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Review

Enzymes and genes involved in the betalain biosynthesis in higher plants

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Betalains, a class of water-soluble nitrogen-containing pigments, replace anthocyanins and serve the analogous functions in 13 families of the order, caryophyllales. They modulate the attractive appearance of plants and protect them against destructive oxidative damage. Their antioxidant roles, radical scavenging properties in human health and their potential uses in food and pharmaceutical industries have made significant progress achieved in the detection, purification, quantification, structure elucidation of betalains, and in particular in the understanding of biosynthetic pathways of the pigments, the enzymes and their genes involved in the pathways. In this paper, major progress in betalain biosynthesis and the enzymes and genes involved in the biosynthetic pathways in higher plant are reviewed, and the perspectives discussed.

Key words: Betalain, biosynthesis, tyrosinase, DOPA dioxygenase, glucosyltransferase, betacyanin, betaxanthin

INTRODUCTION

Plant pigments are generally classified into 4 major groups: anthocyanins, betalains, carotenoids and chlorophylls. Betalains, as anthocyanins, are responsible mainly for the attractive natural display of flower, fruit, and storage root colour. They are water-soluble, nitrogen containing pigments and comprise two groups of colour different pigments: the red-violet betacyanins and the yellow betaxanthins. The betacyanins are further divided into four sub-groups; and the betaxanthins, three groups (Strack et al., 2003) (Figure 1). Each subgroup contains several compounds except the subgroup, amaranthin in the betacyanins which has only one number (Figure 1).

Although being responsible for the attractive colour of flowers, fruits, and occasional vegetative tissues, betalains, unlike anthocyanins, are present only in 13 families of the order, caryophyllales and in some higher fungi such as fly agaric (*Amanita muscaria*) (Steglich and Strack, 1990; Strack et al., 2003; Grotewold, 2006; Moreno et al., 2008). More interestingly, betalains are mutually exclusive with anthocyanins in the angiosperms, and the betalains and anthocyanins have never been observed in the same plants (Mabry and Dreiding, 1968; Kimler et al., 1970; Clement and Mabry, 1996; Lee and Collins, 2001; Strack et al., 2003; Stintzing and Carle, 2004a, Cai et al., 2005; Grotewold, 2006; Moreno et al., 2008), for which the reason remains mysterious.

In natural plant, betalains play important roles in physiology, optical attraction for pollination and seed dispersers (Piattelli, 1981). They also function as reactive oxygen species (ROS) scavengers, protect plants from damages caused by wounding and bacterial infiltration as seen in red beet (*Beta vulgaris* subsp. *vulgaris*) (Sepúlveda-Jiménez et al., 2004), function as UV-protecter in ice plant (*Mesembryanthemum crystallinum*) (Vogt et al., 1999a). In human sociality, since synthetic dyes are becoming more and more critically assessed by the consumer, betalains, as food additives, receive more and more attention with the growing interest in the use of natural pigments for food colouring. As food additives, in particular as low acid food additives, betalains do not only improve food appearance, but also contribute to consumer health. It has been reported that betalains are a class of compounds with antioxidant and radical scavenging properties (Escribano et al., 1998; Pedreño and Escribano, 2000;

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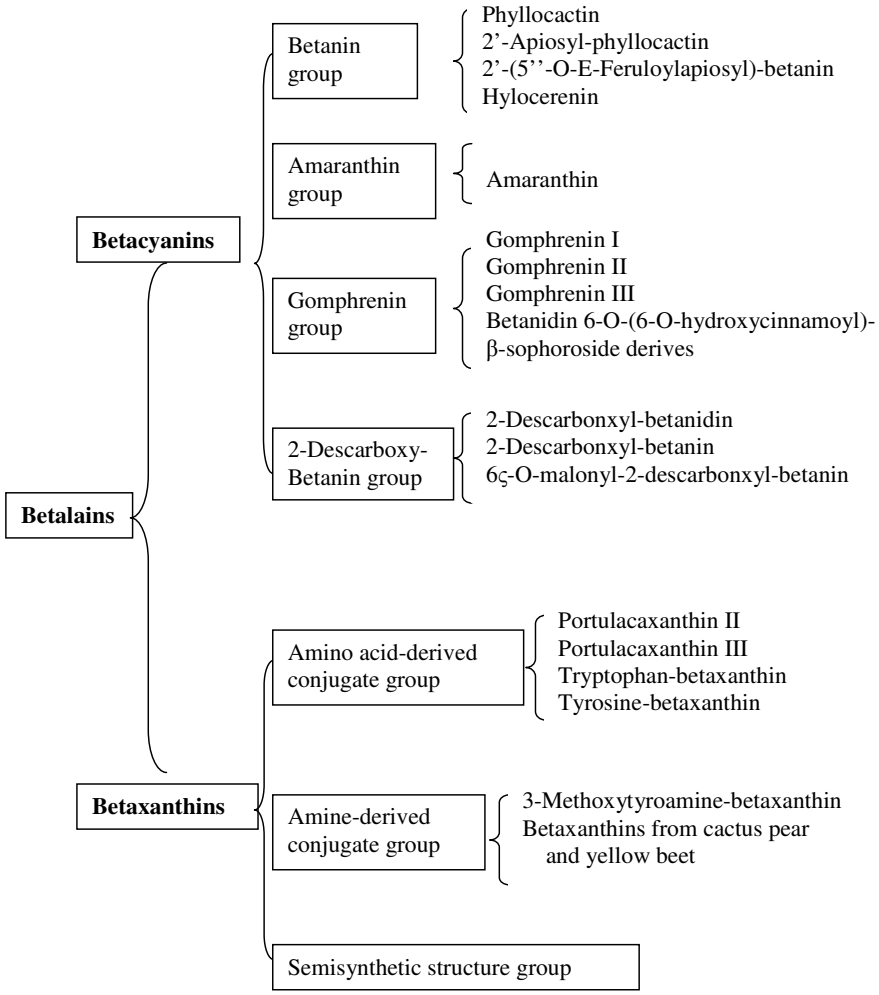

