Chapter 6
The Betalain Secondary Metabolic Network

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Introduction

Beets are red because of an evolutionary left turn. Virtually, all extant flowering plants produce the red/violet, phenylalanine-based, secondary metabolic anthocyanin pigments, with the exception of a handful of families in a single order, the Caryophyllales. These families produce unrelated red/violet/yellow betalain or beet pigments. While the families of almost all the major crops produce anthocyanins, the betalain families contain crop species that are widely grown and form important staples in many agricultural economic systems including beets, Swiss chard, spinach, *Amaranthus*, *Chenopodium*-quinoa, and prickly pear.

The betalain pigments are mutually exclusive with anthocyanins; no known taxa produce both pigments [1]. And, where they are produced, betalains physically and functionally replace the anthocyanins in all biological contexts. Betacyanins, the red betalains, have a visible absorption spectrum very similar to anthocyanins. Betalain biosynthesis is developmentally regulated in flowers and fruits to produce diverse colors to attract pollinators and seed dispersal animals. The betalain pathway responds in the same way to the same biotic and abiotic signals that regulate anthocyanins. These signals include light, temperature, pathogens, and other environmental signaling events. Like the phenylpropanoids such as the anthocyanins, betalains are free oxygen radical scavengers and are nutritionally beneficial in ways similar to the phenylpropanoids. Betalain pigments were originally referred to as "nitrogenous anthocyanins" in recognition that these nitrogen-containing compounds have replaced the biological functions of anthocyanins in the plants where they occur.

While the betalain pathway is considered agriculturally and nutritionally important, and fascinating from an evolutionary perspective, relatively little is known at the molecular level about the enzymatic steps in the betalain pathway, about

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how steps in the network are regulated, or about how this new metabolic pathway evolved. A complete set of structural and regulatory network genes has not yet been identified, our understanding of the regulation, both environmental and developmental, is at its infancy, and the evolution of the pathway is still largely a black box.

The phytochemical literature is full of papers identifying the structures of specific betalain compounds (reviewed in [2]), measuring their antioxidant properties [3], measuring their stability (reviewed in [4]), and other chemical properties. These will not be reviewed here.

Phylogenetic Restriction

As noted, within plants, betalain pigments are restricted to the Caryophyllales order and within this order to the monophyletic core families as defined by Cuenoud et al. [5]. A recent analysis overlaid the occurrence of betalains, anthocyanins, or the absence of pigments within this monophyletic group on a molecular phylogeny [6]. The analysis led to the conclusion that betalains may have arisen either one time or two times within this single restricted group, with equal probability for either scenario. Betalains and anthocyanins can occur in sister families within the larger group and over time one or both pigment pathways have been lost multiple times independently. It should be noted that the molecular phylogenies for this group were constructed with markers that had no relationship to the betalain pathway. It will be interesting, and will soon be possible, to produce phylogenies based on the actual betalain biosynthetic and regulatory genes.

Betalain pigments are also produced in basidiomycete fungi, notably the red, orange, or yellow capped *Amanita muscaria*, or fly agaric. Molecular evidence indicates that this occurrence is the result of an independent evolutionary origin unrelated to the plant pigments ([7], and discussed below).

What are the Betalains

The chemical precursors, intermediates, and products of the betalain ring structure biosynthetic pathway have been determined. Unlike the unrelated phenylalanine-based anthocyanins, betalains derive from tyrosine. The betalains are water-soluble, vacuole-localized pigments that range from red/violet betacyanins, to yellow betaxanthins. The synthesis of the betalain ring structure is proposed to require three enzyme-mediated steps (Fig. 6.1). A molecule of L-3,4-dihydroxyphenylalanine (L-DOPA) is the substrate in step 2, the first ring biosynthetic gene reported, where a ring-opening extradiol cleavage enzyme, DOPA-4,5-dioxygenase (DODA) produces 4,5-seco-DOPA which spontaneously cyclizes to betalamic acid [7]. The gene/enzyme responsible for step 3 in Fig. 6.1 has been recently identified [8] as a cytochrome P450 enzyme, CYP76AD1. This enzyme also uses L-DOPA as a substrate

