments for the food industry. The low market price of beet extract put nevertheless a limit to the possible factory production of such pigments. Specific requests for pure yellow water soluble pigments (betaxanthins) to replace potentially carcinogenic compounds have never materialized.

Beet cell cultures can be manipulated to produce high amount of pure pigment (Leathers et al., 1992); yields of total betacyanins can be as high as 28 mg per g dry weight which is well above the content of cultivated red beet. Hairy root cultures have been shown to produce similar amount of pigments in air-lift reactors (Shin et al., 2002). In an experiment with *B. vulgaris* cells cultivated on solid medium (callus) it was shown that the amount of total betacyanins in the violet cell lines is slightly higher (28 mmoles/ g DW) than the amount present in storage organs of the plant (21 mmoles/ g DW). In the orange or red cell lines the amount of total betaxanthin can reach more than 12 mmoles/ g DW (Girod and Zryd, 1991b); violet betacyanin producing cell cultures contain a large amount of dopamine; it would be probably necessary to shut down the biosynthetic pathway leading to dopamine to further increase fluxes from DOPA toward betalain biosynthesis.

Regulatory mechanisms of betacyanin biosynthesis in suspension cultures of *Phytolacca americana* were investigated in relation to cell division activity. By inhibiting cell division, incorporation of radioactivity from labeled tyrosine into betacyanin is reduced (Sakuta et al., 1994); the conclusion was that conversion of tyrosine to DOPA is associated to cell division. By using the DNA-methylation inhibitor 5-azacytidine, (Girod and Zryd, 1991b) demonstrated that methylation plays a key role in the repression of genes encoding enzymes involved in betacyanin biosynthesis. Yellow cells (accumulating only betaxanthins) could be switched to red cells (containing both betacyanins and betaxanthins) by reducing the concentration of the auxin 2,4-D in the culture medium. The chain of events occurring during this change is stimulated by the methylation inhibitor; this indicates gene activation of normally repressed genes. 3-methoxybenzamide, a poly (ADP-ribosyl) polymerase inhibitor, was found in same experiments

to inhibit color transition. That chromatin rearrangement and DNA-methylation are both involved in the regulation of betalain color genes doesn't come as a surprise.
Betalain synthesis had been reported to be modified in diseased plants after insect, pathogen or virus attacks. Phytopathologists (Steddom et al., 2003) used multi-spectral canopy reflectance to study the physiological differences between healthy sugar beets and beets infested with Beet Necrotic Yellow Vein Virus. It was shown that the ratio of betacyanins to chlorophyll, estimated from canopy spectra, was increased in symptomatic beets at four of seven sampling dates. Differences in betacyanin levels appeared to be related to disease impact and to the development of rhizomania associated with this virus attack. Wounding and infiltration with *Pseudomonas syringae* or *Agrobacterium tumefaciens* also induced the synthesis of betacyanins in red beet leaves (Sepúlveda Jiménez et al., 2003).

Genetic of betalains

We will consider here only two of the main studied species with respect to betalain biosynthesis: *Portulaca grandiflora* and *Beta vulgaris*.

Portulaca grandiflora – large flowered purslane

Large-flowered purslane Portulaca grandiflora was described for the first time by Hooker (Hooker, 1829). This plant originates from South America and can be found there in two forms: one with and deep orange flowers and the other with deep red flowers. The description by Hooker is lively: *«… On the western side of the Rio Desaguardo plants were in great profusion, giving to the ground over which they were spread a riche purple hue, here and there marked with spots of orange color, from the orange-colored variety which grew intermixed with the other.»*; he concludes: *«… I am happy [..] having the opportunity of giving an analysis of what I cannot but think a new species of the genus, and one, the beauty of whose flowers must render it a desirable inhabitant of the cool stove or greenhouse.»*
The beauty and the large spectrum of color and flower shape attracted Japanese geneticists since the beginning of the twentieth century (Yashi, 1920, Ikeno, 1921, Adachi, 1972). Most of those works were done at a time when the nature of the pigments was unknown. More recently, based on an increasing biochemical knowledge, a genetic model of betalain pigmentation based on the analysis of crosses of inbred lines of *P. grandiflora* was published (Trezzini and Zryd, 1990). The resultant segregation patterns indicated that a minimum of three loci *C* (Color), *R* (Red) and *I* (Inhibitory) were involved in petal pigmentation (Table 3).

	Violet	**Orange-Red**	**Deep Yellow**	**Pale Yellow**	**White**
C	*C-*	*C-*	*C-*	*C-*	*cc*
R	*R-*	*R-*	—	—	—
I	*I-*	*ii*	*ii*	*I-*	—

Table 3. A three genes model of *Portulaca grandiflora* flowers color.

The expression of the dominant *C* locus, responsible for the conversion of DOPA to betalamic acid, leads to plants with colored shoots and petals. The expression of the dominant *R* locus is necessary for the production of violet betacyanins correlated with the availability of cyclo-DOPA or its glucosylated form. The dominant *I* locus inhibit (but to not prevent) the accumulation of betaxanthins. In the absence of the dominant *R* locus, *Portulaca* flowers are either pale yellow in presence of the dominant I and deep yellow in the homozygous recessive ii background. The

presence of the dominant *I* locus is essential for the production of pure violet hue, otherwise, flower will display an orange-red color due to the high level of yellow betaxanthin over a violet betacyanin background. The *I* and *R* locus are strongly linked at ~ 5.3 cM (Trezzini, 1990). The *I* locus prevent the conjugation of amino acid or imino residues (others than cyclo-DOPA) with betalamic acid, thus decreasing the synthesis of betaxanthins and leading to the accumulation of betacyanins in the violet phenotype (dominant *R* locus). It is hypothesized that the chromophore is formed in the cytoplasm, transported in the vacuole where conjugation to betaxanthins will take place spontaneously (Trezzini, 1990). This hypothesis postulates the existence of a specific carrier for betalamic acid, which would be partially or totally shutdown in presence of the product of locus *I*. A small amount of yellow pigment was always produced despite the expression of the inhibitor I in the pale-yellow *CCrrII* phenotype. In those plants a high amount of free betalamic acid is present, as well as traces of dopaxanthin, but no miraxanthin V (dopamine) can be detected (Trezzini and Zryd, 1991a). The identity of the products of the *R* and *I* locus are still unknown and will need further investigations.
Coloration of *Portulaca* petals shows an instability which is modulated by the activity of transposable elements (Rossi-Hassani and Zryd, 1995, Rossi-Hassani and Zryd, 1994)

Beta vulgaris – table beet

Beet fleshy hypocotyl is the major source of betalain pigments for the food industry. Betalains are stored in the huge fleshy hypocotyl which is the storage organ of the plant (often misidentified as root); they can also be found in the shoot and leaf veins (swiss chard varieties). Many pigmented cultivars have been selected during the long history of beet domestication. Since the cultivation of beet as an alternative source of sucrose at the beginning of the nineteenth century huge effort have been made to improve sucrose content and disease resistance. The genetics of *Beta vulgaris* pigmentation has been comparatively less studied. The presence of dominant alleles at two linked loci (*R* and *Y*) conditions the qualitative production of betalain pigment in the beet plant (Wolyn and Gabelman, 1989, Goldman and Austin, 2000). Red-pigmented hypocotyls are observed only in the presence of dominant alleles at both the *R* and *Y* loci, while white hypocotyls are conditioned by recessive alleles at the *Y* locus, and yellow hypocotyls by the genotype *rrY*-. Therefore the beet *Y* locus should correspond to the *C* locus and the beet *R* locus to the *R* locus of *P. grandiflora*. Alleles at the *R* locus determine the ratio of betacyanin and betaxanthin (Wolyn and Gabelman, 1989).
In *B. vulgaris* cell lines cultivated «in vitro», careful clonal selection leads to five phenotypes that mimic purslane flower colors (Girod and Zryd, 1991b). The red and violet cell phenotypes were shown to contain 2 to 3 times more pigments than the orange and yellow cell phenotypes. This is due mainly to the accumulation of high amount of betanin over a quite stable background content of betaxanthins. The authors suggested that the synthesis of cyclo-DOPA (gene *R*) is coordinated with that of betalamic acid (gene *C*).
A regulatory gene named ‘blotchy’ (*bl*) that condition a blotchy or irregular pigment patterning in either red or yellow hypocotyls has been characterized (Goldman and Austin, 2000). There are linkage relationships between the *R* and *Y* loci and the *bl* gene. The two-point linkage estimate between the *R* and *Y* loci was estimated ~ 7.4 cM. The *R-Y-bl* genomic region is therefore important in the genetic control of betalain biosynthesis in Beta vulgaris. The linkage relationships demonstrated both in purslane (*R* & *I*) and beet plants (*R* & *Y*) could indicate a strong pressure for co-evolution of the betalain biosynthesis syndrome. The *bl* gene shows some transmission distortion suggesting among other an epigenetic control (Austin and Goldman, 2001)
Careful field selection of red beet populations for high total betalain pigment concentration (Goldman and Austin, 2000) lead to at total pigment increase of about 200%; betalain pigment concentration responds favorably to recurrent selection. Since betalain pigments are formed

following glycosylation of cyclodopa and betalamic acid it could be possible to associate sugar biosynthesis with pigment biosynthesis. RAPD markers associated with genes controlling pigment in red beet have been identified (Eagen and Goldman, 1996).
In one habituated red beet cell line it has been shown that the block in betalain biosynthesis (leading to white cells) is at the level of tyrosine hydroxylation (Zryd et al., 1982); no DOPA is formed; this observation suggest a possible «silencing» of a gene coding for a multifunctional polyphenol oxidase in the white cell lines.