Figure 1. Major components of betalains.

Kanner et al., 2001). They can prevent oxidative processes and thus contribute to the onset of several degenerative diseases and the protection against certain oxidative stress-related disorders in human (Kanner et al., 2001; Tesoriere et al., 2004; Sembries et al., 2006). Besides, the red-violet betalains, betacyanin, may contribute to the antimalarial activities determined from betalains-containing *Amaranthus spinosus* L. and *Boerhaavia arecta* L (Hilou et al., 2006). The details in the biological and health-promoting properties of betalains can be found in the reviews of Strack et al. (2003) and Moreno et al. (2008).

With attractive colour, beneficial bioactivity and health promoting properties, good performance in food processing and the taxonomic significance, the betalains have been the subject to interest. According to Nilsson (1970) and Piattelli (1976), a major red pigment in the betacyanins group, betanin, first described as "Betacyane", was isolated from red beet as early as in 1918 and the yellow pigment, betaxanthins, in 1964 from prickly pear (*Opuntia. ficusindica*) fruits. In the past decades, investigations on betalains were prompted and the isolation, purification, identification, quantification and structure elucidation were extensively studied and the progress was reviewed (Schoefs, 2004; Stintzing et al., 2002a, b, 2004b, c, 2006). In recent years, the biosynthetic pathway of betalains was described in some publications, some enzymes involved were characterized, and the progress has been systematically reviewed by Strack et al. (2003) and redescribed and/or renewed by Grotewold (2006) and Moreno et al. (2008). The review of Strack et al. (2003), to our understanding, were mainely based on the tyrosine as starting point of the pathway. Grandia-Herrers and co-workers (2005a) discovered that the tyroamine, instead of tyrosine, was a starting point for betalains biosynthesis. This discovery and the results published after 2003 in the identification and characterization, in more plant species, of the enzymes involved in betalains biosynthesis pathway make the pathway far more complicated than what it was thought. Fortunately, some genes encoding the enzymes involved in pathway were recently cloned and functionally