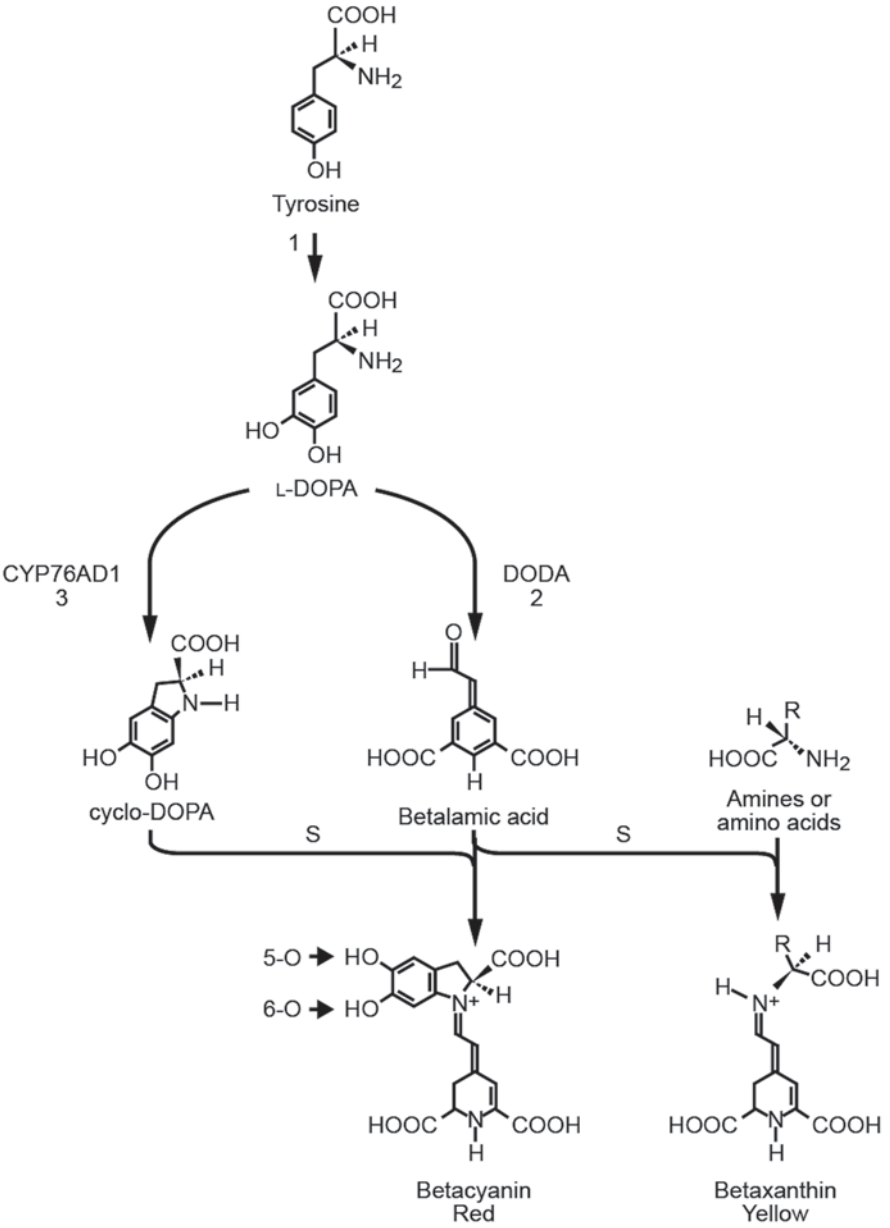

Fig. 6.1 The proposed betalain pathway. Tyrosine is converted by an unknown enzyme to L-DOPA. L-DOPA is the substrate in two enzyme-mediated steps to produce cyclo-DOPA and betalamic acid by CYP76AD1 and DODA, respectively. Betalamic acid then spontaneously condenses with either an amine to produce the yellow pigment betaxanthin or with cyclo-DOPA to produce the red pigment betacyanin

molecule to produce cyclo-DOPA via a DOPAquinone intermediate. The gene(s) or enzyme(s) responsible for step 1, the conversion of tyrosine to L-DOPA (tyrosinase), has not been positively identified.

