Biotechnology Advances 43 (2020) 107600

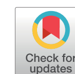
Contents lists available at ScienceDirect

Biotechnology Advances

journal homepage: www.elsevier.com/locate/biotechadv

Research review paper

Anthocyanins, multi-functional natural products of industrial relevance: Recent biotechnological advances



Tarun Belwal[a,*], Gopal Singh[b,c], Philippe Jeandet[d], Aseesh Pandey[e], Lalit Giri[b], Sudipta Ramola[f], Indra D. Bhatt[b], Petras Rimantas Venskutonis[g], Milen I. Georgiev[h,i], Christophe Clément[d], Zisheng Luo[a,j,k,*]

[a] Zhejiang University, College of Biosystems Engineering and Food Science, Zhejiang Key Laboratory for Agri-Food Processing, Key Laboratory of Agro-Products Postharvest Handling of Ministry of Agriculture and Rural Affairs, Hangzhou 310058, People's Republic of China
[b] G.B. Pant National Institute of Himalayan Environment, Kosi- Katarmal, Almora 263643, India
[c] Biotechnology Division, CSIR-Institute of Himalayan Bioresource Technology, Palampur 176061, Himachal Pradesh, India
[d] Research Unit, Induced Resistance and Plant Bioprotection, EA 4707, SFR Condorcet FR CNRS 3417, Faculty of Sciences, University of Reims Champagne-Ardenne, PO Box 1039, 51687 Reims Cedex 2, France
[e] G.B. Pant National Institute of Himalayan Environment, Sikkim Regional Centre, Pangthang, Gangtok 737101, Sikkim, India
[f] College of Chemical Engineering, Zhejiang University of Technology, Hangzhou 310014, China
[g] Department of Food Science and Technology, Kaunas University of Technology, Radvilėnų pl. 19, Kaunas LT-50254, Lithuania
[h] Center of Plant Systems Biology and Biotechnology, Plovdiv, Bulgaria
[i] Laboratory of Metabolomics, The Stephan Angeloff Institute of Microbiology, Bulgarian Academy of Sciences, Plovdiv, Bulgaria
[j] National-Local Joint Engineering Laboratory of Intelligent Food Technology and Equipment, Zhejiang R&D Center for Food Technology and Equipment, Zhejiang University, Hangzhou 310058, People's Republic of China
[k] Ningbo Research Institute, Zhejiang University, Ningbo 315100, People's Republic of China

ARTICLE INFO

Keywords:
Anthocyanins
In vitro culture
Callus culture
Cell culture
Biosynthesis
Metabolic engineering
Transcription factors
Regulatory genes
Microbial cell factory
Biotechnological methods

ABSTRACT

Anthocyanins, the color compounds of plants, are known for their wide applications in food, nutraceuticals and cosmetic industry. The biosynthetic pathway of anthocyanins is well established with the identification of potential key regulatory genes, which makes it possible to modulate its production by biotechnological means. Various biotechnological systems, including use of *in vitro* plant cell or tissue cultures as well as microorganisms have been used for the production of anthocyanins under controlled conditions, however, a wide range of factors affects their production. In addition, metabolic engineering technologies have also used the heterologous production of anthocyanins in recombinant plants and microorganisms. However, these approaches have mostly been tested at the lab- and pilot-scales, while very few up-scaling studies have been undertaken. Various challenges and ways of investigation are proposed here to improve anthocyanin production by using the *in vitro* plant cell or tissue culture and metabolic engineering of plants and microbial culture systems. All these methods are capable of modulating the production of anthocyanins , which can be further utilized for pharmaceutical, cosmetics and food applications.

1. Introduction

Anthocyanins, color pigments present in plants, are highly utilized flavonoid group of compounds known for their physical, chemical and biological properties. Various nutraceutical, pharmaceutical, and cosmeceutical industries utilize anthocyanins as natural colorants and/or for their biological functions. Besides, these are also employed as natural preservatives, flavor scavengers as well as protecting food ingredients against environmental stresses during storage and transportation (Shipp and Abdel-Aal, 2010; Liu et al., 2018). Fruits, berries, and some vegetables as well as flowers are the major natural source of these compounds and are selectively synthesized depending on genetic, climatic and edaphic factors (Pourcel et al., 2010; Borochov-Neori et al., 2011; Iorizzo et al., 2019). The higher demand for anthocyanins could be assessed from their high market value, which was estimated to be USD 305 million in 2018 and is projected to grow at a compound annual growth rate (CAGR) of 4.7% from 2018–2023 (Global Anthocyanin Market report, 2020-2025). The increasing use of