Enzymology and biotechnology

Betalains derive from tyrosine; that distinguishes them clearly from the phenylalanine-derived anthocyanins (Miller et al., 1968). The betalain biosynthetic pathway is rather simple; only three to four enzymes are needed for the synthesis of the most simple betaxanthin or betacyanin (see Fig.6): they will catalyze a) a ring opening reaction leading to the chromophore betalamic acid, b) the formation of cyclo-DOPA c) a further glycosylation step in betacyanin synthesis and finally d) the transport of betaxanthins in the vacuole. Betacyanins synthesis requires a minimum of two tyrosines as precursors, whereas one is sufficient for betaxanthins. Feeding experiments with ^{14}C-radiolabeled tyrosine showed that the entire C_6-C_3-skeleton of this amino acid is incorporated into betalamic acid and cyclo-DOPA molecules (Liebisch and Bohm, 1981). Tyrosine hydroxylation by a tyrosinase produces 3,4-dihydroxyphenylalanine (DOPA), a reaction which is very common in the plant kingdom.

Polyphenol – tyrosinase reactions

Tyrosinases (polyphenoloxydases – PPOs) are widespread among plants and fungi. They are copper-containing enzymes that catalyze hydroxylation of phenols to o-diphenols (EC 4.14.18.1 – monophenol : monooxygenase) and their subsequent oxidation to o-quinones (EC 1.10.3.1. – o-diphenol : oxygen oxidoreductase). An other PPO called catechol oxidase catalyzes only the oxidation of o-diphenols.
The first step in betalain biosynthesis is the formation of 3(3,4dihydroxyphenyl)alanine (DOPA) from tyrosine. DOPA is an important metabolic product of a large number of plant families and accumulates in large amount in some Leguminosae species (*Vicia faba*) (Guggenheim, 1913). In betalain plants DOPA is then oxidized through a further diphenolase reaction to dopaquinone (rearranged to cyclo-DOPA) and modified by a ring-opening extradiol dioxygenase to seco-DOPA (rearranged to betalamic acid) (Fig. 7).
A tyrosinase activity closely linked to the biosynthesis of betalamic acid and muscaflavin has been characterized in *Amanita muscaria* (Mueller et al., 1996); this tyrosinase was not specific for tyrosine but has higher affinity and lower Km value (0.3 mM) for this compound compared to analogs; the protein is an heterodimer with a molecular weight ~ 50’000 kDa. It is strictly localized in the colored part of the fungus where DOPA and betalains accumulate and not found anywhere else. In plants, a PPO transcript correlating with betalain biosynthesis was found in *Phytolacca americana* fruits (Joy et al., 1995). Recently, a plant tyrosinase involved in betalain synthesis has also been isolated from callus cultures of *P. grandiflora* and *B. vulgaris* (Steiner et al., 1999, Steiner et al., 1996); this enzyme has a molecular weight of 53’000 kDa. It was shown that both the fungal and plant PPOs are a truly bifunctional enzymes producing not only DOPA but also able to use DOPA as a substrate. A bifunctional PPO catalyzing the hydroxylation and oxidation of chalcones to aurones has also been found in flowers of the anthocyanin producing plant *Anthirrhinum majus* (Sato et al., 2001, Nakayama et al., 2000). A putative tyrosine-hydroxylase activity from betacyanin producing callus cultures from *Portulaca grandiflora* was separated from a polyphenol-oxidase activity (Yamamoto et al., 2001). The purified enzyme catalyzed the formation of DOPA from tyrosine and was activated by Fe^{2+} and Mn^{2+}, and inhibited by metal chelating agents. Unfortunately the authors didn’t give details about the

substrate specificity of this enzyme and therefore the question of the existence of a plant tyrosine hydroxylase similar to the mammalian enzyme remains open.