Betalamic acid is itself a yellow and fluorescent compound that is required to produce both the yellow and red betalains. Betalamic acid condenses with the product of step 3, cyclo-DOPA, to form the red betanidin pigment, or condenses with amino acids or other amine groups to form the yellow fluorescent betaxanthin pigments [9]. The ring structure is often glycosylated or acylated on the cyclo-DOPA moiety (see 5-O and 6-O arrows in Fig. 6.1) and genes encoding UDP glycosyltransferases (UGT) with in vitro activity on betanidin are known from *Dorotheanthus* [10] and beet [11] though their specificity is not high. In vitro studies have also shown that the cyclo-DOPA structure can be glycosylated [12] and that the glycosylated-cyclo-DOPA can participate in the spontaneous condensation with betalamic acid. The order of modification and condensation does not appear to be fixed.

Steps 1 and 3 were earlier proposed to be performed by the same enzyme, a polyphenol oxidase (PPO), because a single PPO-type enzyme, tyrosinase, performs both reactions during melanogenesis in animals [13], and PPO enzymes will perform steps 1 and 3 in vitro [14]. But, it has not been demonstrated, genetically or otherwise, that these steps are performed by a PPO in the plant [15]. Arguments against a PPO enzyme performing steps 1 and 3 are: all plant PPO enzymes are reported to be plastid localized [16] while the betalain pathway is cytoplasmic, and it is relatively common to obtain mutants that only make yellow betaxanthin pigments (Fig. 6.2), i.e., they are missing step 3, but step 1 is intact. Yellow mutants are

Fig. 6.2 Betalain genetic phenotypes. *Top row*, 4 o'clocks, *left to right*: RR CC, rr CC, rr cc. *Bottom row*, table beets, RR YY, rr YY, RR yy

known from many betalain species, perhaps all that have been in cultivation for any length of time, including beet, *Celosia*, 4 o'clock, *Bougainvillea*, cactus, *Portulaca*, and others. Yellow should be a rare or nonexistent mutant type if a single enzyme were responsible for both steps.

Genetics of Betalains-Beets, 4 o'clocks, and Portulaca

Wesley Keller performed a genetic analysis of beets (*Beta vulgaris*) in the 1930s and identified two loci, R and Y, involved in the production of betalains [17]. R alleles are responsible for the red versus yellow shift (Fig. 6.2) corresponding to step 3 in Fig. 6.1. The R locus is now identified as encoding CYP76AD1 ([8]; discussed in detail below). Red table beets contain a dominant R allele while yellow beets are homozygous for the recessive r allele.

Y alleles direct whether pigment or no pigment is produced in the interior of the beetroot, regardless of whether the pigment is red or yellow (Fig. 6.2). White beets, which include virtually all sugar beets, are homozygous for the recessive y allele; however, it should be noted that "white" beets are still competent to produce betalains and that the pigment is largely restricted to the epidermal layers. The big Y allele responsible for red table beets appears to be a dominant gain-of-function allele that was discovered during cultivation.

Similarly, in 4 o'clock (*Mirabilis jalapa*), there was classic work from Correns (published in German in the early 1900s and reviewed by Rheinberger [18]) and more recent, but still old work [19–21] demonstrating two main betalain loci, also called R and Y. Again, the R alleles dictate red versus yellow (Fig. 6.2), and the yellow (rr) mutant has been shown to be complemented to red by expression of the beet R gene [8]. Dominant Y versus recessive y 4 o'clock alleles are responsible for the presence (either red or yellow) or complete absence of pigment, respectively (white flowers and absence of betalains in leaves and stems (Fig. 6.2)), not tissue placement. Thus, for both beets and 4 o'clock, the R locus is consistent with step 3 in the betalain biosynthetic scheme (Fig. 6.1). The 4 o'clock Y locus is consistent with step 2, the DODA gene, because 4 o'clock yy mutants do not make any betalain pigments; while the beet Y locus is more consistent with some type of upstream regulator because yy beets are fully capable of producing epidermally restricted red betalain pigments. The beet recessive y allele can be considered to direct the "normal" pigment state that mimics the epidermal placement of anthocyanins in the vast majority of flowering plants.