* Corresponding authors at: Zhejiang University, College of Biosystems Engineering and Food Science, Zhejiang Key Laboratory for Agri-Food Processing, Key Laboratory of Agro-Products Postharvest Handling of Ministry of Agriculture and Rural Affairs, Hangzhou 310058, People's Republic of China.
E-mail addresses: research@thetbellab.in (T. Belwal), luozisheng@zju.edu.cn (Z. Luo).

https://doi.org/10.1016/j.biotechadv.2020.107600
Received 20 March 2020; Received in revised form 6 July 2020; Accepted 10 July 2020
Available online 18 July 2020
0734-9750/ © 2020 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY license
(http://creativecommons.org/licenses/BY/4.0/).

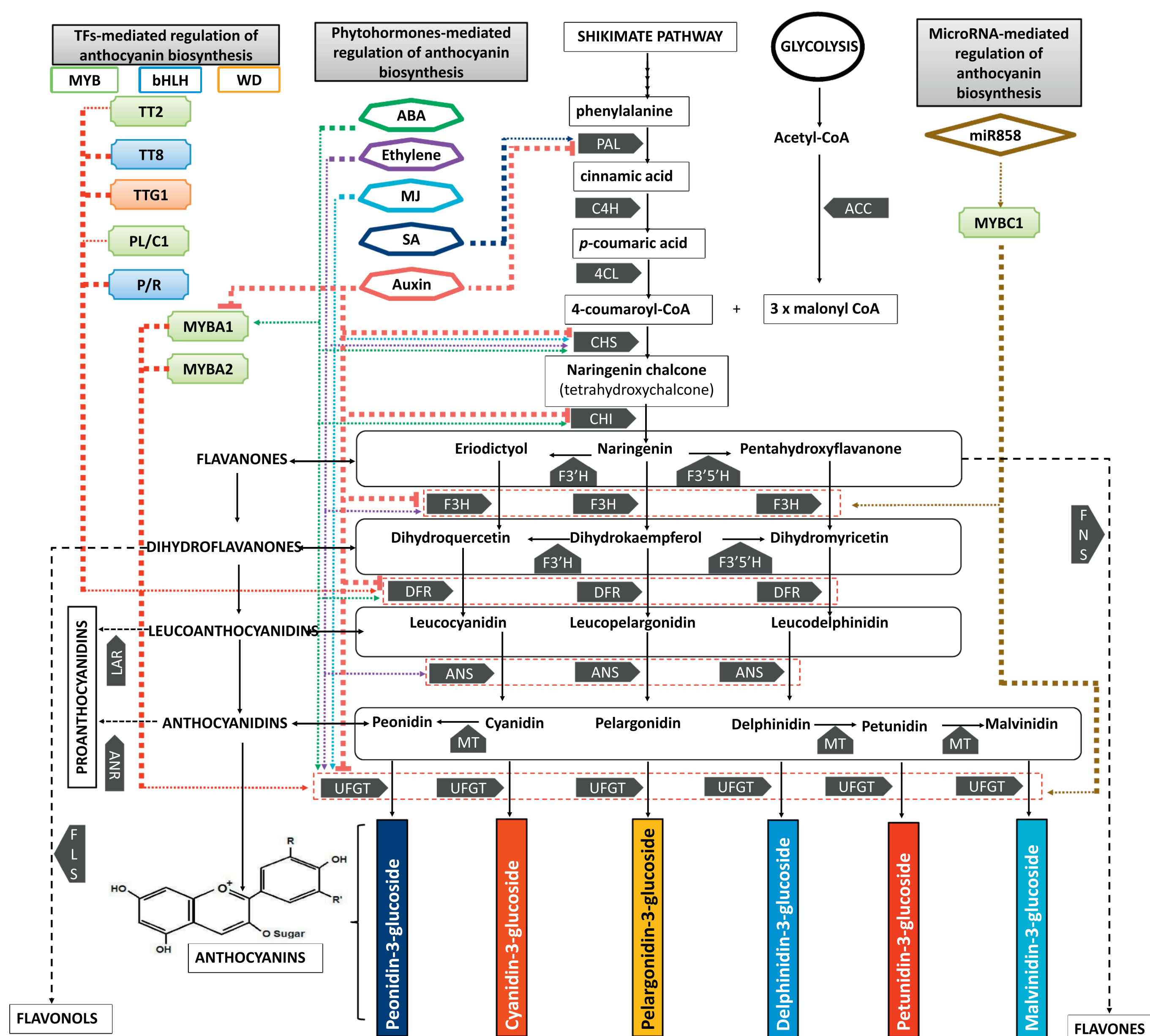


Fig. 1. Schematic representation of the biosynthetic pathway of anthocyanidins and anthocyanins. Enzymes, catalyzing each step, are indicated by abbreviated capital letters in black boxes and most of them loosely bound to the endoplasmic reticulum. Dotted arrows indicating multi-step process. In each step, end products are indicated (left) by capital letters, except for the multi-step end products *i.e.* flavonols and flavones. The end products viz. proanthocyanin and anthocyanin stored in vacuoles (anthocyanoplasts). Small dotted lines denote regulation of anthocyanin biosynthesis through the action of various transcription factors of MBW complex, phytohormones and anthocyanin-specific microRNA. *PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL,* para*-coumaroyl:CoA ligase; CHS, chalcone synthase; CHI, chalconeisomerase; F3H, flavanone3-hydroxylase; F3'H, flavonoid-3' -hydroxylase; F3'5'H, flavonoid-3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; ANS, anthocyanidin synthase; UFGT, UDP-glucose flavonoid 3-O-glucosyl transferase; FLS, flavonol synthase; FNS, flavone synthase; MT, O-methyltransferases; LAR, leucoanthocyanidinreductase; ANR, anthocyanidinreductase; ACC, acetyl-CoA carboxylase; ABA, abscisic acid; MJ, Methyl jasmonate; SA, salicylic acid; TT2/TT8, transparent testa 2/8; TTG1, transparent testa glabra1*

anthocyanins as natural colorants, largely in beverages and food products as well as bioactive compounds in health, nutraceuticals and cosmetics has extensively boosted their market requirement. The high demand for these valuable natural compounds, along with climate change and other environmental issues exert tremendous pressure on the availability of raw materials (plants) and its supply chain; thus, needed alternative solutions.

