Advances and future directions in betalain metabolic engineering

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Summary

Betalains are nitrogenous red and yellow pigments found in a single order of plants, the Caryophyllales, and in some higher fungi. They are responsible for the colors observed in many ornamental plants, as well as in various food products, where they are used as natural colorants. Their nutritional properties and attractive colors make them an appealing target for metabolic engineering. This is further heightened by the limited availability of natural betalain sources, arising from their relative scarcity in the plant kingdom, particularly in edible plants. Recent progress in decoding their biosynthetic pathway has facilitated stable heterologous production of betalains in several plant and microbial systems. Here we provide a brief review of recent advances and discuss current approaches and possible future directions in betalain metabolic engineering, including expanding the chemical diversity of betalains and increasing their yield, exploring new host organisms for their heterologous production and engineering their secretion from the cell.

Key words: betalains, plant pigments, secondary metabolism, metabolic engineering, biosynthesis

I. Introduction

Betalains are water-soluble, tyrosine-derived pigments produced in plants belonging to the Caryophyllales order (e.g. the Cactaceae family), where they play important roles in pollinator attraction and defense against various abiotic and biotic stress factors (Gandia-Herrero & Garcia-Carmona, 2013; Jain & Gould, 2015). Their potential beneficial roles in human health and nutrition have also been widely studied (Gandia-Herrero *et al.*, 2016; Khan, 2016), driven in part by their reported antioxidant properties. They are generally classified into two groups, the red-violet betacyanins and the yellow-orange betaxanthins.

Our understanding of betalain biosynthesis has largely remained behind in comparison to other major groups of plant pigments such as anthocyanins and carotenoids. Nonetheless,

recent discoveries have led to the characterization of the last unknown steps in the core betalain biosynthetic pathway, offering new opportunities in betalain metabolic engineering. This may provide new sources and uses for these pigments in basic research (e.g. as chemical biosensors (DeLoache *et al.*, 2015), protein-labeling fluorophores (Cabanes *et al.*, 2016), or genetic transformation markers), as well as in commercial applications (e.g. as food colorants and dietary supplements, or in development of new ornamental plant varieties). The potential exploitation of betalain engineering is further elaborated in a recent review (Polturak & Aharoni, 2018)..

The betalain biosynthetic pathway (Fig. 1) holds several features that make it particularly amenable to metabolic engineering; it involves only few enzymatic steps, alongside spontaneously-occurring cyclization and conjugation reactions, the latter offering great versatility with regards to the possible end-products that can be formed and the target species that can be utilized for their production. Moreover, flexibility within the pathway is found in its order of reactions (e.g. glucosylation followed by conjugation *or vice versa*), and the varied catalytic activity of the cytochrome P450 enzymes involved in the pathway, which enables increased control over the produced betacyanin/betaxanthin ratio. These, and other attributes of the betalain pathway will be discussed, together with a brief summary of recent efforts aimed at their engineering.

II. Advances in betalain engineering in plants and microbes

Efforts to metabolically engineer betalains have progressed alongside new discoveries in the betalain biosynthetic pathway. Identification of the *DOD* gene (Mueller *et al.*, 1997; Christinet *et al.*, 2004), followed by later identification of homologs in additional betalain-producing species, first enabled *in-vitro* production of betaxanthins by recombinant expression in *Escherichia coli* of *Mirabilis jalapa DOD* (Sasaki et al., 2009; Sekiguchi et al., 2010), or beet *DODA* (Gandia-Herrero & Garcia-Carmona, 2012), though a different beet homolog, *BvDODA1*, is the major contributor to DOPA-4,5 dioxygenase activity *in-planta* (Hatlestad *et al.*, 2012; Chung *et al.*, 2015). Heterologous betalain production in plants supplemented with L-DOPA was also reported (Harris *et al.*, 2012). The subsequent discovery of *CYP76AD1* in red beet, enabled the production of betanidin in L-DOPA-fed baker's yeast (*Saccharomyces cerevisiae*) when co-expressed with *BvDODA1* (Hatlestad *et al.*, 2012). More recently, characterization of the first committed step of the pathway, namely the hydroxylation of tyrosine to L-DOPA, allowed the first examples of heterologous production of betalains without substrate feeding, both in microbes and plants (Fig. 2) (DeLoache *et al.*, 2015; Polturak *et al.*, 2016; Sunnadeniya *et al.*, 2016; Polturak *et al.*, 2017; Grewal *et al.*, 2018).