In beets, Keller [17] reported that the Y and R loci were linked at about 7 cM and this distance has more recently been verified [22]. The work of Engels et al. [21] appears to show that the 4 o'clock Y and R loci are not linked.

In *Portulaca*, three loci direct flower color, C, R, and I. Dominant C is required to make any pigment, red/violet or yellow, dominant R is required to make red/violet (rr flowers are yellow), while dominant I inhibits the formation of yellow pigments [23]. The white or cc mutant is complemented by expression of DOPA dioxygenases from either *Amanita* mushrooms [24] or *Portulaca* [7]. So, the C locus

undoubtedly encodes the DODA gene, though the *Portulaca* DODA gene has not yet been shown to be genetically linked to the C locus and mutations giving rise to any c alleles have not yet been reported. The *Portulaca* R locus probably encodes a paralog of the beet and 4 o'clock R loci, encoding a cytochrome P450 protein, as the r mutation has the same yellow phenotype in all three species. However, no molecular analysis has been reported. Nothing is presently known about the identity of the *Portulaca* I gene.

External Signals are Transduced to Output of Betalain Pigment Phenotypes

The regulation of betalain biosynthesis by environmental signals and applied bioactive compounds has been investigated by several groups. A commonly used system for these studies is hairy root cultures of beets and other species. Most of these studies were conducted with an eye towards maximizing pigment production in culture for potential commercial use. Blue plus far red light [25], cytokinin, nitric oxide [26], methyl jasmonate [27], Ca2+[28], and phosphate [29] have all been shown to strongly influence betalain pigment accumulation in cultures. There are fewer studies using whole seedlings or plants, but those generally agree with the in vitro studies. For example, high white or UV-A light induced betalain biosynthesis in ice plant [30]. There is a single gene expression paper showing that a DODA gene homolog in calli of the betalain producer, *Suaeda salsa,* is upregulated by light [31].

Cloning the Betalamic Acid Biosynthetic Gene, 4,5-DOPA-dioxygenase

The first betalain ring structure biosynthetic gene to be cloned was DODA from the mushroom, *A. muscaria* [32]. To clone this gene, Zrÿd and coworkers purified the protein from mushroom caps, raised antibodies against it, and used the anti-DODA antibodies to screen an *Amanita* cDNA expression library in *Escherichia coli*. They went on to show that expression of this mushroom gene complemented white *Portulaca* tissue (cc genotype), allowing it to produce pigment, either red or yellow depending on the genotype at the R/r locus [24].

This same group was successful in cloning the first plant betalain 4,5-DOPA-dioxygenase (DODA; [7]). Unfortunately, the mushroom gene and protein were not similar enough to the plant versions to allow the use of the antibody or the mushroom gene sequence as probes. They produced subtractive libraries to isolate differentially expressed cDNAs and identified genes highly expressed in red *Portulaca* flowers. Through sequencing of clones, they identified a putative translation product with high similarity to the LigB ring cleavage domain of prokaryotic proteins with potential extradiol 4,5-dioxygenase activity, the same activity that a

betalain 4,5-DOPA-dioxygenase was predicted to have. They went on to show that expression of this gene in white *Portulaca* petal tissue resulted in red or yellow pigmentation, depending on the genetic background, showing that they had the right gene. Sequence analysis of this gene indicated that it has a different phylogenetic origin than the mushroom gene. They also point out that all plants have homologs of this gene and that there are a common set of amino acid changes near the putative active site that separates the betalain and nonbetalain-producing plant DODA genes. It remains to be conclusively shown whether these amino acid changes are responsible for a change in substrate specificity or are the result of the common ancestry of these caryophyllaceous plants.