In recent times, efforts have been made to meet up the demand of anthocyanins and provide sustainable means for their production utilizing various biotechnological advancements. As such, plant cell or tissue culture (PCTC) involve culturing of plant cells or tissues for the vegetative propagation of desired plants and/or the biosynthesis of secondary metabolites for multiple uses. Plant cells have special ability (called "totipotency") to divide and differentiate into multiple cell types by simply varying growth medium and culture conditions. Plant cell suspension cultures provide an excellent means for modulation of the secondary metabolites production under varied culture conditions independent of their natural sources. The conditions, such as culture medium, pH, agitation rate, air flow, etc. need to be precisely selected and optimized for higher production of targeted compounds in any of the plant or cell cultures. Various enzymes involved in the biosynthesis of anthocyanins have been reported to be regulated by precursors/

elicitors/substrates and culture conditions (light, pH, temperature, plant growth regulators, macro and micro nutrients), resulting in substantial changes in the quality of the end products (Simões et al., 2012). The production of anthocyanins in PTCT displays various advantages such as all-time availability, possibility of modification of the anthocyanin skeleton, better control of culture conditions, climate independency, and higher yield. In order to provide an alternative means for meeting the higher market demand of anthocyanins or to provide phenotypic changes, genetic modification/manipulation in plants have also been conducted.

Microbial cell factories (MCF) are regarded as one of the most promising systems for large-scale production of anthocyanins (Marienhagen and Bott, 2013; Pandey et al., 2016). By altering the culture conditions, it is possible to modify the anthocyanin biosynthetic pathways for the production of novel compounds (Ochoa-Villarreal et al., 2016). Use of advanced genetic and metabolic engineering approaches, for manipulation or introduction of any specific biosynthetic gene(s)/pathways into different heterologous hosts (especially microorganisms such as bacteria and yeast) is another potential approach to improve the production of secondary metabolites (Yuan and Grotewold, 2015; Pandey et al., 2016; Chouhan et al., 2017; Jeandet et al., 2018; Marchev et al., 2020). These techniques showed promising applications in terms of higher yield and quality production of anthocyanins under various culture conditions. Advancements in culture techniques such as the use of novel elicitors, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR/Cas) gene editing, bioreactors are leading to encouraging results.