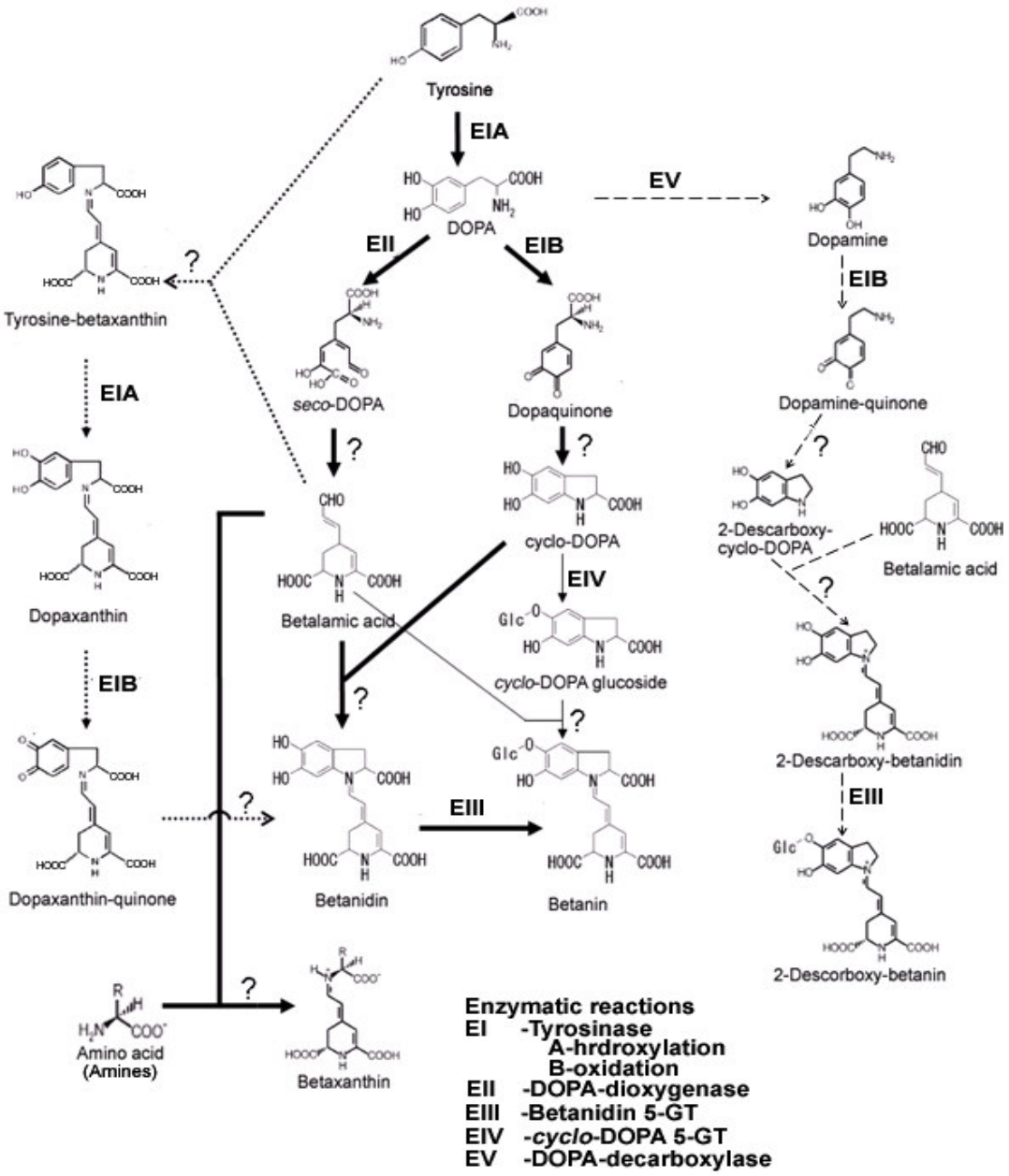

Figure 2. Tyrosine-based biosynthetic pathway of betalains.

analyzed, which would help to elucidate the pathway. In this paper, we outline the biosynthetic pathway of betalains with emphasis on the enzymes and genes involved in the pathway in higher plants. Well evaluation of the biosynthetic and metabolic pathway of betalains will benefit and prompt their wide use in food and pharmaceutical industry.

BETALAIN BIOSYNTHETIC PATHWAY

Betalains biosynthesis can be classified into two major pathways: 1, tyrosine-based pathway in which the tyrosine is used as starting substrate; 2, tyramine-based pathway in which the tyramine is used as initial substrate. The first one is classic, and the second one, new.

Tyrosine-based biosynthesis

The basic scheme of tyrosine-based betalains biosynthesis was described in the review of Strack et al. (2003) and the enriched one is presented in Figure 2. The biosynthesis is initiated by the hydroxylation of tyrosine, through which the tyrosine is converted into DOPA (dihydroxyphenylalanine). This reaction is reported catalyzed

by tyrosinase with hydroxylase activity (EIA). The resulting DOPA has 3 outlets: A. It is oxidized to dopaquinone by the tyrosinase with polyphenol-oxidase activity of (EIB). B. It is converted into *seco*-DOPA with the catalysis of DOPA dioxygenase (EII). C. It undergoes decarboxylation and forms dapamine catalyzed by DOPA-decarboxylase (EV).

The dopaquinone issued from the outlet A of DOPA is cyclizated to produce *cyclo*-DOPA, which was postulated spontaneous (Strack et al., 2003) because of lack of information about the responsible enzyme(s). The condensation of *cyclo*-DOPA with betalamic acid gives rise to betanidin (betacyanin formation), which is also postulated spontaneous (Strack et al., 2003). The resulting betanidin is glucosylated with the aid of betanidin-5-glucosyltransferase (EIII) and forms betanin which is later transported into and stored in the vacuole of cytoplasm. The *cyclo*-DOPA can be also glucosylated directly under the catalysis of *cyclo*-DOPA-5-glucosyltransferase (EIII) and forms *cyclo*-DOPA glucoside (Sasaki et al., 2005a, b). This new product is condensated with betalamic acid and gives rise to betanin formation directly without passing by betanidin.

The *seco*-DOPA issued from the outlet B of DOPA is converted into betalamic acid, for which the catalyzing enzyme remains unknown. The betalamic acid has 5 routes, so far to know, to the formation of betacyanins and betaxanthinins. The first two routes are classic, as reviewed by Strack et al. (2003), that is, betalamic acid is condensed with cyclo-DOPA to form betanidin, or condensed with amino acid and/or amine to give rise to betaxanthin (shown by bold solid arrow in the Figure 2). The third is that betalamic acid is condensed with cyclo-DOPA glucoside and forms betanin directly (Sasaki et al., 2005a, b), as shown in the outlet A of the DOPA. The fourth, betalamic acid is combined with 2-descarboxy-cyclo-DOPA and results in formation of 2-descarboxyl-betandin (Kobayashi et al., 2001), as shown in the right side of the Figure 2 with longer-dashed arrow. The fifth, betalamic acid is back-condensed with tyrosine, the starting molecular of the pathway, and forms tyrosine-betaxanthin directly (Grandia-Herrers et al., 2005b, c) as presented in the left side of the Figure 2.