III. Current approaches and future directions in betalain engineering

Several strategies may be considered for further development of betalain metabolic engineering in plants and microbes, which stem from some of the key features and unique characteristics of the betalain biosynthetic pathway (Fig. 1):

1. Increasing yield. Betalains are synthesized from the aromatic amino acid tyrosine. Increasing betalain yields in a heterologous expression system may entail optimization of the pathway from tyrosine to the final betalain product, through selection of the best-performing isozymes from betalain-producing species and the optimal combination of regulatory elements that drive their expression. Yield optimization can alternatively be focused on increasing flux towards tyrosine production. Both in plants and microbes, tyrosine is derived from chorismate, the end product of the shikimate pathway (Schenck & Maeda, 2018). This pathway has been well-studied, enabling implementation of different strategies to increase tyrosine production. One such strategy would be the use of feedback-insensitive forms of enzymes that take part in tyrosine biosynthesis. For example, expression of a feedback-insensitive bacterial form of the shikimate-pathway gene, *AroG,* leads to an increase in tyrosine and tyrosine-derived metabolites in plants (Tzin *et al.*, 2013; Oliva *et al.*, 2015). Similarly, expression of a feedback-insensitive plant arogenate dehydrogenase (ADH), which converts arogenate to tyrosine, could be used for the same purpose. Indeed, development of an ADH isoform that is partially insensitive to tyrosine inhibition seems to be an evolutionary strategy that has been largely adopted by betalain-producing plants of the Caryophyllales order (Lopez-Nieves *et al.*, 2018). In a recent report, transient expression of this de-regulated ADH isoform (*BvADHα*) resulted in a 7-fold increase in betalain production, when expressed together with betalain biosynthetic genes in *N. benthamiana* (Timoneda et al., 2018). It remains to be seen whether this approach could also efficiently increase production of betalains or other tyrosine-derived metabolites in stably transformed plants; a detrimental effect to the plant may arise from diverting flux towards tyrosine production on the expense of the competing path to phenylalanine and the derived phenylpropanoids. A decrease in phenylalanine accumulation was indeed observed following *BvADHα* transient expression in *N. benthamiana* (Lopez-Nieves *et al.*, 2018). Similar approaches for engineering increased flux through the shikimate pathway have also been explored in various microbes (Rodriguez *et al.*, 2014; Averesch & Krömer, 2018). While there are currently no known examples of feedback inhibition in the betalain pathway downstream to tyrosine, future discovery of transcriptional or enzymatic inhibition mechanisms in this pathway could provide additional strategies for increasing betalain production.

2. Exploring new target organisms. To date, stable heterologous production of betalains has been reported in a limited number of plant species and microbes. Attaining betalain production in additional plant species, or other organisms, may be of interest with regards to development of novel ornamental varieties or exploration of new potential sources for betalains. It is plausible that betalain production could be engineered in a wide variety of organisms, as betalains are formed only two or three enzymatic steps downstream from a ubiquitously present precursor, tyrosine. However, tyrosine availability varies between different species and can be a limiting factor for betalain production in some cases; visible

red pigmentation in Arabidopsis seedlings expressing *CYP76AD1* and a *Portulaca grandiflora DOD* was obtained only with supplementation of tyrosine (Sunnadeniya *et al.*, 2016). Similarly, a weak pigmentation phenotype in flowers of transgenic betalain-producing *Petunia hybrida* (Polturak *et al.*, 2017) was accentuated when flowers were fed with tyrosine (unpublished data). Where levels of free tyrosine are sufficient, expression of only two or three genes is essentially required for heterologous production of betaxanthins or glucosylated betacyanins, respectively. By contrast, engineering heterologous anthocyanin production, for example, can at times present a more elaborate task, where in some cases expression of 13 to 14 genes is required for this purpose (Zhu *et al.*, 2017; Levisson *et al.*, 2018). In other cases, however, induction of anthocyanin biosynthesis in plant tissues can be more easily achieved through expression of transcription factors that upregulate the naturally occurring anthocyanin pathway genes (Lloyd *et al.*, 1992; Borevitz *et al.*, 2000; Butelli *et al.*, 2008). A similar approach might also be considered for engineering betalains. A MYB-type transcription factor acting as a positive regulator of betalain biosynthesis, *BvMYB1*, has been identified in red beet (Hatlestad *et al.*, 2015). However, heterologous expression of *BvMYB1* in other betalain-producing plant species in means of increasing betalain production has not been reported, to date.