Due to higher demand and applications for anthocyanins as food and beverage supplements, nutraceuticals, pharmaceuticals, and cosmeceutical agents, various biotechnological tools and techniques have largely been explored and applied for improving their biosynthesis and accumulation. The present review thus highlights the advantages and challenges for the use of cell cultures and engineered cells (plants/microbes) in the production of anthocyanins with regards to efficiency, feasibility, and reproducibility. Future recommendations and scientific attentions on the better use of these biotechnological tools and techniques for the efficient production of anthocyanins are also made.

2. Anthocyanins chemistry, biosynthesis and its regulation

2.1. Anthocyanins chemistry

Anthocyanins are naturally-occurring pigments belonging to the group of flavonoids and more than 600 anthocyanins have been identified so far (Smeriglio et al., 2016). These are mostly located inside cell vacuoles (Liu et al., 2018) and the presence of an etherified sugar (usually glucose) at the C3 position ensures their aqueous solubility (Fig. 1). The light absorption of anthocyanins is attributed to overall ring structure and conjugated double bonds (Khoo et al., 2017). Changing in the intravacuolar environment [*i.e.*, co-existing colorless compounds (flavones and flavanols), metal ions, and pH] bring variation in the color property of anthocyanins (Liu et al., 2018). The Na^{+}/H^{+}-antiporter (structural gene that regulates the vacuolar pH with relevance to color) was identified in Japanese morning glory (*Ipomea nil*), and expressed prior to flower opening in order to increase the vacuolar pH and consequently affording blue colored flowers (Fukada-Tanaka et al., 2000).

Anthocyanidins represent sugar-free counterparts of anthocyanins and anthocyanidins (aglycones) having sugar and acyl conjugates represent anthocyanins (Stommel et al., 2009). Structurally, the anthocyanin skeleton is based on the 2-phenylbenzopyrilium cation (Fig. 1). More than 20 anthocyanidins are known, and only six viz. cyanidin, delphinidin, malvidin, pelargonidin, peonidin and petunidin (Fig. 1) are prevalent in plants (Kong et al., 2003; Zhao et al., 2014) particularly in flowers, fruits, and tuber parts (Khoo et al., 2017). Diversification of anthocyanins is mainly influenced by its backbone (anthocyanidin) and the position of conjugated sugar, quantity, structure, and other moieties such as the presence of methyl groups also play an important role (Liu et al., 2018). For instance, a single *O*-methylation on 3-*O*-glucoside position of cyanidin resulted in the formation of peonidin. Similarly, single and double methylation of delphinidin results into petunidin and malvidin, respectively (Fig. 1) Depending on the number of hydroxyl groups at the B-ring, anthocyanidins showed different color hues. For instance, cyanidin, delphinidin and pelargonidin, showed red to magenta, violet to blue and orange to red color hues, respectively (Tanaka and Ohmiya, 2008). Further, the content of anthocyanin in different parts of plant depends on the regulatory bodies (precursors and enzymes) of biosynthetic and degradation pathways. Moreover, metabolism of anthocyanins is also regulated by different developmental, environmental and genetic factors (Liu et al., 2018).

Pyranoanthocyanins are minor plant anthocyanins having an additional pyran ring (ring D) between the C-4 and hydroxyl group of the C-5 in anthocyanin skeleton, which are found in wines, strawberry, grape pomace, black carrot and blood orange juice at a low quantity. Vitisin A and vitisin B, respectively resulting from the addition of pyruvic acid and ethanal to malvidin-3-*O*-glucoside are the most common types of pyranoanthocyanins found in red wine (Hillebrand et al., 2004; Akdemir et al., 2019; Morata et al., 2019; Ruta and Farcasanu, 2019). Similarly, vinylphenolic pyranoanthocyanins are usually formed by condensation of free hydroxycinnamic acids and anthocyanins during fermentation and red wine aging (Morata et al., 2019). Their enhanced stability and resistance to SO_2 bleaching compared to the parent anthocyanins have increased commercial interest in these minor anthocyanins. This could be evident during red wine aging wherein conversion of anthocyanins to pyranoanthocyanins leads to enhancement in wine flavours and antioxidant properties (Quina and Bastos, 2017; Akdemir et al., 2019).