The dopamine that came from the outlet C of DOPA is converted to dopamine-quinone under the catalysis of tyrosinase with oxidation activity (EIB). Resulting dopamine-quinone is 2-descarboxylated and cyclizated or cycli- zated with 2-descarboxylated to form 2-descarboxy-cyclo- DOPA (Kobayashi et al., 2001), for which the order of descaboxylation and cyclization and the catalyzing enzymes remain clarified. The 2-descarboxy-cyclo-DOPA is then condensed with the betalamic acid issued from the outlet B of the DOPA and yields 2-descarboxyl-betandin which is further glucosylated to produce 2-descarboxyl-betanin (Kobayashi et al., 2001), as shown in the right part of Figure 2. The glucosylation is proposed, catalyzed by betatanin 5-glucosyltransferase (EIII), while unknown remains for the condensation.

Recently, Grandia-Herrers and co-workers (2005b, c) found that the betalamic acid issued from the outlet B of DOPA may back-condensed with tyrosine, the starting molecular of the pathway, and form tyrosine-betaxanthin directly. The newly forming tyrosine-betaxanthin is converted to dopaxanthin-quinone after hydroxylation and oxidation catalyzed by tyrosinase with hydroxylation (EIA) and oxidation (EIB) activity, respectively, as presented in the left part of Figure 2. The dopaxanthin-quinone is subsequently transformed into the stable product, but the reaction detail is unknown up till now, although it was non-enzymatic.

In summary, tyrosine-based biosynthesis of betalains has 4 pathways known up to date, and all pathways pass by the condensation of betalamic acid, a common chromophore of betacyanins and betaxanthins, with a derivative of DOPA or with the tyrosine itself. Whether the condensation reaction is enzymatic or spontaneous remains unclarified (Grandia-Herrers et al., 2005b, c; 2006; Grotewold, 2006), although the spontaneous is speculated (Strack et al., 2003; Moreno et al., 2008).

Tyramine-based biosynthesis

Tyramine-based biosynthetic pathway of betalains was first proposed by Kobayashi et al. (2001) mainly according to the observations that one sub-group of red-violet pigment betacyanins, 2-descarboxy-betacyanins, are found in flowers of *Carpobrotus acinaciformis* (Aizoaceae) (Piattelli and Impellizzeri, 1970), in hairy root cultures of yellow beet (Schliemann et al., 1999; Kobayashi et al., 2001) and in *Celosia sp*. inflorescences (Schliemann et al., 2001). The tyramine, instead of tyrosine, as the initiated substrate, was directly hydroxylated to dopamine by hydroxylase-type tyrosinase (EIA). Then, the dopamine is oxidized to produce dopamine-quinone under the catalysis of polyphenoloxidase-type tyrosinase (EIB). The dopamine-quinone is sequently converted to 2-descarboxy-*cyclo*-DOPA which further condenses with betaminic acid to form 2-descarboxy-betanidin, as shown in the upright part of Figure 3. The information about the enzymes responsible for these two reations is still lacking, and this resulted in a spectulation that the reactions are spontaneous, although the condensation of 2-descarboxy-*cyclo*-DOPA with betalamic acid was suggested as the decisive reaction to give rise to 2-descarboxy-betanidin, based on a short-term dopamine administration experiment with fodder beet seedlings (Kobayashi et al., 2001). Glucosylation of tyramine-resulting to 2-descarboxy-betanidin is considered the same as that from tyrosine, and the reaction is proposed catalyzed by betatanin 5-glucosyltransferase (EIII) (Figure 3).

Another branch of tyramine-based biosynthetic pathway of betalains (left part of Figure 3) was recently proposed

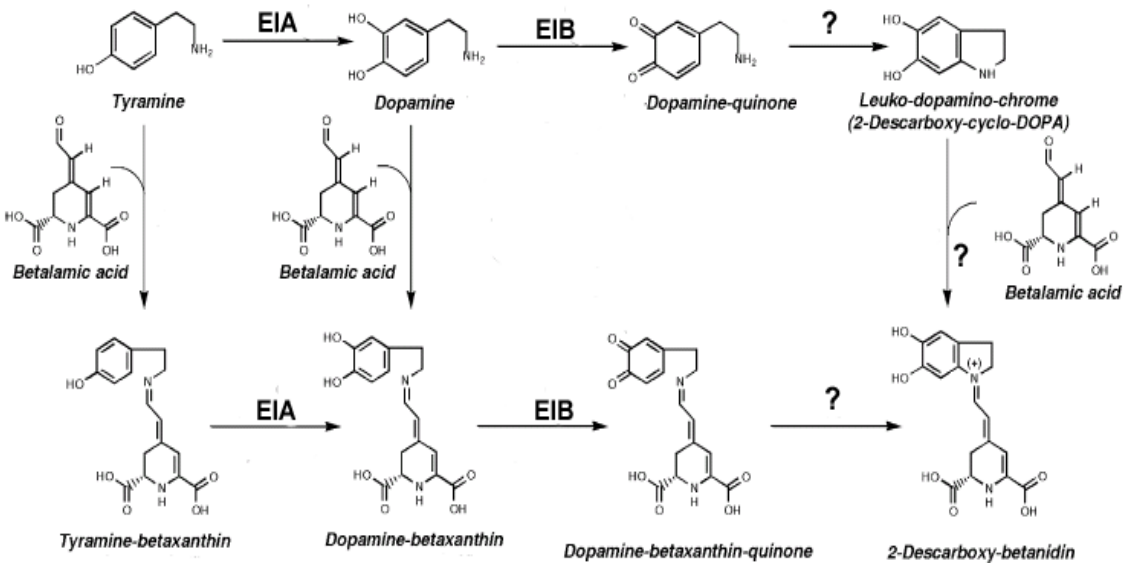

Figure 3. Tyramine-based biosynthetic pathway of betalains.