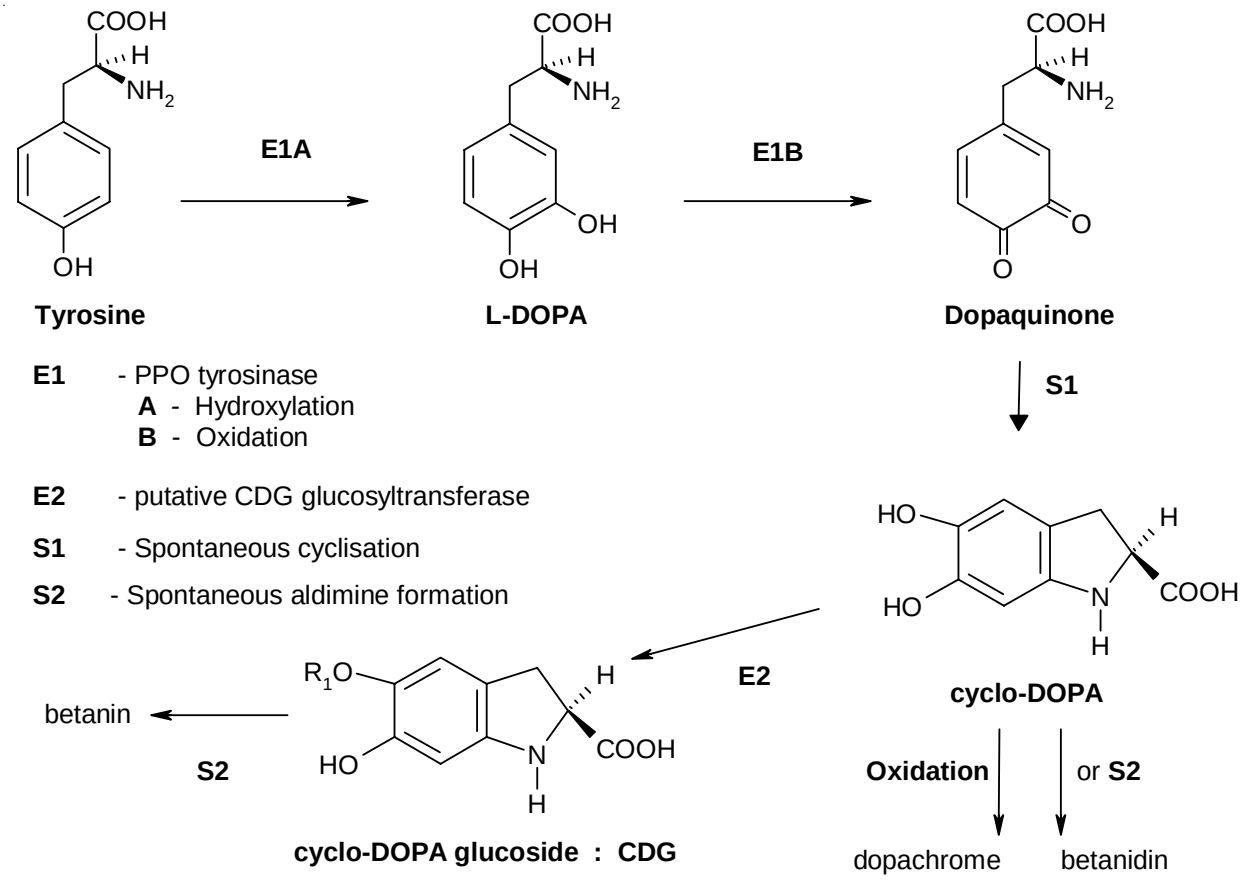


Figure 7 The PPOs reactions and the possible fates of the unstable cyclo-DOPA

The ring opening reaction

The fungal (*Amanita muscaria*) enzyme

Betalamic acid is formed through an enzymatic cleavage of the DOPA aromatic ring at the position 4,5; this produce an unstable seco-DOPA intermediate (Fischer and Dreiding, 1972, Terradas and Wyler, 1991b) which spontaneously close on itself (Schliemann et al., 1998). In *Amanita muscaria* an active DOPA extradiol -dioxygenase catalyze this extradiol cleavage both in 4,5 and 2,3 position (Terradas and Wyler, 1991a, Girod and Zryd, 1991a). Depending of the pH, part of the substrate undergo a 2,3 cleavage leading to the formation of the fungal pigment muscaflavin (see figure 8). The purified enzyme is an iron containing homo-multimeric protein with a 22'000 kDa subunit. This enzyme do not show a strict specificity toward DOPA (Km = 3.9 mM) and is able to use caffeic acid (Km = 0.9 mM), dopamine (Km = 6.3 mM) or catechol as substrates. The gene for this enzyme has been isolated (Hinz et al., 1997); this *DODA* gene codes for a 228 amino acid protein which shows no homology with any sequence yet published; the closest matches at the protein level are sequences from *Burkholderia fungorum, Botrytis cinerea* (ESTs) and *Nostoc sp.* (complete sequencing). The recombinant enzyme displays Km et Vmax values close to those of the native enzyme and is also able to produce both betalamic acid and muscaflavin (Mueller et al., 1997b).

Interestingly, the fungal enzyme is able to complement the betalain pathway in *P. grandiflora cc* genotypes deficient in the gene *C*, that was postulated to code for a plant ring-opening

dioxygenase (Mueller et al., 1997a). This successful complementation was obtained by biolistic transformation of petals using a pNco DODA vector; the construct being the *DODA* cDNA under the control of a CaMV 35S promoter and terminator cloned into a pUC18 vector. The main pigments found in transformed, colored petal cells were dopaxanthin, betanin and miraxanthin V, depending of the genetic background; muscaflavin specific to fungi and normally absent from plants (Barth et al., 1979) was also found, showing that the fungal enzyme was working properly in a plant cell environment retaining its full catalytic properties.

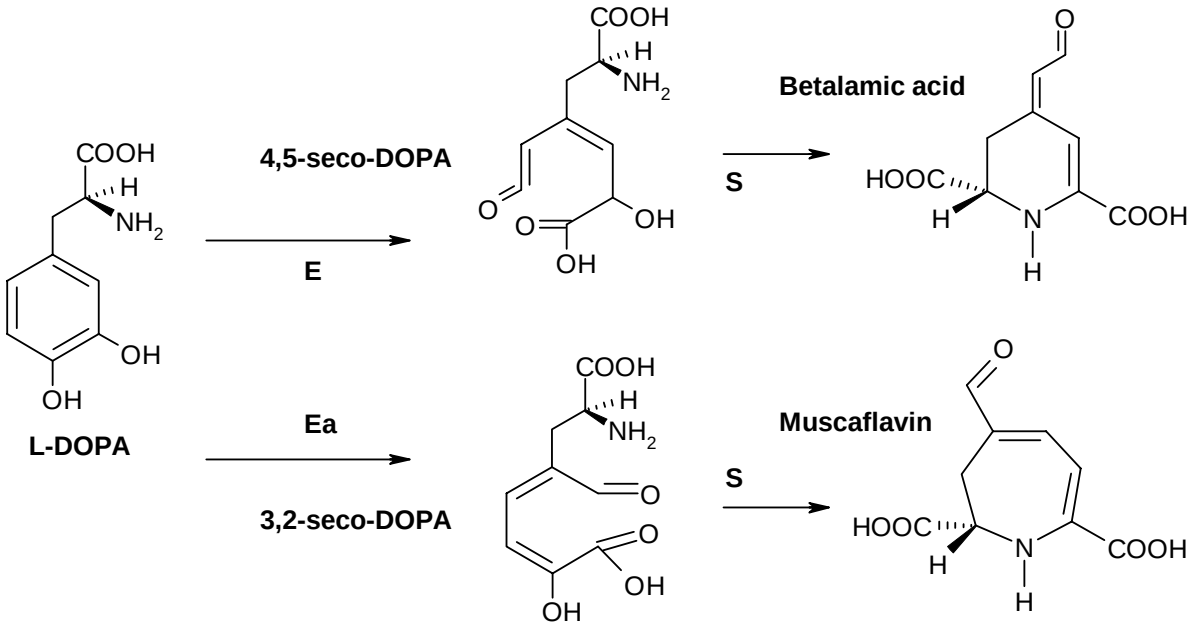


Figure 8 : The DOPA 4,5 and 3,2 extradiol ring-opening reactions in betalain containing plants and in the fungus *Amanita muscaria* and the spontaneous cyclization products

The plant (*Portulaca grandiflora*) enzyme

The complementation experiments performed on *Portulaca grandiflora* with the *Amanita* enzyme confirmed simultaneously that the plant dioxygenase, could be expected to be different from the fungal enzyme and probably totally unrelated to it. The plant enzyme escaped all isolation attempts; despite the effort of many research groups no enzymatic activity was ever detected in plants.

Based on the availability of *Portulaca grandiflora* inbred lines (Trezzini and Zryd, 1990), subtractive libraries where constructed in order to isolate directly plant genes associated with betalain biosynthesis (Zaiko, 2000). After PCR-Select® cDNA subtraction, betalain-specific transcripts present in yellow or violet flowered (*C*-) genotypes and not in white (*cc*) flowered genotypes where obtained. Putative interesting transcripts where selected for their strong expression during the early stages of flower development when betalain rate of synthesis is at its maximum. Corresponding full-length cDNAs where obtained using RACE amplification strategy. A potentially interesting candidate showing strong homology at the level of translated protein with the LigB domain present in the bacteria *Sphingomonas paucimobilis* protocatechuate extradiol 4,5- dioxygenase was identified (Christinet et al., 2004). This single copy gene was expressed only in colored flower petals and stem epidermal cells and not in non pigmented plant tissues. The function of this gene in the betalain biosynthetic pathway was

confirmed by using biolistic genetic complementation of *P. grandiflora* petals (Mueller et al., 1997a).The recombinant iron protein has yet to be produced.
This gene named *DODA* is the first characterized member of a novel family of plant non-heme dioxygenases phylogenetically distinct from *Amanita* DOPA-dioxygenase; not only the DODA protein shares no homology with the fungal protein but its catalytic activity is that of a genuine 4,5- extradiol dioxygenase with no 2,3- activity. Homologues of DODA are present not only in betalain producing plants but also, albeit with some changes near the catalytic site, in other angiosperms and in the bryophyte *Physcomitrella patens*. These homologues are part of a novel conserved plant gene family that could be involved in aromatic compound metabolism. Due to the broad distribution of sequences similar to the *P. grandiflora* DODA across terrestrial plants it can be hypothesized that the betalain pathway originated by recruitment of an existing metabolic pathway compensating for loss(es) in the capacity of synthesizing colored anthocyanins in plants of the order Caryophyllales. A conserved amino-acid motif (HNL-R/G) part of the catalytic site is present in the DODA like protein of all organisms tested except in plants synthesizing betalains. In these plants, a completely different conserved motif is present: the highly conserved catalytic amino acid His177 is followed by the pattern P-(S,A)-(N,D)-x-T-P. Both motifs begin with the strictly conserved His177, which is essential for the catalytic activity of extradiol dioxygenase class III enzyme like *Portulaca* DODA (Sugimoto et al., 1999). 3D-modeling of the enzyme based on the crystal structure from the protocatechuate 4,5-dioxygenase LigAB, revealed that the amino acids following His177 control the access of the substrate. The *Sphingomonas* enzyme involved in lignin degradation use a substrate with a lateral chain shorter than that of the DOPA molecule specific for DODA enzyme from *P. grandiflora* (Fig 9).