2.2. Anthocyanins biosynthesis

Biosynthesis of anthocyanins begins from phenylpropanoid pathway through the transformation of phenylalanine into *para*-coumaroyl-CoA, which is the first precursor molecule in the flavonoid biosynthetic pathway (Fig. 1). The first specific enzyme working on the anthocyanin pathway is chalcone synthase (CHS), which produces chalcone skeletons from which all flavonoids are derived (Ferreyra et al., 2012). The CHS catalyzes the synthesis of 2',4,4',6'-tetrahydroxy chalcone (THC) using one molecule of *para*-coumaroyl-CoA and three molecules of malonyl CoA. The THC is hastily and stereo-specifically isomerized to the colorless naringenin via the chalcone isomerase (CHI). Enzymes associated to the phenylpropanoid biosynthesis are equipped into macromolecular complexes and related with endomembranes (Kutchan, 2005). Naringenin is hydroxylated at the 3rd position by the flavanone 3-hydroxylase (F3H) to produce hydroflavonols, i.e., dihydrokaempferol. F3H belongs to the 2-oxoglutarate-dependent dioxygenase (OGD) family, which catalyzes the hydroxylation of eriodictyol and pentahydroxylflavanones to dihydroquercetin and dihydromyricetin, respectively (Fig. 1). Flavonoid 3'-hydroxylase (F3'H) and flavonoid 3', 5'-hydroxylase (F3'5'H) are cytochrome P450 enzymes, catalyzing the hydroxylation of dihydrokaempferol to dihydroflavonols (viz. dihydroquercetin and dihydromyricetin, respectively). Both F3'H and F3'5'H determine the hydroxylation pattern of the B-ring of flavonoids including anthocyanins, and are essential for the production of cyanidin and delphinidin. It is reported that plant species such as rose (*Rosa hybrida*), and chrysanthemum (*Chrysanthemum morifolium*) do not produce delphinidin, consequently lack violet/blue color varieties, and that can be attributed to the lack of the F3'5'H gene (Tanaka et al., 2008). However, transgenic blue/violet roses are being developed through the heterologous expression of the F3'5'H gene (Chandler and Tanaka, 2007; Katsumoto et al., 2007), thus underlying its importance. Further, by the action of dihydroflavonol 4-reductase (DFR) enzyme, dihydroflavonols are reduced to the corresponding 3,4-*cis*-

leucoanthocyanidins. In some plant species (e.g. petunia and cymbidium), DFR displays a strict substrate specificity and cannot utilize dihydrokaempferol; hence, these species lack pelargonidin-based anthocyanins, resulting in no brick red/ orange color flowers (Tanaka et al., 2008). Anthocyanidin synthase (ANS, also referred to as leucoanthocyanidin dioxygenase), belongs to the OGD family and catalyzes the synthesis of consequent colored anthocyanidins.

Additionally, conjugation of free anthocyanins with other metabolites (pyruvate, acetaldehyde, hydroxycinnamic acids, and vinylphenols) are reported to be responsible for carboxypyranoanthocyanins biosynthesis. This cyclic condensation occurs between C-4 and –OH group at C-5 of basic anthocyanin skeleton and double bonded enolic form of pyruvate followed by subsequent dehydration and rearomatization steps (Morata et al., 2007; Quina and Bastos, 2017). Interestingly, addition of vinylphenol in red wine anthocyanins was found to introduce a bathochromic shift in their absorption maxima towards 540 nm wavelength which corresponds to a bluish red colored pigmentation (Schwarz et al., 2003; Morata et al., 2019).

Most of the anthocyanin-synthesizing enzymes are loosely bound to the endoplasmic reticulum (EPR), whereas their end products like anthocyanins and proanthocyanidins accumulated in vacuoles (Winkel-Shirley, 2001). Studies indicate that CHS and CHI in *Arabidopsis* have been localized in the nucleus (Saslowsky et al., 2005). Moreover, F3'H in soybean has been localized in the tonoplast (hilum region) of the immature seed coat (Toda et al., 2012). The mechanics of anthocyanin trafficking from the EPR to their storage sites (i.e. vacuoles) can be explained by two models, i) the ligandin transport, and ii) the vesicular transport (Grotewold and Davis, 2008; Zhao and Dixon, 2010; Ferreyra et al., 2012). The ligand in transport has genetic evidences, indicating that in maize, petunia and *Arabidopsis* (AtTT19), glutathione transferase (GST)-like proteins are required for the vacuolar impounding of pigments (Marrs et al., 1995; Alfenito et al., 1998; Ferreyra et al., 2012). Wherein, the vacuolar transportation of anthocyanins in maize requires a multidrug resistance associated protein-type (MRP) transporter located on the tonoplast membrane whose expression is co-regulated with the structural anthocyanin genes (Goodman et al., 2004). Moreover, the vesicular transport mechanism supposes that the anthocyanins which accumulated in the cytoplasm in distinct vesicle-like structures (*i.e.*, anthocyanoplasts), undergo a transportation process into the vacuole through an autophagocytic mechanism (Pourcel et al., 2010).