Cloning the Cyclo-DOPA Biosynthetic Gene, CYP76AD1

The enzymatic steps 1 and 3 (Fig. 6.1) were proposed to be performed by a single enzyme, a PPO. This prediction was supported by the fact that a single PPO-like enzyme, tyrosinase, performs both of these reactions during the formation of dark melanin pigments in animals, and the fact that plant PPO enzymes will also perform these reactions in vitro [14]. Arguments against a PPO are that all known plant PPO enzymes are chloroplast localized while the betalain pathway is cytoplasmic, and that it is commonplace to obtain mutants that only make yellow betaxanthin pigments (in beets, cockscomb, 4 o'clocks, *Portulaca*, cactus, and others), suggesting that these mutants have an intact step 1 but cannot supply the cyclo-DOPA ring structure required for red pigment in the proposed step 3.

Recently, a cytochrome P450 enzyme (CYP76AD1) was identified that is required for the conversion of L-DOPA to the cyclo-DOPA moiety in beet (step 3; [8]). CYP76AD1 was identified through analysis of next generation sequencing of red beet transcripts, highlighting the value of high-throughput next-generation sequencing in studies on nonmodel organisms. cDNA from red table beet seedling hypocotyl sections was sequenced using Roche 454 pyrosequencing. These stem sections produce high concentrations of betalain pigments from the outer epidermis to the inner core and eventually give rise to the swollen red beet. It was hypothesized that betalain pathway genes would be highly represented among these transcripts. This was proven to be true as the previously identified betalain biosynthesic gene, DODA, was the 14th most expressed contig in the data set of nearly 10,000 contigs.

The 454 database was queried for other highly expressed genes that could be candidates for enzymatic steps 1 and/ or 3. PPO- and laccase-encoding cDNAs were found but they were not expressed at the high levels expected for betalain biosynthetic genes. The contig database was also searched for cytochrome P450-encoding sequences because step 1 resembles the canonical cytochrome P450 reaction and it was possible that step 3 could be performed by an unusual cytochrome P450 activity [33]. A cytochrome P450 cDNA, CYP76AD1, was identified as the 33rd most highly expressed contig.

After similar transcript profiling was performed in several other betalain-producing Caryophyllales, CYP76AD1 became the founding member of a new subfamily of cytochrome P450 enzymes that are most similar to CYP76T and CYP76C. It is predicted that CYP76AD2 and CYP76AD3 are responsible for the same step in *Amaranthus* and 4 o'clocks, respectively.

CYP76AD1's role in the betalain pathway was verified through a series of genetic and biochemical experiments. Its expression is correlated with the red phenotype, CYP76AD1 is expressed at high levels in red beets but low levels in yellow and white beets. Mutant analysis using virus-induced gene silencing (VIGS) to suppress gene expression in very red beets resulted in the loss of the red pigment and appearance of yellow pigment. The change from betanin (red) to betaxanthin (yellow) pigments was verified using mass spectrometry.

As already stated there are many yellow mutants and CYP76AD1 was able to complement yellow mutants in beets, cockscomb, and 4 o'clocks. In each species, overexpression of CYP76AD1 resulted in the loss of the yellow phenotype and the production of red betalain pigments.

These data showed that CYP76AD1 is responsible for the enzymatic step 3 in the pathway, biosynthesis of cyclo-DOPA from L-DOPA. Loss of step 3 should result in the inability to synthesize red pigments but should not affect the ability to synthesize yellow pigments consistent with the function of the beet R gene. This led to the hypothesis that CYP76AD1 was the beet R gene genetically defined by Keller in 1936 (discussed above, [17]).