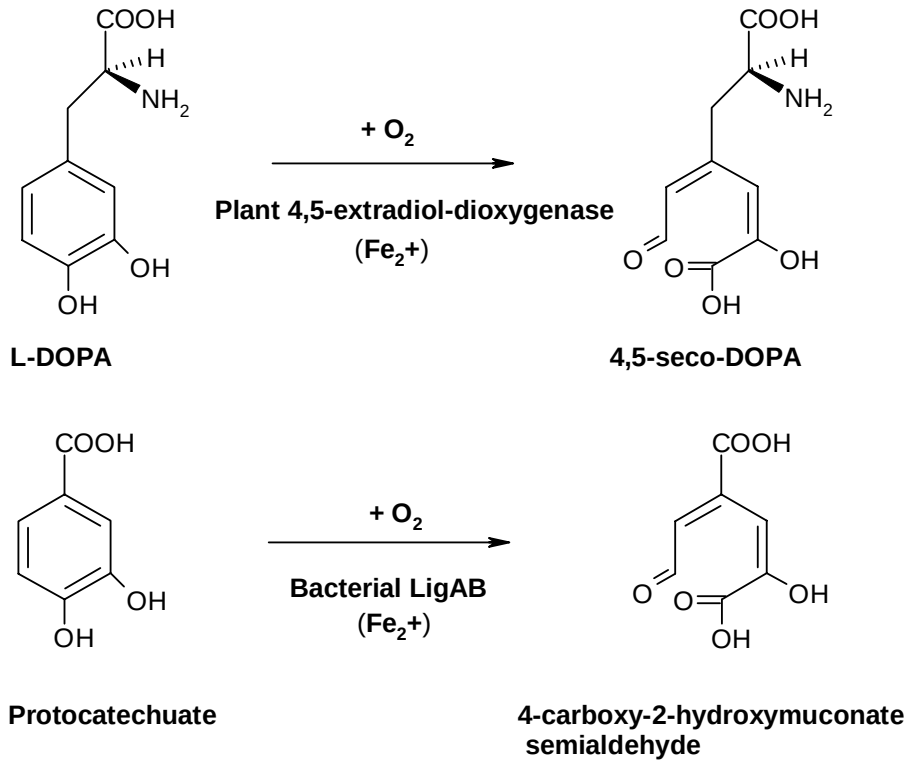


Figure 9 : The two homologues reactions catalyzed by the plant DOPA 4,5-extradiol-dioxygenase and by the protocatechuate 4,5-extradiol-dioxygenase from the bacteria *Sphingomonas paucimobilis*

It is worth remembering here that, in Fabaceae, where DOPA is present at high concentration (5% in the seeds from the genera *Mucuna* and *Stizolobium* (Yang et al., 2001)) another DOPA ring-cleavage activity is present; those plants have been shown to contain stizolobic or

stizolobinic acid (Saito and Komamine, 1978). The synthesis of those compounds needs a supplementary reduction step that a classical DOPA-dioxygenase does not catalyze alone. Both compounds are naturally present in *Amanita* (Saito and Komamine, 1978).

Glycosylation and other modifications

Most betacyanins are obtained by a final step involving the 5- or 6-*O*-glucosylation of betanidin or isobetanidin (Kobayashi et al., 2001) (Fig. 10). Two distinct enzymes have been isolated from *Dorotheanthus bellidiformis* cell suspensions (Heuer et al., 1996, Vogt et al., 1997). Those enzymes catalyze the indiscriminate transfer of glucose from UDP-glucose to hydroxyl groups of betanidin, flavonols, anthocyanidins and flavones. Sequence comparison shows that these enzymes have only 19% amino acid sequence identity, suggesting a paraphyletic origin of these two glucosyltansferases (GT). Their kinetic properties are similar to other known GTs related to flavonoid biosynthesis (Vogt et al., 1997).

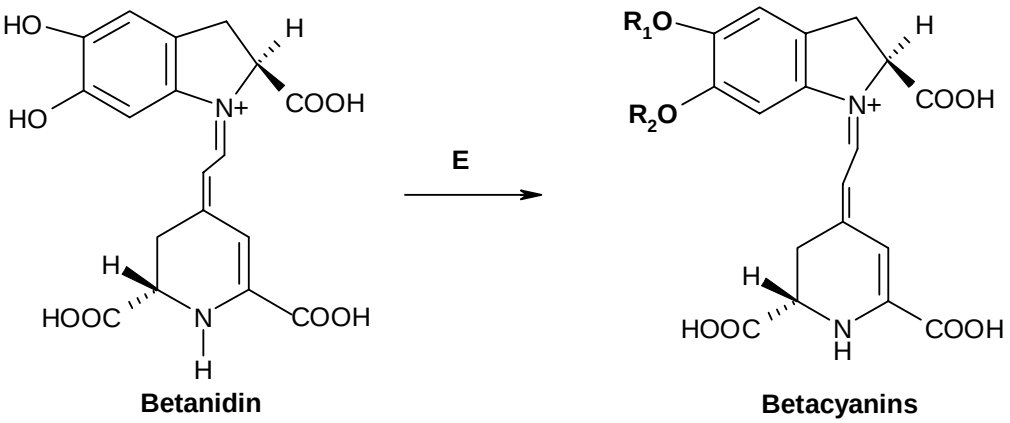


Figure 10: The formation of betanins from betanidin through the action of 5-*O*- and 6-*O*-glucosyl-transferases

It was suggested that cyclo-DOPA glycoside, observed at trace level only, is an artifact that originates from the hydrolysis of betanin (Strack et al., 2003) and that glycosylation occurs at a later step, on betanidin itself by mean of specific GTs. Nevertheless, labeled cyclo-DOPA glycoside is formed in red beet cell culture after addition of radiolabeled tyrosine or DOPA to the culture medium (Zryd et al., 1982, Bauer, 2001); this would indicate that glucosylation occurs before the condensation step with betalamic acid. Careful compartmental kinetic analysis using ^{3}H labeled tyrosine feeding of *Beta vulgaris* cell suspensions showed that glucosylated cyclo-DOPA accumulates before its incorporation into betanin. Rate constant for the formation of cyclo-DOPA glucoside from DOPA is 0.005 s^{-1} whether the rate constant for the formation of betanin is only 0.0029 s^{-1}(Bauer, 2001). A fast and early glycosylation of cyclo-DOPA would make sense in view of the well known instability of this molecule. We already know that DOPA is oxidized to DOPA-quinone which itself spontaneously rearrange into 5, 6- dihydroxyindole-2-carboxylate (cyclo-DOPA); this unstable compound could either be further oxidized or stabilized by glycosylation (see fig 7). In intact, not-wounded, plant cells neither cyclo-DOPA neither pigmented (black) oxidation compounds have ever be detected. The violet phenotypes of *Beta vulgaris* cell cultivated in vitro are very sensitive to mechanical stress and cell disruption and become rapidly black if wounded, this is not the case with the yellow phenotypes which do not

synthesize cyclo-DOPA. It seems therefore that rapid glucosylation of cyclo-DOPA to CDG could protect cells against damages from oxidation products. The question of the place of the glucosylation step(s) in betanin biosynthesis remains open to further investigations.
Celosia argentea var. plumosa contains in addition to the known compounds amaranthin and betalamic acid, three yellow pigments that are immonium conjugates of betalamic acid with dopamine, 3-methoxytyramine and (S)-tryptophan and 2-descarboxy-betanidin, a dopamine-derived betacyanin (Schliemann et al., 2001)
Betanins are, like flavonoids, frequently acylated via an ester linkage to the sugar moiety. An acyltransferase from cell cultures of *Chenopodium rubrum* was purified (Bokern et al., 1992). This enzyme catalyzes the transfer of hydroxycinnamic acids from 1-0-hydroxycinnamoyl-beta-glucose to the C-2 hydroxy group of glucuronic acid of amaranthin (betanidin 5-*O*-glucuronosylglucose). The in vivo products formed are celosianin I (4-coumaroylamaranthin) and celosianin II (feruloylamaranthin).

DOPA decarboxylation

Dopamine-derived betalains like the miraxanthin V or the 2-descarboxy-betanin need a supplementary decarboxylation step of the DOPA into dopamine (Dunkelbl et al., 1972, Kobayashi et al., 2001). Protein extract from red beet revealed the presence of a decarboxylase, transforming DOPA into dopamine, thus supporting the synthesis of dopamine-derived betacyanins as observed previously (Terradas, 1989).