3. Accessing the betalain color range. Members of the *CYP76AD1*-like subfamily of cytochrome P450s that take part in betalain biosynthesis, play a major role in directing the pathway towards betacyanin or betaxanthin production. Consequently, controlling their activity may allow access to the entire color palette of betalains observed in plants. Within this subfamily, genes can be further divided into clades based on their sequence (Brockington *et al.*, 2015). Enzymes belonging to the α-clade (e.g. CYP76AD1/2/3/4) catalyze conversion of tyrosine to L-DOPA and subsequently to *cyclo*-DOPA, while those of the β-clade (e.g., CYP76AD5/6/15) catalyze merely the hydroxylation of tyrosine to L-DOPA (Hatlestad *et al.*, 2012; Polturak *et al.*, 2016; Sunnadeniya *et al.*, 2016; Polturak *et al.*, 2018). The difference in activity between the two enzyme groups eventually affects the relative yellow to red pigmentation attained; while betaxanthins are formed by spontaneous conjugation of the L-DOPA derived betalamic acid with any free amino acid, betacyanin formation is also dependent on the presence of *cyclo*-DOPA or its derivatives. Thus, betalain engineering can effectively be directed towards production of predominantly red or yellow pigmentation, through expression of an α- or β- clade member, respectively. Combined expression of CYPs from both groups can lead to formation of other colors in between the red to yellow spectrum (Polturak *et al.*, 2017). The hues eventually obtained may possibly be further fine-tuned by adjusting gene expression levels or catalytic activity of the different CYPs. In that regard, it will be of interest to explore the possibility to manipulate pigmentation in natural betalain-producing plants through precise genome editing of the *CYP76AD1*-like loci, in means of altering expression or enzymatic activity. For example, a single amino acid mutation in CYP76AD1 was shown to hinder its L-DOPA oxidation activity while retaining tyrosine hydroxylase activity, leading to accumulation of betaxanthins instead of betacyanins when co-expressed with *DOD* in yeast (DeLoache *et al.*, 2015).

4. Expanding the chemical diversity of betaxanthins. Another key feature of the betalain pathway stems from its pigment-forming step, in which betalamic acid spontaneously conjugates with free amino acids and other amines to form betaxanthins, or with *cyclo*-DOPA and its derivatives to form betacyanins. The fact that these reactions occur spontaneously, averts the specificity typically constrained by enzymatic catalysis, and thus contributes to the diversity of naturally-occurring betalains. Moreover, it adds great versatility to the possible betalain molecules that can be biosynthesized artificially. Notably, the absorption spectral range typically exhibited by naturally-occurring betalains can be significantly widened, as the condensation of amines with betalamic acid, in itself a chromophore, can affect the number and position of conjugated bonds in the formed molecule. This was demonstrated in engineered betalain-producing yeast that were fed with different amines, which led to formation of pigments ranging from orange to blue-violet color (Grewal *et al.*, 2018). Nevertheless, in contrast to the flexibility offered by the spontaneous condensation reaction, it can also present a major challenge in cases where production of a specific type of betalain compound is desired. This challenge may partially be overcome by (i) increasing the availability of a specific amine conjugate for condensation with betalamic acid (e.g. through feeding or genetic modification of a metabolic pathway), thereby enhancing formation of a specific product-of-interest or (ii) *in-vitro* production of a single betaxanthin product, for example by reacting L-DOPA with a DOD enzyme and the amine conjugate (Sekiguchi *et al.*, 2010), or by utilizing an amine polymer-based betalamic acid-derivatized support (Cabanes *et al.*, 2014).