2.3. Regulation of anthocyanin biosynthesis

Biosynthesis of anthocyanins usually takes place under strict cellular regulation especially through the interplay of various transcription factors of MYB-bHLH-WDR (MBW) complex and phytohormones (Fig. 1; He et al., 2010; Saigo et al., 2020). The regulation of structural genes of anthocyanins is suggested to be differentially modulated in dicots and monocots, although they are controlled by the same MBW complex that commonly includes two imperfect myeloblastosis protein repeats (R2R3MYB) transcription factors, basic helix-loop-helix (bHLH), and WD40 proteins (Grotewold, 2005; Petroni and Tonelli, 2011; Zhang et al., 2019). In dicots, the initial enzymatic step leading to proanthocyanidin biosynthesis is catalyzed by an anthocyanidin reductase (DFR) enzyme encoded by the *BANYULS* gene (BAN) and this specific expression pattern is mainly bestowed by TT2, a R2R3MYB transcription factor encoded by the *TRANSPARENT TESTA2* gene (Baudry et al., 2004; Xie et al., 2004. For instance, Nesi et al. (2000) have characterized two additional regulatory genes, namely *TRANSPARENT TESTA8* (TT8) and *TRANSPARENT TESTA GLABRA1* (TTG1) that participate in the control of *BAN* expression. Likewise, other members of MYB (*PRODUCTION OF ANTHOCYANIN PIGMENT 1, 2; PAP1, PAP2*), and bHLH (*GLABRA3*, *GL3*, and *ENHANCER OF GLABRA3*, *EGL3*) have also been studied to regulate anthocyanin biosynthesis in *A. thaliana* (Baudry et al., 2004; Feller et al., 2011; Petroni and Tonelli, 2011). In addition, *PAP1* (R2R3 MYB) gene was also identified to regulate anthocyanin biosynthesis by binding directly to G- and ACE-boxes in the promoter region of HY5 (a component of light-signaling pathway) in *A. thaliana* (Shin et al., 2013). On the other hand, PL/C1 (MYB) and B/R (bHLH) have been identified to modulate DFR expression in monocot (Carey et al., 2004). The cooperative action of bHLH protein R and MYB protein C1 (C1/R complex) has been suggested to be critical for A1 (*DFR*) gene expression through increased acetylation of H3 histone (K9/K14) at its promoter binding site (Hernandez et al., 2007).

Several other members of the MYB transcription factor family (*VvMYBA1* and *VvMYBA2*) have also been found to regulate the expression of *UFGT* gene of anthocyanin biosynthesis (Cutanda-Perez et al., 2009; He et al., 2010). Interestingly, the presence of a retrotransposon (*Gret1*) in the 5'-flanking region of the *MYBA1* homolog in white grapes have been validated to lead its functional abnormality; however, deletion of this retrotransposon was reported to cause a bud mutation for white to red skins due to restoration of anthocyanin biosynthesis (Kobayashi et al., 2005). Similarly, two R2R3 MYBs (McMYB12a and McMYB12b) have been found to regulate the expression of proanthocyanin biosynthesis in *Malus* crabapple. Overexpression of these two genes in tobacco resulted in an improved anthocyanin biosynthesis as evidenced by pigmented petals, while silencing reduced its level (Tian et al., 2017). Similarly, two *StMYB44* genes (R2R3 MYB transcription factor) have also been identified to integrate environmental conditions (high temperature) and anthocyanin biosynthesis in potato tuber (Liu et al., 2019a). The transient expression of these two genes in tobacco leaves caused downregulation of anthocyanin biosynthetic pathway genes; however, during heat stress conditions, high activity of the basic phenylpropanoid pathway shifted metabolic fluxes towards lignin or chlorogenic acid synthesis (Liu et al., 2019a). Recently, two MYBs (*DcMYB6* and *DcMYB7*) were studied to interact with other members of the MBW complex to control anthocyanin biosynthesis in purple-pigmented carrots (Xu et al., 2019). The overexpression of *DcMYB7* genes leads to the appearance of a purple pigmentation throughout the carrot root tissues as well as in transgenic *A. thaliana*. However, a complete depigmentation was observed in a knockout mutant with yellowish pigmented roots confirming its vital role for purple phenotype in carrot roots (Xu et al., 2019). Interestingly, the function of another R2R3 MYB (*SlMYBTV*), a SlAN2-like anthocyanin repressor gene has been identified to be crucial for anthocyanin biosynthesis and purple-pigmented phenotype in tomato, as it is absent in cultivated tomatoes and show purple pigmentation in transgenic tomato (Sun et al., 2020).