Degradation

In vivo betalain degradation albeit an important factor in the maintenance of betalain steady state and in the recycling of nitrogen has been somehow a neglected field. Some of our knowledge comes from «in vitro» or post-harvesting studies (Cai and Corke, 2001). It is known that betanin can be degraded spontaneously into betalamic acid and cyclo-DOPA-5-O-b-D-glucoside (Schwartz and von_Elbe, 1983); this could happen when cells are damaged but also under normal condition. Degradation of betacyanin is reversible and could be accompanied by synthesis of betaxanthins in presence of the proper amine. Degradation of betanin to betanidin is probably catalyzed by a b-glucosidase (Zakharova and Petrova, 2000).
An enzyme catalyzing the discoloration and breakdown of betacyanins was isolated and purified from beet hypocotyls *Beta vulgaris* (Soboleva et al., 1976). The enzyme activity induced the oxidative discoloration of betanin and betanidin. The enzyme is membrane associated, degrades pigments in presence of oxygen and is inhibited by chelating agents and could well be a PPO. Peroxidases (EC 1.11.1.7) have been suspected to be involved in betalain degradation (Martinez-Parra and Munoz, 2001). A protein fraction with peroxidase activity against guaiacol from *Beta vulgaris* L. hypocotyls oxidized both betanidin and betanin. Betanidin quinone was formed as the only product in the course of enzymatic betanidin oxidation, whereas betalamic acid and several oxidized cyclo-DOPA-5-*O*-b-D-glucoside polymers were generated during the oxidation of betanin; the apparent Km for the reaction was 0.46 mM.

Evolution of betalains

Betalains replace anthocyanins in most families of the order Caryophyllales (Clement and Mabry, 1996), except in the families Caryophyllaceae and Molluginaceae where anthocyanins are still exclusively present (Table 4). Mutual exclusion of betalains and anthocyanins pigments (Wyler and Dreiding, 1961) and their respective biosynthetic pathways (Kimler et al., 1971) provides the main chemotaxonomic criterion to differentiate Chenopodiaceae from Caryophyllaceae.
Betalains have also been identified in the pilei from a very restricted number of basidiomycetes species belonging to *Amanita* and *Hygrocybe genera* (Döpp and Musso, 1973b, von Ardenne et

al., 1974).
Betalains and anthocyanins have a similar localization in plant and both use a large spectra of color which is part of the attraction syndrome toward insects, mammals and birds involved in pollination and seed dispersal (Clement and Mabry, 1996). The origin of betalains in plant is a fascinating question. The biological role of betalain in fungal species is unknown.

Suborder	Family	Examples of genus
Chenopodiineae Betalain-producing Anthocyanin-free taxa	Achatocarpaceae Aizoaceae Amaranthaceae Basellaceae Cactaceae Chenopodiaceae Didiereaceae Halophytaceae Hectorellaceae Nyctaginaceae Phytolaccaceae Portulacaceae Stegnospermataceae	*Achatocarpus* *Dorotheanthus, Mesembryanthemum, Carpobrotus* *Amaranthus, Iresine, Gomphrena* *Basella* *Mammillaria, Opuntia, Pereskia* *Beta, Chenopodium, Spinacia* *Didierea* *Halophytum* *Hectorella* *Bougainvillea, Mirabilis* *Phytolacca, Gisekia* *Portulaca, Claytonia* *Stegnosperma*
Caryophyllineae Betalain-free Anthocyanin-producing taxa	Caryophyllaceae Molluginaceae	*Dianthus, Silene* *Mollugo, Limeum*

Table 4: Caryophyllales and the distribution of betalain and anthocyanin producing taxa

Ehrendorfer (Ehrendorfer, 1976) was the first to propose a general hypothesis for the presence of betalains in Caryophyllales. Ancestral group of plants may have evolved under environmental conditions (drought) that could have induce a preference for anemochory (wind pollination) and in the same time the loss of insect attracting capability (pigmentation). It is remarkable that the majority of today betalain containing plants are mostly drought and salt resistant. Subsequently the acquisition of betalain type pigments would have permitted a new evolution back to zoochory (animal linked pollination). In recent phylogenetic studies of the Caryophyllales (Cuenoud et al., 2002), it was shown that the Caryophyllaceae which possesses anthocyanins are closely related to the Amaranthaceae (Chenopodiaceae s. str. & Amaranthaceae s. str.) which synthesize betalains but are also known also for their inconspicuous flowers and for their predominant anemophily. Evolution of new type of highly colored flowers (in Portulacaceae, Cactaceae and others) from almost flower-less plants is also coherent with this hypothesis. Amaranthaceae have small sepals but petals are absent, the flowers of Cactaceae have numerous separate large and colorful tepals, the lower-most of which may appear sepal-like. Throughout most of the order Caryophyllales flowers have true sepals but no true petals. Some families that display what appear to be petals, such as Portulacaceae and Nyctaginaceae, are thought to have greatly modified sepals, in which case the sepal-like appendages are then interpreted as bracts. For a description of floral ontogeny in *Portulaca* see papers by Soetiart (Soetiart and Ball, 1969, Soetiart and Ball, 1968).
Substrate specificity for DOPA of the specific ring opening dioxygenase DODA should have occurred during the evolution of early betalain-synthesizing plants. Only an extensive analysis across the phylogenetic tree will answer the question of the point of divergence. We could expect to find a few plants with intermediate sequences, at the interface of betalain producing and non-producing plants, which have escaped current investigations; those plants could help to solve

some critical aspects of the molecular phylogeny of Caryophyllales (Cuenoud et al., 2002). Examination of the phylogenetic tree of DODA-type proteins (Christinet et al., 2004), puts Chenopodiaceae (*Beta vulgaris*) at the base of the branch of betalain producing plants. DODA-like proteins are present not only in plants but also in eubacteria and archea, they are notably absent from the fungal and animal kingdoms (Fig 11).

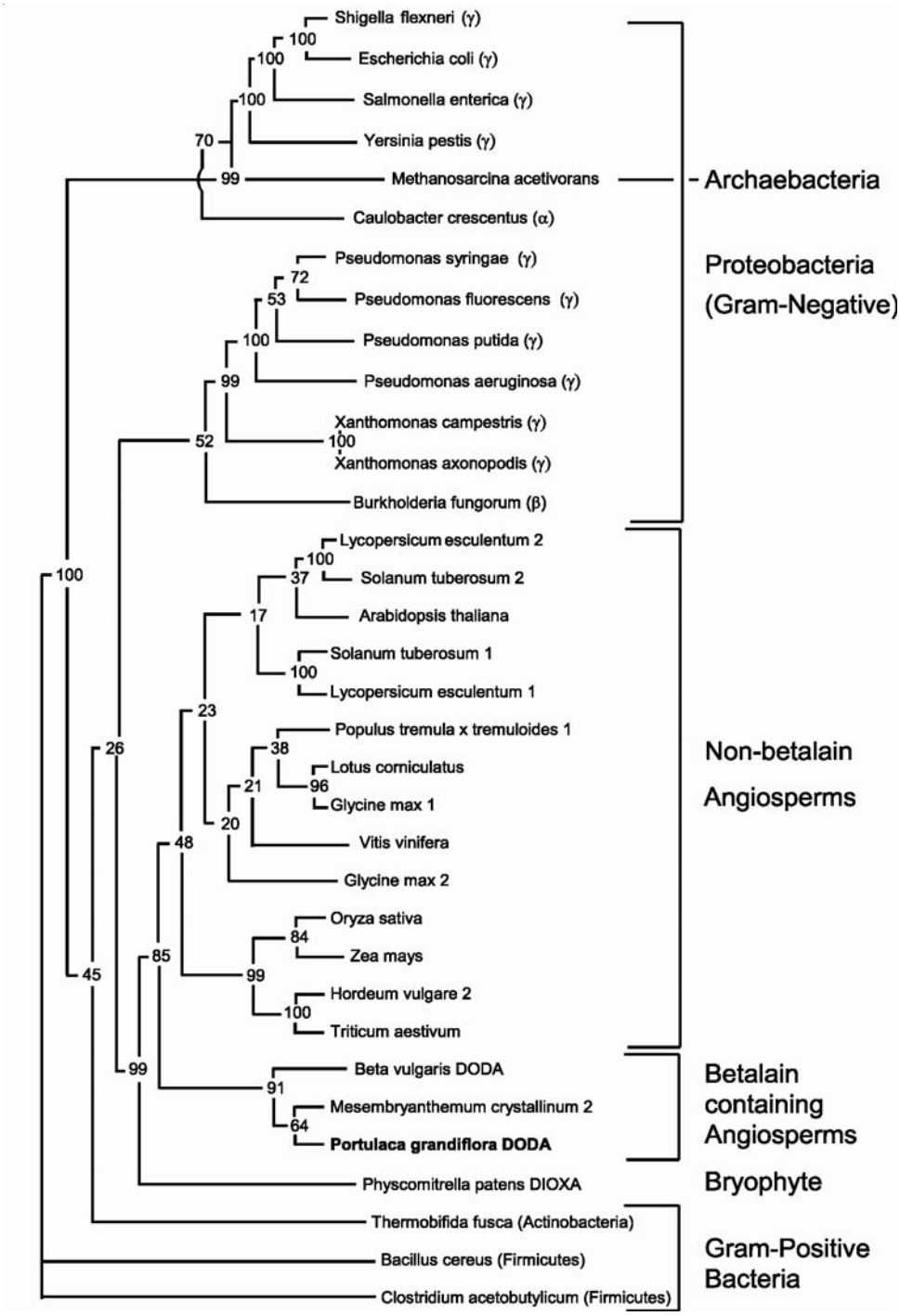


Fig 11 – Phylogeny analysis of *Portulaca grandiflora* DOPA-dioxygenase. This strict consensus phylogenetic tree has been build across all prokaryotic and eukaryotic genomes in which sequence homology was found (note the absence of animals and fungi). The bootstrap values at the forks indicate the number of times out of 100 trees that this grouping occurred.