The CYP76AD1 alleles were sequenced in a sugar beet variety, C869 [34], which segregates red and yellow hypocotyls (segregating for *R*/*r*) [17, 22]. A 5-bp insertion was identified, 325 bp before the stop codon in the yellow (rr) segregants (TAAAT), that shows complete linkage to the R phenotype. This insertion and the resulting frameshift introduced an early stop codon, which causes the deletion of the heme-ring-binding site and results in an inactive protein. On the basis of this genetic data and the above described functional data, *CYP76AD1* has been identified as the *R* locus described more than 70 years ago [8].

Modification Enzymes

Betalain pigments, like Anthocyanins, are usually highly decorated. In different species, there is a vast array of different combinations of modifications that produce pigment end products (reviewed in [2]). These modifications are most easily observed on the stable final products; however, there is some debate about when these decorations are added. Two groups have identified enzymes from betalain-producing species that are 5-*O*-glucosyltransferases [10, 12]. One is predicted to modify the unglycosylated betacyanin, betanidin, and the other is predicted to modify cyclo-DOPA, an unstable intermediate (see Fig. 6.1). It is likely that both scenarios are occurring simultaneously.

Enzymes that modify the final product betanidin consist of two types so far, 5-*O*-glucosyltransferase (betanidin 5-GT) and 6-*O*-glucosyltransferase (betanidin 6-GT; *arrows* in Fig. 6.1 point to the 5-O and 6-O positions). Betanidin 5-GT transfers

glucose from UDP-glucose to the 5-hydroxyl group of betanidin. Betanidin 6-GT performs the glucose transfer to the 6-hydroxyl group. However, both betanidin 5-GT and 6-GT are not very specific and can transfer glucose to several flavonols and anthocyanidins, and, based on activities and protein sequence, it has been hypothesized that these enzymes evolved from flavonoid GTs [10, 35].

There is one enzyme/cDNA identified in 4 o'clock and cockscomb that is able to glycosylate cyclo-DOPA in vitro, UDP-glucose:cyclo-DOPA 5-*O*-glucosyltransferase, cDOPA5GT [12], and it has been proposed to perform this same function in vivo. Again, this activity was not highly specific for cyclo-DOPA and it was speculated that these types of GTs evolved from flavonoid GTs.

The added glucose molecules can be further decorated at multiple positions with a variety of groups including acyl, malonyl, apiosyl, feruloyl, glucosyl, hydroxylcinnamoyl, and other moieties. Although some biochemistry has been performed on some of these reactions, nothing has yet been reported on the genes or genetics associated with these reactions.

Expression in Heterologous Species

Evolution has restricted betalain pigments to the order Caryophyllales and a few fungi; however, there has been work to transfer the betalain pathway to heterologous species such as *E. coli*, yeast, *Solanum tuberosum* (potato), *Antirrhinum majus* (snapdragon), and *Arabidopsis thaliana*. Most of the work to express betalains in heterologous species has centered around the DODA gene. The first attempts made with plant betalain pathway genes in *E. coli* proved unsuccessful [7]. However, Sasaki and coworkers were finally able to produce betalamic acid using an *E. coli* expressed DODA gene from 4 o'clocks (MjDODA) and its substrate L-DOPA [36]. They also reported preliminary experiments expressing DODA in yeast**;** however, they did not show any data from these experiments. Gandía-Herrero and García-Carmona recently expressed a *B. vulgaris* DODA gene with its codons optimized for *E. coli* and they also successfully produced betalamic acid and the yellow betaxanthin pigments [37]. The method used by this group was to express the DODA in a heterologous system and then produced the pigment in vitro using protein extracts.

Betalains were first produced in vivo in heterologous systems in 2012 by two different groups. Harris and colleagues expressed a DODA gene from *Portulaca grandiflora,* PgDODA, in cell cultures of *S. tuberosum* (potato) and petals of *A. majus* (snapdragon) using biolistic introduction of the overexpression constructs [38]. They also created stable transgenic lines of *A. thaliana* expressing PgDODA using *Agrobacterium* transformation. Upon feeding the transgenic tissues with L-DOPA, yellow, orange, and red betalain pigments were formed in vivo. It is interesting that with just the addition of one gene (DODA) and one substrate (L-DOPA), nonbetalain-producing plants can be transformed to produce both yellow and red betalain pigments. This suggests that anthocyanin producing plants contain an activity to perform step 3, the production of cyclo-DOPA from L-DOPA. At present, it is unknown how this is being accomplished in the anthocyanin plants.