by Gandía-Herrero and co-workers in 2004-2005, based on the kinetic characterization of the tyrosinase-catalyzed oxidation of tyramine-betaxanthin and dopamine-betaxanthin which had been found in *Mirabilis jalapa* flowers (Piattelli et al., 1965; Gandía-Herrero et al., 2005d), in orange callus cultures of *Beta vlugaris* (Girod and Zrÿd, 1991a) and in red Swiss chard (Kluger et al., 2004). The enzymatic oxidation of tyramine-betaxanthin and dopamine-betaxanthin resulted in formation of dopamine-betaxanthin-quinone as the intermediate product identified with HPLC and ESI-MS and its more stable species, 2-descarboxy-betanidin for example, as the final one (Gandía-Herrero et al., 2005a). Therefore, in Gandía-Herrero and co-workers proposed branch of tyramine-based biosynthetic pathway of betalains (Figure 3), the starting substrate, tyramine, is converted to dopamine under the catalysis of hydroxylase-type tyrosinase (EIA) which is the same as proposed by Kobayashi et al. (2001), and the dopamine is, then, condensed directly with betalamic acid to form dopamine-betaxanthin. The starting substrate, tyramine can also be condensed directly with the betalamic acid to give rise to tyramine-betaxanthin, and the resulting tyramine-betaxanthin is then hydroxylated by hydroxylase-type tyrosinase (EIA) to produce dopamine-betaxanthin. The dopamine-betaxanthin is further oxidized by the tyrosinase with phenoloxidase activity (EIB) to yield dopamine-betaxanthin-quinone which is finally converted to 2-descarboxy-betanidin (Figure 3) via intramolecular cyclization.

This proposal left several questions unanswered: where does the betalamic acid come from? How does the condensation of the betalamic acid with tyramine or with dopamine take place? In other word, is the condensation enzymatic or spontaneous? Is the intramolecular cyclization of dopamine-betaxanthin-quinone enzymatic or spontaneous? And if the questioned reaction is enzymatic, what enzyme is responsible for it?

ENZYMES AND GENES INVOLVED IN THE BATALAIN BIOSYNTHETIC PATHWAY

In betalain biosynthetic pathways as shown in Figures 2 and 3, some steps are enzymatically catalyzed and the others are assumed spontaneous because the responsible enzyme has not been identified and/or characterized. The enzymes characterized and involved in the betalain biosynthesis in higher plants are summarized in Table 1.

Tyrosinase

Tyrosinase is a class of copper-containing, PPO-type bifunctional enzymes. They catalyze hydroxylation of phenols to *o*-diphenols (EC 1.14.18.1, monophenol: monooxygenase) and oxidize subsequently the diphenols to corresponding *o*-quinones (EC 1.10.3.1, *o*-diphenol: oxygen oxidoreductase) (Kaim and Rall, 1996; Strack et al., 2003). They are present widely in and characterized from bacteria, fungi, plants and animals (Gandia-Herrero et al., 2004).

In plant, higher PPO-type tyrosinase activity in betanin-producing than non-producing callus cultures of *Beta vulgaris* var. *crassa* was first detected by Constabel and Haala in 1968. Then, the tyrosinase involved in betalain biosynthesis was described from callus cultures of common portulaca *(Portulaca grandiflora)* (Endress, 1979) and plants of the portulaca and red beet (*B. vulgaris* subsp. *vulgaris*) (Steiner et al., 1996, 1999). Presence of PPO transcripts correlating with betacyanin accumulation

Table 1. Key Enzymes involved in betalain biosynthesis in higher plants.

Name	Plant sources		Reference
	Species	Common name	
Tyrosinase*	*Portulaca grandiflora*	Bigflower purslane	Steiner, 1999
Tyrosinase*	*Beta vulgaris*	Red beet	Gandia Herrero et al., 2004
Tyrosinase*	*Lampranthus productus*	Ice plant	Gandia Herrero et al., 2007
DOPA-dioxygenase	*P. grandiflora*	Bigflower purslane	Christine et al., 2004
DOPA-dioxygenase	*Suaeda Salsa*	Saline seepweed	Ruan, 2008
DOPA-dioxygenase (CAE47100)	*B. vulgaris*	Red beet	Zyrd et al., 2003
DOPA-decarboxylase*	*B. vulgaris*	Red beet	Terradas, 1989
Betanidin 5-GT**	*Dorotheanthus bellidiformis*	Livingstone daisy	Vogt et al., 1999
Betanidin 6-GT	*D. bellidiformis*	Livingstone daisy	Vogt, 2002
cyclo-DOPA 5-GT	*Mirabilis jalapa*	Four o'clock	Sasaki et al., 2005
cyclo-DOPA 5-GT	*Celosia cristata*	Feather cockcomb	Sasaki et al., 2005
GT	*B. vulgaris*	Red beet	Sepulveda-Jimenez et al., 2003
GT	*Phytolacca americana*	American pokeweed	Nakayama et al., 2008
GT	*P. americana*	American pokeweed	Nakayama et al., 2008
GT	*P. americana*	American pokeweed	Nakayama et al., 2008

*Coding gene not cloned in betalain-producing plants, up till now.
**GT: Glucosyltransferase

in the fruits of *Phytolacca americana* (Joy et al., 1995) provided a molecular biology evidence of tyrosinase involvement in betalain biosynthesis.