Social and economic value

Due to their colorant properties, betalains can be used in various applications in the food and agro industries. People are more often in contact with betalains than thought, while eating red beet or strawberry yoghourt colored with beet juice, admiring cactus or bougainvillea flowers. Betacyanin producing plants, mainly in the Chenopodiaceae family (beet) and in the Amaranthaceae family (*Amaranthus paniculatus, Amaranthus hypochondriacus*), have been used traditionally as food, medicine and as well as cult items in sacred ceremonies. The Aztec have used red-violet amaranths leaves or seed heads (.huauhtli in classic nahuatl) to symbolize human blood during sacrificial ceremonies or to make colored (bleeding) idols either as a substitute for human sacrifice or simply as representation of harvest gods; in a apparently paradoxical way this could be related with the use of red wine (anthocyanidins) as representation of Christ blood in the Christian Eucharist. In fact the use of Amaranthus as food crop was eventually forbidden by the Catholic Church and it is only recently that cultivation and use of this plant has resumed in Central America. In Eastern Europe red beet species were not related directly to specific religious ceremony; nevertheless red beet soup (borsch) fermented or not is often offered in special occasions (Christmas, Jewish celebrations - Pesach etc.). On the negative side, it has been reported in an ethnological study in Sri Lanka, that red colored food, including red beet, are considered as inappropriate for pregnant women (De Silva, 1996). As the beginning of twentieth century, red beet hypocotyl (root) was highly valued as a remedy against cancer, those belief have been partly confirmed recently (Kapadia et al., 1998, Kapadia et al., 1996, Wettasinghe et al., 2002).

The betaxanthin colored fungus *Amanita muscaria* is used by Siberian shaman as a potent healing drug which is not given to the patient but used by the medium (Eliade, 1968) . The hallucinogenic properties of *A. muscaria* are not related to its betalain content but to its ibotenic acid and muscimol content. It is possible nevertheless that its attractive, unique pattern of color was a major factor in its use as a revered and popular medicine.

In Europe, Russia and Northern America the main betalain containing plant with an economic value is red beet. Beet has been cultivated in Europe since the antiquity. Today it is used either as a salad (cooked or raw) in Western Europe or as a basic soup ingredient (borsch) in Poland and Russia; In USA red beet is sold mainly canned. The use of raw or refined beet extract as a coloring food additive represent, in EU (with France as the major producer), a market of more than 10 million € for a production of 4000 tons a year. Red beet extract (N° C.E.E.: E162) is used mainly in dairy food products, confectionery, jams, sausages, pâté and cannery.

Among other interesting food products, the juices from cactus (*Opuntia ficus-indica, Myrtillocactus geometrizans, Hylocereus polyrhizus*) are a readily available source of various betalain pigments (Stintzing et al., 2001). The fact that those plants are adapted to semi-arid regions adds a further value to this source of betalains in the perspective of sustainable development. It has been suggested that, through the application of the relatively simple technique of semi-synthesis, betaxanthins can be used as a mean to introduce essential amino-acids into food giving rise to «essential dietary colorant» (Leathers et al., 1992).

In horticulture there is no betalain containing plants in the top cultivated species; nevertheless, there is a large amateur market for Cactaceae and various species of *Bougainvillea, Mirabilis, Phytolacca, Portulaca, Mesembryanthemum, Amaranthus, Iresine* (*Celosia*) and *Beta* are widely used as ornamentals.

Prospectives

Considering the actual state of the art it is now possible to use recombinant DODAs (either from *Amanita* or from *Portulaca*) to boost the level of betalamic acid in order to increase pigment content either for ornamental or nutritional purposes. The limiting factor will probably

be DOPA. The general availability of amino acid and other amines will potentially allows flux toward the synthesis of betaxanthins. The limiting factor in this case would probably be the vacuolar transport system. For betacyanin synthesis there is a strong requirement for cyclo-DOPA (or cyclo-DOPA-glucoside). We should realize that unfortunately we are still far from a clear understanding of those specific steps (corresponding to the *R* and *I* gene locus in *Portulaca grandiflora*); nevertheless transformation experiments can and should be done with the DODA containing vectors as we know already that their products are correctly expressed in competent cells. As long as enough DOPA is present, DODA could be used as colored reporter molecule in transgenic plants (either as a transient marker or as an integrated one) and other organisms. It has the advantage to be non-toxic and to be detectable in vivo by simple spectroscopic means or microscopic multispectral analysis (Fernandez et al., 1995). It could be used for the «in situ» quantitative analysis of DOPA, not only in plant cells but also in animal cells and specifically in the nervous system. DODA genes and DODA-like genes of the same family are now at the reach of interested investigators; studies on the characteristics of those genes and their evolutionary relationship will certainly help us in designing new strategies in the field of the biotechnology of aromatic compounds.
Among other betalain specific enzyme glucosyl transferases have been characterized enough to be potentially useful (Vogt et al., 1999a). If a suitable PPO can be engineered to produce DOPA, the whole betalain pathway will be available for optimization and expression in new plants.
To complete the picture of today situation we could add that P. grandiflora transformation is possible with some further optimization (Rossi-Hassani et al., 1995) and that transgenic beet have already been produced (Jacq et al., 1993, Dhalluin et al., 1992, Lindsey and Gallois, 1990) and tested in the field (Meier and Wackernagel, 2003). Large collections of EST are available for Beta vulgaris (Bellin et al., 2002) and Mesembryanthemum crystallinum (Andolfatto et al., 1994) for gene identification and isolation; in the case of B. vulgaris, the Gabi-beet program (http://www.mips.biochem.mpg.de/proj/gabi/projects/gabibeet.htm) has put among other important gene to be mapped the R gene for red hypocotyl.

Aknowledgments

We would like to dedicate this review to all previous members of the Laboratory of Plant Cell Genetics of the University of Lausanne, whose dedication, hard work and enthusiasm have significantly contributed to our understanding of the betalain biosynthetic pathway. Some of their work have been published in peer-reviewed journals, some in PhD thesis, some in difficult to access congress reports; in any case all pieces of this puzzle where needed to get a better picture of this fascinating biological enigma.