Hatlestad et al. [8] also had success with heterologous expression in vivo, this time in yeast and with both the DODA and the newly discovered step 3 enzyme, CYP76AD1. They expressed a beet DODA, BvDODA1, in yeast and fed L-DOPA, which resulted in the production of yellow betalain pigments. CYP76AD1 was also expressed by itself in yeast as a check of its enzymatic function. CYP76AD1 was shown to use L-DOPA to produce cyclo-DOPA, consistent with the proposed step 3 activity. In addition they were able to recreate part of the betalain pathway in vivo in yeast by expressing the BvDODA1 and CYP76AD1 genes, simultaneously and feeding with L-DOPA, resulting in the production of betanidin, the undecorated red beet pigment [8].

Genetic and Genomic Resources for Betalain Research

Unfortunately, none of the betalain-producing species has the resources of a model genetic organism. However, there are resources available and the amount of these resources is slowly growing. The National Center for Genetic Resources Preservation (NCGRP) maintains extensive germplasm collections and seeds can be requested through Germplasm Resources Information Network (GRIN; http://www.ars-grin.gov) administered by the United States Department of Agriculture. The *B. vulgaris* collection includes table beet, sugar beet, swiss chard, fodder beet, wild or sea beet (the undomesticated wild precursor), as well as segregating populations from controlled crosses. These five distinct plant types are all the same species. They also maintain extensive collections of *Amaranthus* including cultivated varieties and many species, and collections of *Chenopodium* including *C. quinoa* (the pseudograin, quinoa) accessions as well as other *Chenopodium* species. GRIN also maintains representative collections of other betalain-producing genera like *Opuntia*, *Portulaca*, *Boehravia*, *Tetragonia*, and *Basella*. The second largest *Beta* germplasm collection is maintained at the Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany (http://eurisco.ecpgr.org/).

Beet recombinant inbred lines (RILs) have been produced from a sugar beet/table beet cross and these are available by request. The RILs were developed from a cross used to produce a molecular-based genetic map [39]. In addition, many other RIL populations are in earlier stages of completion by this same research group.

The McGrath group has also produced a sugar beet bacterial artificial chromosome (BAC) library that can be screened by PCR to identify genomic clones for genes [40].

The Gene Index Project maintains a database of beet ESTs and beet genes that can be searched by basic local alignment search tool (BLAST; http://compbio.dfci.harvard.edu/cgi-bin/tgi/gimain.pl?gudb=beet). They also maintain a database for *Mesembryanthemum crystallinum* (ice plant) a betalain producer in the Aizoaceae family.

The Genome Analysis of the Plant Biological System (GABI) is a public/private group with several goals regarding the sugar beet genome including a BAC-based physical map and a complete annotated genome (http://www.gabi.de/projekte-alle-projekte-neue-seite-144.php). The beet genome is approximately 758 Mb and

886 cM [41]. The GABI-based group has assembled 535 chromosomally anchored contigs that comprise about 600 Mb [41]. These can be accessed through (http://bvseq.molgen.mpg.de/index.shtml).

Future Needs

There is much still to be discovered about betalains. This is not limited to the minutiae of the pathway, but major elements, including biosynthetic steps and regulation are still to be discovered. Currently, there is nothing known about the gene(s) required for the first step in the synthesis of betalains, the tyrosine hydroxylase or L-DOPA synthesis step, or the genes involved in the regulation of the pathway. There is very little known about the genes involved in the modification of the pigments, and nothing known about the transport of these pigments into the vacuole, nor any possible degradation pathways.