Tyrosinase involved in the betalain biosynthesis was first purified and characterized from mushroom *Amanita muscaria* by Mueller et al. (1996). In higher plant, the tyrosinase was partially purified from betacyanin-producing callus cultures of *P. grandiflora*, and it appeared to be a monomer with a molecular mass of 53 kDa (Steiner, 1999). Gandia-Herrero et al. (2004) purified tyrosinase from beet root and found that betaxanthins, tyrosine-betaxanthin and dopaxanthin fed were used as substrates to synthesize betalains by the tyrosinase, which disclosed the monophenol activity of the tyrosinase. This finding and supplement results (Gandia-Herrero et al., 2005a,b,c) allow them to propose a new pathway of betalain biosynthesis, as described above in the section 2 (Betalain biosynthetic pathway). They also extracted and purified tyrosinase from *Lampranthus productus* violet flowers and characterized its oxidation activity on betanidin by HPLC-MS (Gandia-Herrero et al., 2007). We purified and characterized a tyrosinase from red Swiss chard (*B. vulgaris* subspecies *cicla)* leaf and the enzyme appeared as a monomer protein with molecular weight of 41 kDa and showed higher diphenolase activity than monophenolase one (Gao et al., submitted).

Although PPO-type tyrosinases, a class of proposed key enzymes responsable for betalain biosynthesis, have been purified and partially or fully characterized in numbers of betalain-producing and non-producing plants or their callus cultures, most of them display much higher diphenolase activity than monophenolase one in the betalain-producing plants as in non-producing ones. Almost all attempts, if not all, to convert the tyrosine to L-DOPA *in vitro* with purified tyrosinase(s) from higher plants, including betalain-producing plants, failed. This arises the question: whether the tyrosine is indeed a starting substrate for betalain biosynthesis as thought classically.

Up to date, the gene encoding for betalain-specific tyrosinase has been cloned only from fungi *A. muscaria.* In betalain-producing higher plants, Joy et al. (1995) obtained two PPO transcripts correlating with betacyanin accumulation in *P. americana* fruits, from which the deduced amino acid sequences contained typical copper-binding domains and transit peptide sequences. We cloned PPO cDNAs from red-leaf amaranth (Gao et al., unpublished) and red Swiss chard (Gao et al., unpublished) and the amino acid sequences deduced from them contained also the typical copper-binding domains, but without transit peptide sequence.

DOPA dioxygenase

Two types of extradiolic DOPA dioxygenase have been identified in plants. One is 2,3-DOPA dioxygenase from fungi *A. muscaria* (Girod and Zryd, 1991b). It is responsible for 2,3-cleavage of DOPA to form 2,3-*seco*-DOPA, which recyclizes assumedly spontaneously and gives rise to muscaflavin. Another is 4,5-DOPA dioxygenase from higher plant. It catalyzes extradiolic 4, 5-cleaving, the aromatic ring of DOPA to produce an unstable 4,5-*seco*-DOPA which "spontaneously" precedes an intramolecular condensation to betalamic acid (Strack et al., 2003). Although the function of DOPA-dioxygenase in the betalain biosynthesis seems clear, the natural enzyme has been

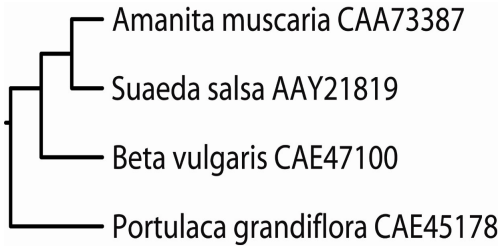

Figure 4. Phylogenic scheme of DOPA-dioxygenases in betalain-producing plant based on amino acid sequences. The tree was created with PHYLIP.

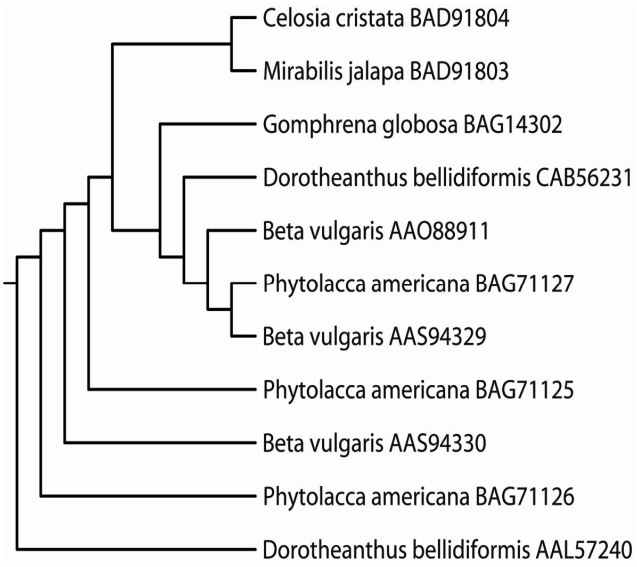

Figure 5. Phylogenic scheme of glucosyltransferases in betalain-producing plant based on amino acid sequences. The tree was created with PHYLIP.

purified only from fungi *A. muscaria* (Girod and Zryd, 1991b; Terradas and Wyler, 1991), and its purification from higher plant is not yet available up till now.