Bibliography

Adachi, T. (1972) ***Bull Lab Plant Breed Miyazaki University, Japan,*** 4, **1-98.**

Andolfatto, P., Bornhouser, A., Bohnert, H. J. and Thomas, J. C. (1994) ***Physiologia Plantarum,*** 90, **708-714.**

Austin, D. and Goldman, I. L. (2001) ***Journal of the American Society for Horticultural Science,*** 126, **340-343.**

Barth, H., Kobayashi, M. and Musso, H. (1979) ***Helvetica Chimica Acta,*** 62, **1231-1235.**

Bauer, J. A.(2001) ***Etude des voies métaboliques conduisant à la bétanine dans des cultures de «Beta vulgaris L.» par incorporation de tyrosine et de dihydroxyphenylalanine*** **Thesis: Université de Lausanne, pp. 198.**

Bellin, D., Werber, M., Theis, T., Schulz, B., Weisshaar, B. and Schneider, K. (2002) ***Plant Biology,*** 4, **700-710.**

Biddington, N. and Thomas, T. H. (1973) ***Planta,*** 111, **183-186.**

Bigot, C. (1968) ***Comptes rendus hebdomadaires des séances de l'Académie des Sciences Série D,*** 266, **349-&.**

Bokern, M., Heuer, S. and Strack, D. (1992) ***Botanica Acta,*** 105, **146-151.**

Cai, Y., Sun, M. and Corke, H. (2001) ***Journal of Agricultural and Food Chemistry,*** 49, **1971-8.**

Cai, Y. Z. and Corke, H. (2001) ***Journal of Food Science,*** 66, **1112-1118.**

Christinet, L., Burdet, F., Zaiko, M., Hinz, U. and Zryd, J. P. (2004) ***Plant Physiology,*** 134, **in press.**

Clement, J. S. and Mabry, T. J. (1996) ***Botanica Acta,*** 109, **360-367.**

Cuenoud, P., Savolainen, V., Chatrou, L. W., Powell, M., Grayer, R. J. and Chase, M. W. (2002) ***American Journal of Botany,*** 89, **132-144.**

De Silva, W. I. (1996) ***The Journal of Family Welfare,*** 41, **18-26.**

Dhalluin, K., Bossut, M., Bonne, E., Mazur, B., Leemans, J. and Botterman, J. (1992) ***Bio-Technology,*** 10, **309-314.**

Döpp, H. and Musso, H. (1973a) ***Naturwissenschaften,*** 60, **477-478.**

Döpp, H. and Musso, H. (1973b) ***Chemische Berichte,*** 106, **3473-3482.**

Dunkelbl, E., Dreiding, A. S. and Miller, H. E. (1972) ***Helvetica Chimica Acta,*** 55, **642-&.**

Eagen, K. A. and Goldman, I. L. (1996) ***Molecular Breeding,*** 2, **107-115.**

Ehrendorfer, F. (1976) ***Plant. Syst. Evol.,*** 126, **99-105.**

Eliade, M. (1968) ***Le chamanisme et les techniques archaïques de l'extase,*** **Payot, Paris.**

Endress, R., Jager, A. and Kreis, W. (1984) ***Journal of Plant Physiology,*** 115, **291-295.**

Fernandez, G., Kunt, M. and Zryd, J.-P. (1995) In ***Workshop on Physics-Based Modeling in Computer Vision*** **Cambridge, USA, pp. 166-172.**

Fischer, N. and Dreiding, A. S. (1972) ***Helvetica Chimica Acta,*** 55, **649-&.**

Girod, P. A. and Zryd, J. P. (1987) ***Plant Cell Reports,*** 6, **27-30.**

Girod, P. A. and Zryd, J. P. (1991a) ***Phytochemistry,*** 30, **169-174.**

Girod, P. A. and Zryd, J. P. (1991b) ***Plant Cell Tissue and Organ Culture,*** 25, **1-12.**

Giudici De Nicola, M., Amico, V., Sciuto, S. and Piattelli, M. (1975) ***Phytochemistry,*** 14, **479-481.**

Goldman, I. L. and Austin, D. (2000) ***Theoretical and Applied Genetics,*** 100, **337-343.**

Guggenheim, M. (1913) ***Z. Physiol. Chem.,*** 88, **276–284.**

Heuer, S., Richter, S., Metzger, J. W., Wray, V., Nimtz, M. and Strack, D. (1994) ***Phytochemistry,*** 37, **761-7.**

Heuer, S., Vogt, T., Bohm, H. and Strack, D. (1996) ***Planta,*** 199, **244-250.**

Heuer, S., Wray, V., Metzger, J. W. and Strack, D. (1992) ***Phytochemistry,*** 31, **1801-1807.**

Hinz, U. G., Fivaz, J., Girod, P. A. and Zryd, J. P. (1997) ***Molecular & General Genetics,*** 256, **1-6.**

Hooker, W. J. (1829) In ***Curti's Botanical Magazine*****, Vol. III, pp. 2885.**

Ibdah, M., Krins, A., Seidlitz, H. K., Heller, W., Strack, D. and Vogt, T. (2002) ***Plant Cell and Environment,*** 25, **1145-1154.**

Ikeno, S. (1921) ***Journal of the College of Agriculture, Imperial University Tokyo,*** 8, **93-133.**

Impellizzeri, G., Piattelli, M. and Sciuto, S. (1973) ***Phytochemistry,*** 12, **2293-2294.**

Jacq, B., Lesobre, O., Sangwan, R. S. and Sangwannorreel, B. (1993) ***Plant Cell Reports,*** 12, **621-624.**

Joy, R. W., Sugiyama, M., Fukuda, H. and Komamine, A. (1995) ***Plant Physiology,*** 107, **1083-1089.**

Kapadia, G. J., Tokuda, H., Konoshima, T. and Nishino, H. (1996) ***Cancer Letters,*** 100, **211-4.**

Kapadia, G. J., Tokuda, H., Sridhar, R., Balasubramanian, V., Takayasu, J., Bu, P., Enjo, F., Takasaki, M., Konoshima, T. and Nishino, H. (1998) ***Cancer Letters,*** 129, **87-95.**

Kimler, L., Larson, R. A., Messenge.L, Moore, J. B. and Mabry, T. J. (1971) ***Journal of the Chemical Society D - Chemical Communications,*** **1329-&.**

Kishima, Y., Shimaya, A. and Adachi, T. (1995) ***Plant Cell Tissue and Organ Culture,*** 43, **67-70.**

Kobayashi, N., Schmidt, J., Nimtz, M., Wray, V. and Schliemann, W. (2000) ***Phytochemistry,*** 54, **419-26.**

Kobayashi, N., Schmidt, J., Wray, V. and Schliemann, W. (2001) ***Phytochemistry,*** 56, **429-36.**

Kubota, S., Imamura, H., Hisamatsu, T. and Koshioka, M. (1999) ***Journal of Plant Physiology,*** 155, **133-135.**

Leathers, R. R., Davin, C. and Zryd, J. P. (1992) ***In Vitro Cellular & Developmental Biology - Plant,*** 28P, **39-45.**

Liebisch, H. W. and Bohm, H. (1981) ***Pharmazie,*** 36, **218-218.**

Lindsey, K. and Gallois, P. (1990) ***Journal of Experimental Botany,*** 41, **529-536.**

Mabry, T. J. (2001) ***Journal of Natural Products,*** 64, **1596-604.**

Martinez-Parra, J. and Munoz, R. (2001) ***Journal of Agricultural and Food Chemistry,*** 49, **4064-8.**

Meier, P. and Wackernagel, W. (2003) ***Transgenic Research,*** 12, **293-304.**

Miller, H. E., Rosler, H., Wohlpart, A., Wyler, H., Wilcox, M. E., Frohofer, H., Mabry, T. J. and Dreiding, A. S. (1968) ***Helvetica Chimica Acta,*** 51, **1470-&.**

Minale, L. (1967) ***Phytochemistry,*** 6, **703-&.**

Mueller, L. A., Hinz, U., Uze, M., Sautter, C. and Zryd, J. P. (1997a) ***Planta,*** 203, **260-263.**

Mueller, L. A., Hinz, U. and Zryd, J. P. (1996) ***Phytochemistry,*** 42, **1511-1515.**

Mueller, L. A., Hinz, U. and Zryd, J. P. (1997b) ***Phytochemistry,*** 44, **567-569.**

Nakayama, T., Yonekura-Sakakibara, K., Sato, T., Kikuchi, S., Fukui, Y., Fukuchi-Mizutani, M., Ueda, T., Nakao, M., Tanaka, Y., Kusumi, T. and Nishino, T. (2000) ***Science,*** 290, **1163-1166.**

Piattelli, M., Giudici De Nicola, M. and Castrogiovanni, V. (1969) ***Phytochemistry,*** 8, **731-736.**

Piattelli, M. and Imperato, F. (1970) ***Phytochemistry,*** 9, **2557-&.**

Piattelli, M. and Minale, L. (1964) ***Phytochemistry,*** 3, **307-311.**

Piattelli, M., Minale, L. and Nicolaus, R. A. (1965a) ***Phytochemistry,*** 4, **817-823.**

Piattelli, M., Minale, L. and Nicolaus, R. A. (1965b) ***Rend. Accad. Sci. Fis. Mat. Napoli,*** 32, **55-56.**

Piattelli, M., Minale, L. and Prota, G. (1964) ***Tetrahedron,*** 20, **2325-&.**

Piattelli, M., Minale, L. and Prota, G. (1965c) ***Phytochemistry,*** 4, **121-125.**