5. Modifying betacyanin decoration. Recent attempts to heterologously produce betacyanins were principally directed towards the most prevalent betacyanin, betanin. Production of this single-glucosylated compound requires the expression of a UDP-glucosyltransferase which can glycosylate either *cyclo*-DOPA or betanidin at the 5-*O* position. Betanidin-5-*O* and *cyclo*-DOPA-5-*O* glucosyltransferases have previously been identified from several betalain-producing plants (Vogt *et al.*, 1999; Sasaki *et al.*, 2005; Sepulveda-Jimenez *et al.*, 2005) and may be used for engineered production of betanin. Biosynthesis of more highly decorated betacyanins may also be of commercial interest, considering that some betacyanins exhibit improved stability or color-retention properties compared to betanin (Reynoso *et al.*, 1997; Schliemann & Strack, 1998; Herbach *et al.*, 2005). However, to access the wide variety of naturally-occurring betacyanins, more research will be needed in order to identify genes responsible for decorating reactions on betanin, including additional glycosylations and acylations. Similarly, gomphrenin I (betanidin 6-*O*-β-glucoside) may essentially be produced by expression of the known betanidin-6-*O*-glucosyltransferase (Vogt, 2002). Yet, downstream genes required for the production of other gomphrenin-type betacyanins remain unknown. Additionally, acylation and glycosylation of heterologously produced betalains can also occur through activity of endogenous enzymes in the host plant, resulting in inadvertent formation of various betalains, including possibly new-to-nature compounds such as glycosylated betaxanthins (Polturak *et al.*, 2016; Polturak *et al.*, 2017).

6. Engineering secretion of betalains. An ultimate aspect to be considered is the release of betanin, or other end products, from the cell into the extracellular surroundings. In the case of plant cells, attempts to produce betalains in tobacco BY-2 and *Arabidopsis* T87 cells have shown that both betacyanins and betaxanthins largely remain inside the cell (Nakatsuka *et al.*, 2013; Polturak *et al.*, 2017). This is consistent with cell or hairy root cultures derived from naturally-producing plants, where betalain release must be achieved by permeabilization of the cells using various physical or chemical approaches including oxygen limitation, use of surfactants or acids, temperature stress, and sonification (Thimmaraju *et al.*, 2003; Georgiev *et al.*, 2008). Application of these methods may also be considered in the case of betalain production *via* engineered transgenic cell cultures. Conversely, betalains produced in yeast were found to be spontaneously released from the cells, including betaxanthins, betanidin and betanin (Hatlestad *et al.*, 2012; Polturak *et al.*, 2016; Sunnadeniya *et al.*, 2016; Grewal *et al.*, 2018). It is currently unclear whether the compounds are passively released from the yeast cells or actively secreted via membranal transporters. Future identification and overexpression of such transporters in yeast may result in enhanced secretion and productivity. In plant cells, betalains are deposited in vacuoles, where various specialized metabolites are commonly stored to prevent degradation or formation of adverse metabolic derivatives. Identification of dedicated transporters from betalain-producing plants (e.g. transporters involved in vacuolar sequestration of betalains), may also be utilized to enhance betalain secretion from the cellular membrane in a microbial or plant cell system, or alternatively to increase pigment accumulation in plant cell cultures by facilitating sequestration into the vacuole. In any case, enhancement of secretion would likely enable an increase in overall yield, as well as facilitate purification of the compounds-of-interest. In this context, optimizing extraction and processing methods, including consideration of factors contributing to betalain degradation, is imperative for obtaining high pigment yields. This topic has been well studied and was previously reviewed (Esquivel, 2016).

IV. Conclusions

While some genes involved in betalain biosynthesis in plants have been identified over a decade ago, the core biosynthetic pathway has only recently been fully elucidated. Thus, the number of examples for stable heterologous betalain production reported to date is limited. Considering the various potential uses of betalains, taken together with the simplicity and versatility of their biosynthetic pathway, future achievements in betalain metabolic engineering will likely follow. These advances will provide new approaches to study the roles betalains play in plants, and may ultimately lead to novel commercial application, such as developing new varieties of ornamentals, enhancing nutritional value of food crops, and providing new betalain sources for use as colorants in the food, cosmetics or pharmaceutical industries. However, endeavours for commercialization of these future advancements will also be met with challenging regulatory hurdles and consumer acceptance issues. Thus, continued developments in metabolic engineering of betalains, as well as of other high-value metabolites, will have to be accompanied with progress in societal acceptance of synthetic biology applications.