The gene coding for DOPA-dioxygenase was first cloned by Hinz et al. (1997) from *A. muscaria*. The fungal DOPA dioxygenase expressed can catalyze the 2,3- and 4,5-aromatic ring cleaving of the DOPA and give rise to the simultaneous accumulation of betalain and mulscaflavin. When its cDNA was transformed into white petal type of *P. grandiflora* by particle bombardment, the color of petal changed (Mueller et al., 1997). In 2004, Christinet et al. isolated and characterized a colored petal-specific DOPA-dioxygenase gene from *P. grandiflora*. Transient overexpression of this gene in the white petal of *P. grandiflora* resulted in appearing of violet or yellow spots. They also noticed that DOPA dioxygenased from *P. grandiflora* only had the 4, 5-aromatic ring cleaving-activity, which was different from the fungal DOPA dioxygenased. In 2008, Ruan cloned the cDNA and promoter of 4, 5-DOPA dioxygenased from *Suaeda Salsa* and determined the enzymatic activity. The amino acid sequence deduced from the cDNA of *S. Salsa* has a higher similarity to *A. muscaria* than to *B. vulgaris* and *P. grandiflora* (Figure 4). The high diversity of amino acid sequences of DOPA-dioxygenase between *A. muscaria* and *P. grandiflora* may partly explain the difference in aromatic ring cleaving between two enzymes.

Glucosyltransferase

Glucosyltransferases involved in betalain biosynthesis can be classified into 2 groups based on their substrate. The first group is betanidin glucosyltransferase, and the second, cyclo-DOPA glucosyltransferase. The first group has 2 members identified, betanidin 5-*O*-glucosyltransferase (betanidin 5-GT) and betanidin 6-*O*-glucosyltransferase (betanidin 6-GT), while the second group, only one, *cyclo*-DOPA 5-glucosyltransferase (*cyclo*-DOPA 5-GT).

Betanidin glucosyltransferase is responsible for the glycosylation of betanidin and the glucosides are then transported into and stored in vacuoles. Both betanidin glucosyltransferases (betanidin 5-GT and betanidin 6-GT) have been purified and characterized from cell suspension cultures of Livingstone daisy (*Dorotheanthus bellidiformis)* by Vogt et al. (1997). Their coding cDNAs have been cloned from the same plant species and by the same team (Vogt et al., 1999; 2002). The sequence of betanidin 5-GT cDNA shows high identity to those of tobacco and tomato glucosyltransferases. Betanidin 5-GT conducts glucose transfer from UDP-glucose to the betanidin and gives rise to the formation of betacyanins. It can also catalyze the glycosylation of *ortho*-dihydroxylated flavonols and flavones in addition to the natural substrate betanidin. Betanidin 6-GT performs the glucose transfer to the 6-hydroxyl group of betanidin. Betanidin 6-GT is distinctly different from betanidin 5-GT both in amino acid and nucleotide sequences (Figure 5), though they come from the same plant species and share similar substrates. They showed only 19% amino acid sequence identity, which suggests that two enzymes catalyze the transfer of glucose by different mechanisms. In betanidin 5-GT of *D. bellidiformis*, the amino acid Glu378 and His22 are highly conserved (Hans et al., 2004), and substitution of them resulted in complete loss of enzyme activity. In addition, both betanidin 5-GT and 6-GT can transfer glucose with similar efficiency to betanidin and to several flavonoids in transgenic non betalain-producing plants, which suggests the evolutionary links between betalains and carotenoids pathways (Grotewold, 2006). Hans and co-workers (2004) 3D model of betanidin 5-GT would provide a deeper insight into the substrate and regiospecificity of these class of enzymes.

The *cyclo*-DOPA 5-GT activity was first detected in betalain-producing plant four o'clocks (*Mirabilis jalapa*) by Sasaki and co-workers in 2004. One year later, the

cDNAs encoding this enzyme from four o'clock and feather cockscombs (*Celosia cristata*) were successfully isolated, characterized and functionally analyzed (Sasaki et al., 2005a, b). Sasaki and co-workers (2005a) showed the contribution of the cDNA-expressed *cyclo*-DOPA 5-GT to the accumulation of betanin in four o'clock and feather cockscombs and found (2005b) that the amaranthin, one subgroup of betacyanins, is synthesized via glucuronylation at the *cyclo*-DOPA glucoside step in feather cockscombs. That means that the *cyclo*-DOPA 5-GT, different from its counterpart, betanidin 5-GT, catalyzes the glucose transfer to *cyclo*-DOPA instead of to betanidin. This finding allows Sasaki and co-workers to propose a new branch pathway of tyrosine-based biosynthesis of betalains, as shown above in the Figure 2.