The step 1 enzyme that catalyzes the conversion of tyrosine to L-DOPA is unknown and is a glaring hole in our knowledge of how betalains are made. There are reports of biochemical isolation of this activity from betalain plants [14], but no reports on gene or protein identity. This step has long been hypothesized to be a tyrosinase-like or PPO enzyme, as in vitro experiments and analysis of animal systems have shown that these types of enzymes can produce L-DOPA from tyrosine; however, it was also long thought that steps 1 and 3 would be performed by the same enzyme. This last theory was challenged when CYP76AD1 (the beet R locus) was discovered to be required for step 3 but not for step 1. While it has not been demonstrated that CYP76AD1 cannot do step 1 (if it does, it is redundant with other genes), the genetic and heterologous expression data are clear that it is only the R locus that is required for step 3 activity, production of cyclo-DOPA from L-DOPA. At the present time, it is possible that step 1 is performed by another cytochrome P450, or another type of enzyme or enzymes, including PPO-like enzymes. It is interesting and perhaps revealing to note that there are no reported step 1 mutants among the many betalain mutants in several cultivated species. Unless, the step 1 enzyme(s) is somehow required for life, this mutant-deficit implies that there is genetic redundancy for this activity.

There are a few studies about how betalain pigments are produced in response to different stimuli (discussed briefly above) but there is nothing known about the genes responsible for this environmental regulation or the developmental regulation needed to place betalains in specific tissues. Identifying the regulatory genes will provide insight into the evolution of the pathway and understanding of developmental placement and environmental regulation. It will be important for possible improvements to betalain-producing fruits and decorative plants by genetic or molecular means. Because betalains are expressed in the same patterns (temporally/spatially) and respond similarly to the same stresses as anthocyanins, it has been speculated that the betalain pathway may be regulated by the anthocyanin regulatory network [42], implying the co-option of regulatory genes. If true, this would be a fascinating evolutionary phenomenon where a regulatory network has switched

to regulate new genes and end products, but the new pathway is still responsible for the same functions overall, in this case red pigmentation and all the biological functions that it serves.

Betalain pigments do not consist of a single final compound, but instead include many possible chemical modifications that decorate the standard backbone. These modifications may be a contributing factor causing the countless shades and hues of red/violet/pink or yellow/orange that are seen across the betalain-producing families. So far, there are only a few modification genes/enzymes identified and these are limited to the addition of glucose at two positions on cyclo-DOPA moiety, either before or after condensation with betalamic acid. More work is needed in this arena to identify the genes/enzymes responsible for other types of modifications.

It is not completely understood where exactly in the cell the synthesis of betalains takes place. The DODA appears to be soluble while the identification of the step 3 enzyme as a cytochrome P450 implies membrane localization in the ER. However, the locations of these enzymes have not been reported.

There is no information on how the final products end up in the vacuole. With anthocyanin producing plants there are several transporters that are involved in the movement of pigments. It is expected that betalain pigments will have similar requirements for transport into the vacuole. Thus far, there are no such transporters known.

DODA-like genes are found in nearly all life, but this protein type has evolved a novel function in betalain-producing plants. The biochemical functions of DODA gene homologs in other plant species have a yet to be determine. Sequence analysis of betalain pathway and nonpathway DODAs has identified two divergent motifs at the predicted catalytic site, one conserved in betalain species and the other in nonbetalain species. It has been proposed that this motif change at the catalytic site is responsible for the specific biosynthetic ability of DODAs that produce betalains. However, there has been no experimentation to test this theory [7]. It is possible that the conserved motifs are conserved based on phylogeny and not function. Given the current state of sequencing and transgenic technologies, it should be easy to test this.

Above we have outlined some of the obvious holes in our knowledge of the genes involved in the production of betalains. With the introduction of modern sequencing and analysis techniques, and the generation of more genetic tools and resources it should be much easier to find the undiscovered genes and test their functions. The strange occurrence and isolation of the betalains to a single order remain the most fascinating aspect of this pathway. Once found, discovery of these genes will allow an informed look at how the betalain pigment pathway functions and how it came into being.

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