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Figure 1. Approaches in betalain metabolic engineering derived from attributes of the betalain biosynthetic pathway. Tyrosine is 3-hydroxylated to L-DOPA by an *α*-clade (e.g. CYP76AD1 in *Beta vulgaris*) or *β*-clade (e.g. CYP76AD6 in *B. vulgaris*) cytochrome P450 of the CYP76AD1-like subfamily. L-DOPA is converted to *cyclo*-DOPA by an *α*-clade cytochrome P450 or to betalamic acid by DOPA 4,5-dioxygenase (DOD). Betalamic acid undergoes spontaneous conjugation with amino acids and other amines, or with *cyclo*-DOPA to respectively form betaxanthins or the aglycone betacyanin betanidin. Betanidin is next glycosylated by betanidin 5-*O*-glucosyltransferase to form betanin. Alternatively, *cyclo*-DOPA is first glycosylated by *cyclo*-DOPA-5-*O*-glucosyltransferase (cDOPA5GT) and is next conjugated with betalamic acid for direct formation of betanin. Betanin can undergo additional glycosylation and acylation reactions leading to more complex betacyanins. Dashed lines represent an alternative glucosylation path for the formation of betanin. Asterisks denote spontaneous condensation reactions. Illustrations numbered 1–6 are referred to in the corresponding paragraphs in section III 'Current approaches and future directions in betalain engineering' of the main text.

Figure 2. Metabolic engineering of betalains in plants and yeast. (a) Expression of three genes from the betalain core biosynthetic pathway, namely *CYP76AD1* and *BvDODA1* from *Beta vulgaris*, and *cDOPA5GT* from *Mirabilis jalapa*, is sufficient for betacyanin formation in naturally-non-producing plants. Images in clockwise from top left: tobacco hairy root culture, tomato, tobacco BY-2 cells, eggplant. (b) Expression of *B. vulgaris CYP76AD6* and *BvDODA1* enables production of betaxanthins in *Nicotiana benthamiana* (upper) and baker's yeast (lower).

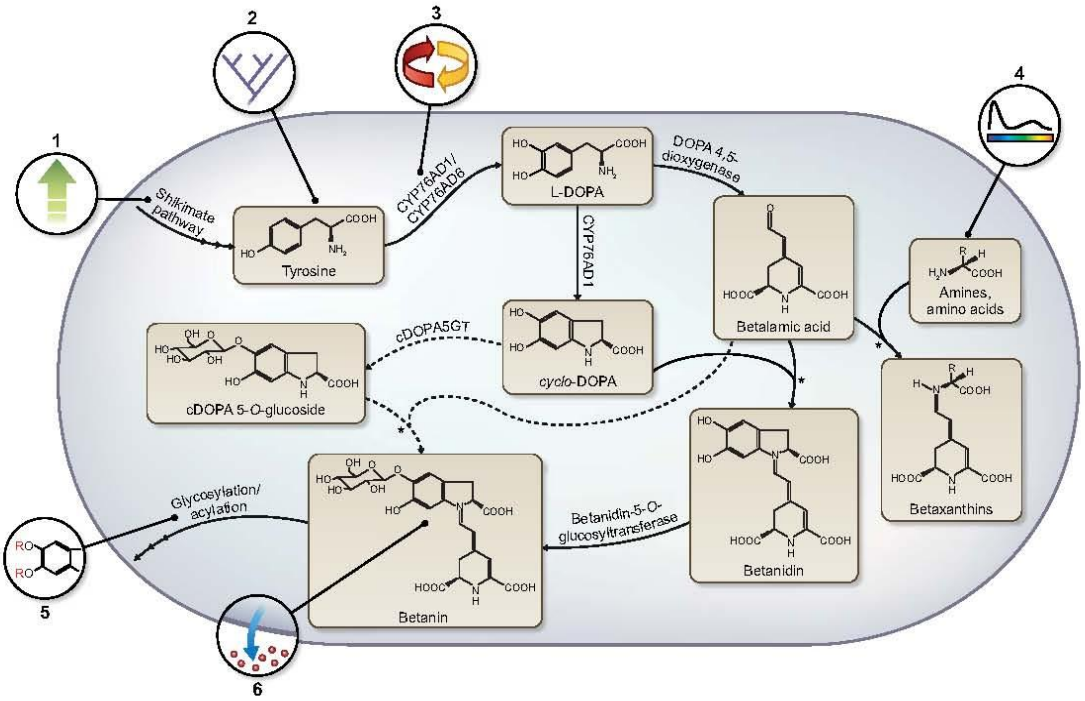

Figure 1

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Figure 2

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