A database and literature survey discloses that up till now there are 11 glucosyltransferase cDNAs or DNAs isolated from 6 species of betalain-producing plants (Table 1), and the enzymes can be phylognetically grouped into 4 based on the deduced amino acid sequence similarity (Figure 5).

Dopa decarboxylase

2-Descarboxy-betacyanins, one sub-group of red-violet pigment betacyanins were found occurrent with dopamine-derived betaxanthins in flowers of *C. acinaciformis* (Aizoaceae) (Piattelli and Impellizzeri, 1970), in hairy root cultures of yellow beet (Schliemann et al., 1999; Kobayashi et al., 2001) and in inflorescences of *Celosia sp*. (Schliemann et al., 2001). From these observations arose the assumption that certain enzymes might be involved in the decarboxylation of DOPA or its precursors. Terradas (1989) detected an enzyme that decarboxylates dopa to dopamine in the protein extracts from cell cultures of red beet. The crude protein extracts from hairy root cultures of yellow beet could convert dopa to dopamine highly efficiently, but not accept the tyrosine as a substrate (Alfred and Willibald, unpublished while cited by Strack et al., 2003). Up till now, DOPA decarboxylase has not been purified and its coding gene(s), not cloned.

PERSPECTIVES

Extensive studies in the biosynthesis of betalains in plant and enzymes and their genes involved in the biosynthesis in the last decades have made great progress in the understanding and use of this class of water-soluble nitrogen-containing pigments. The betalain biosynthesis is far more complicated than it was thought 5 years ago, and even up till now, two quite different biosynthetic pathways, tyrosine-based and tyramine-based pathways have been proposed and/or established (Figures 2 and 3). The tyrosine-based pathway comprises 5 branches, and the tyramine-based one, 3 branches. Most of the enzymes involved in those pathways have been detected and some have been identified, purified or characterized, such as tyrosinases and glucosyltransferases (Table 1). The genes (cDNA and or DNA) encoding DOPA-dioxygenase and glucosyltransferase from at least 8 species of betalain-producing plants have been cloned and/or fuctionally analyzed (Table 1).

Betalain biosynthetic pathways are yet to be clearly elucidated and/or clarified, despite the progress made so far. The tyrosine as the starting material of betalain biosynthesis, as thought classically, is first challenged, because the crude protein extracts from hairy root cultures of yellow beet did not accept it as the substrate (Alfred and Willibald, unpublished while cited by Strack et al., 2003) and the DOPA, a proposed enzymatic product and substrate of tyrosinase in the early reaction, does not accumulate in the plants. The second major question is how the aldimine formation takes place between betalamic acid and cyclo-DOPA or cyclo-DOPA glucoside or 2-descarboxy-cyclo-DOPA (to make red pigment betacyanins), and between betalamic acid and amino acid or amine or dapamine or its (or) their derivatives (to make yellow pigment betaxanthins and their derivatives). This aldimine formation has been postulated spontaneous for long time because of failure in the detection of enzyme activity catalysing this reaction (Strack et al., 2003; Moreno et al., 2008), on one hand, and some indirect observations in amino acid feeding experiments (Schliemann et al., 1999), on the other hand. If the aldimine formation is spontaneous as postulated, the reason of consistent presence of the specific patterns of betalains in the same plant remains unclear (Grotewold, 2006). Some other unresolved questions are marked "?" in the Figures 2 and 3, which are also about whether the reaction is enzymatic or spontaneous. In regard to the enymes requested in the well-known enzymatic reactions of betalain biosynthesis, one of the most important enzymes, tyrosinase, remains purified and characterized in betalain-producing plants. It is the same for another important enzyme, DOPA-dioxygenase, although its coding genes have been cloned and functionally analyzed in some plant species. Identification, purification and characterization of 2-DOPA-descarboxylase(s) are needed to complete and better understand the new pathways of betalain biosynthesis as presented in Figures 2 and 3. Besides, the transport and storage of betalamic acid, a common chromophore of betacyanins and betaxanthins, and the pigments remain unclear.

With rapid development of plant molecular biology and biotechnology, more genes (cDNA and/or DNA) encoding the enzymes involved in the betalain biosynthesis will be identified, cloned and functionally analyzed, in particular the gene(s) for PPO-type tyrosinases. Successful cloning of the gene(s), especially those coding for the tyrosinases will help to identify, purify and characterize the enzymes, for example, by Western blot, on one hand; and to verify the function of the enzyme(s), for instance,

by *in vitro* system, on the other hand. Development and utilization of micro-array, gene chip (cDNA and DNA) and protein fingerprints in betalain-producing plants will help to detect, identify and verify well-known, unknown and proposed genes and/or enzymes involved in the betalain biosynthesis, and in particular those, if there are, involved in the condensation of betalamic acid with various different DOPA or amino acid or amine and/or their derivatives. Better understanding of the biosynthetic pathway and the corresponding regulated mechanism of betalains will contribute to further application of the pigments in the food and pharmaceutical industry.

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