Rast, D., Skrivanova, R. and Wohlpart, A. (1972) ***Ber. Schweiz. Bot. Ges.,*** 82, **213-222.**

Romanov, G. A., Getman, I. A. and Schmulling, T. (2000) ***Plant Growth Regulation,*** 32, **337-344.**

Rossi-Hassani, B. D., Bennani, F. and Zryd, J. P. (1995) ***Genome,*** 38, **752-756.**

Rossi-Hassani, B. D. and Zryd, J. P. (1994) ***Annales de Génétique,*** 37, **53-59.**

Rossi-Hassani, B. D. and Zryd, J. P. (1995) ***Annales de Génétique,*** 38, **90-96.**

Saito, K. and Komamine, A. (1978) ***Eur. J. Biochem.,*** 82, **385-392.**

Sakuta, M., Hirano, H., Kakegawa, K., Suda, J., Hirose, M., Joy, R. W., Sugiyama, M. and Komamine, A. (1994) ***Plant Cell Tissue and Organ Culture,*** 38, **167-169.**

Sato, T., Nakayama, T., Kikuchi, S., Fukui, Y., Yonekura-Sakakibara, K., Ueda, T., Nishino, T., Tanaka, Y. and Kusumi, T. (2001) ***Plant Science,*** 160, **229-236.**

Schliemann, W., Cai, Y., Degenkolb, T., Schmidt, J. and Corke, H. (2001) ***Phytochemistry,*** 58, **159-65.**

Schliemann, W., Joy, R. W., Komamine, A., Metzger, J. W., Nimtz, M., Wray, V. and Strack, D. (1996) ***Phytochemistry,*** 42, **1039-46.**

Schliemann, W., Kobayashi, N. and Strack, D. (1999) ***Plant Physiology,*** 119, **1217-1232.**

Schliemann, W., Steiner, U. and Strack, D. (1998) ***Phytochemistry,*** 49, **1593-1598.**

Schudel, G.(1919) ***Über Alkalischmelzen*** **Thesis: ETHZ, pp. 48.**

Schwartz, S. J. and von_Elbe, J. H. (1983) ***Zeitschrift Für Lebensmittel Untersuchung und Forschung,*** 176, **448-53.**

Sciuto, S., Oriente, G., Piattell.M, Impelliz.G and Amico, V. (1974) ***Phytochemistry,*** 13, **947-951.**

Sepúlveda Jiménez, G., Rueda Benítez, P., Porta, H. and Rocha Sosa, M. (2003) In ***7th International Congress of Plant Molecular Biology*** **Barcelona.**

Shin, K. S., Murthy, H. N., Ko, J. Y. and Paek, K. Y. (2002) ***Biotechnology Letters,*** 24, **2067-2069.**

Soboleva, G. A., Ul_ianova, M. S., Zakharova, N. S. and Bokuchava, M. A. (1976) ***Biokhimiia,*** 41, **968-74.**

Soetiart, S. and Ball, E. (1968) ***American Journal of Botany,*** 55, **715-&.**

Soetiart, S. and Ball, E. (1969) ***Canadian Journal of Botany,*** 47, **133-&.**

Steddom, K., Heidel, G., Jones, D. and Rush, C. M. (2003) ***Phytopathology,*** 93, **720-726.**

Steiner, U., Schliemann, W., Bohm, H. and Strack, D. (1999) ***Planta,*** 208, **114-124.**

Steiner, U., Schliemann, W. and Strack, D. (1996) ***Analytical Biochemistry,*** 238, **72-75.**

Stintzing, F. C., Schieber, A. and Carle, R. (2001) ***European Food Research and Technology,*** 212, **396-407.**

Stintzing, F. C., Schieber, A. and Carle, R. (2002) ***Journal of Agricultural and Food Chemistry,*** 50, **2302-7.**

Stobart, A. K. and Kinsman, L. T. (1977) ***Phytochemistry,*** 16, **1137-1142.**

Strack, D., Schmitt, D., Reznik, H., Boland, W., Grotjahn, L. and Wray, V. (1987) ***Phytochemistry,*** 26, **2285-2287.**

Strack, D., Vogt, T. and Schliemann, W. (2003) ***Phytochemistry,*** 62, **247-269.**

Sugimoto, K., Senda, T., Aoshima, H., Masai, E., Fukuda, M. and Mitsui, Y. (1999) ***Structure with Folding & Design,*** 7, **953-965.**

Suzuki, H., Nakayama, T., Yonekura-Sakakibara, K., Fukui, Y., Nakamura, N., Yamaguchi, M., Tanaka, Y., Kusumi, T. and Nishino, T. (2002) ***Plant Physiology,*** 130, **2142-2151.**

Terradas, F.(1989) ***Etude par voie enzymatique des métabolites des bétalaines dans Beta vulgaris et Amanita muscaria*** **Thesis: University of Lausanne, pp. 147.**

Terradas, F. and Wyler, H. (1991a) ***Helvetica Chimica Acta,*** 74, **124-140.**

Terradas, F. and Wyler, H. (1991b) ***Phytochemistry,*** 30, **3251-3253.**

Trezzini, G. F.(1990) ***Génétique des bétalaïnes chez Portulaca grandiflora Hook*** **Thesis: University of Lausanne, pp. 135.**

Trezzini, G. F. and Zryd, J. P. (1991a) ***Phytochemistry,*** 30, **1897-1899.**

Trezzini, G. F. and Zryd, J. P. (1991b) ***Phytochemistry,*** 30, **1901-1904.**

Trezzini, G. F. and Zryd, J.-P. (1990) ***Acta Horticulturae,*** 280, **581-585.**

Vogt, T., Grimm, R. and Strack, D. (1999a) ***Plant Journal,*** 19, **509-519.**

Vogt, T., Ibdah, M., Schmidt, J., Wray, V., Nimtz, M. and Strack, D. (1999b) ***Phytochemistry,*** 52, **583-92.**

Vogt, T., Zimmermann, E., Grimm, R., Meyer, M. and Strack, D. (1997) ***Planta,*** 203, **349-61.**

von Ardenne, R., Döpp, H., Musso, H. and Steiglich, W. (1974) ***Zeitschrift der Naturforschung,*** 29c, **637-639.**

Wettasinghe, M., Bolling, B., Plhak, L., Xiao, H. and Parkin, K. (2002) ***Journal of Agricultural and Food Chemistry,*** 50, **6704-6709.**

Wilcox, M. E., Wyler, H. and Dreiding, A. S. (1965) ***Helvetica Chimica Acta,*** 48, **1134-&.**

Wohlpart, A. and Mabry, T. J. (1966) ***Plant Physiology***, **R72-&.**

Wohlpart, A. and Mabry, T. J. (1968) ***Plant Physiology,*** 43, **457-&.**

Wolyn, D. J. and Gabelman, W. H. (1989) ***Journal of Heredity,*** 80, **33-38.**

Wybraniec, S., Platzner, I., Geresh, S., Gottlieb, H. E., Haimberg, M., Mogilnitzki, M. and Mizrahi, Y. (2001) ***Phytochemistry,*** 58, **1209-12.**

Wyler, H. and Dreiding, A. S. (1961) ***Experientia,*** 17, **23-&.**

Wyler, H., Mabry, T. J. and Dreiding, A. S. (1963a) ***Helvetica Chimica Acta,*** 46, **1745-1748.**

Wyler, H., Mabry, T. J. and Dreiding, A. S. (1963b) ***Helvetica Chimica Acta,*** 46, **1745-&.**

Yamamoto, K., Kobayashi, N., Yoshitama, K., Teramoto, S. and Komamine, A. (2001) ***Plant & Cell Physiology,*** 42, **969-75.**

Yang, X. H., Zhang, X. T. and Zhou, R. H. (2001) ***Pharmaceutical Biology,*** 39, **312-316.**

Yashi, K. (1920) ***Botanical Magazine Tokyo,*** 34, **55-65.**

Zaiko, M.(2000) ***Colour-specific genes from betalain producing plants*** **Thesis: Université de Lausanne, pp. 129.**

Zakharova, N. S. and Petrova, T. A. (2000) ***Applied Biochemistry and Microbiology,*** 36, **394-397.**

Zryd, J. P., Bauer, J., Wyler, H. and Lavanchy, P. (1982) In ***Proceedings of the 5th International Congress of Plant Tissue & Cell Culture*** **(Ed, Fujiwara, A.) Tokyo, pp. 387-388.**