Phytohormones, a collective terminology for an array of plant specialized metabolites are known to regulate various cellular and developmental processes in plants, including anthocyanin biosynthesis (Fig. 1). Due to their high potency to modulate targeted plant metabolism, these are usually used as chemical elicitors to enhance anthocyanin production under *in vitro* conditions (He et al., 2010). Abscisic acid (ABA), a well-studied phytohormone has been reported to improve anthocyanin biosynthesis as complemented by higher expression of several structural genes (*CHS*, *CHI*, *DFR* and *UFGT*; Jeong et al., 2004). Similarly, ethylene, another well-studied phytohormone was reported for its role in fruit ripening, and also enhanced anthocyanin level alongwith upregulation of *CHS*, *F3H*, *ANS*, *UFGT* genes, though not affecting *DFR* expression (El-Kereamy et al., 2003). Another stress hormone, methyl jasmonate (MJ) also stimulates anthocyanin biosynthesis as well as *CHS* and *UFGT* expression in grape cell suspension cultures (Belhadj et al., 2008). Salicylic acid (SA) is also reported to induce expression of *PAL* gene, thus could be considered as a positive regulator of anthocyanin biosynthesis (Wen et al., 2005). Interestingly, researchers have noticed negative impact of auxin on anthocyanin biosynthesis as 2,4-dichlorophenoxyacetic acid (2,4-D) or 1-naphthaleneacetic acid (1-NAA) reduced the expression of *PAL*, *CHS*, *CHI*, *F3H*, *DFR*, *UFGT* and *VvMYBA1* genes (Jeong et al., 2004; He et al., 2010).

In addition to transcription factors and phytohormone-mediated

regulation of anthocyanin biosynthesis, a recent study in kiwifruit has also revealed the role of miR858 in the regulation of fruit coloring (Li et al., 2019) (Fig. 1). It has been reported that the microRNA miR858 inhibits the expression of the *AaMYBC1* gene, which subsequently regulates *AaF3H* and *AaUFGT* genes to inhibit anthocyanin biosynthesis during the early green stages of kiwifruits. However, during fruit maturity, the expression level of this inhibitory microRNA is too low to express its inhibitory action on downstream targets, consequently leading to high anthocyanin accumulation to produce red pigmentation in ripped fruits (Li et al., 2019). This also indicates the need to investigate in depth the potential of non-coding small RNAs for the regulation of anthocyanin biosynthesis.

3. Anthocyanin production by biotechnological means

In general, there are three main ways to produce anthocyanins or anthocyanin-rich preparations. Anthocyanins are present as ubiquitous plant flavonoids in various natural sources particularly coloured fruits (açaí, blackcurrant, aronia, blackberry, black raspberry, blueberry, cherry, plum, blood orange, redcurrant, pyrus, grape) and vegetables (eggplant, purple corn, red cabbage, Okinawan sweet potato, purple yam) (Biswas and Mathur, 2017; Belwal et al., 2019). Due to high anthocyanin content in blackcurrant (*Ribes nigrum*), elderberry (*Sambucus nigra*) and aronia (*Aronia melanocarpa*), their juices are the best coloring agents and used in various products such as candies, confectionery, ice cream, soft drinks, or other fermented beverages (Vilela and Cosme, 2016). Currently, there are many companies producing and marketing anthocyanin rich extracts and other preparations from berries and vegetables; however, due to lack of natural blue colorants, it is being replacing by synthetic one (Sigurdson et al., 2017). The main disadvantages of using natural raw materials for the isolation of anthocyanins are (i) low abundance (on average, 20–1800 mg/100 g), (ii) environmental, seasonal, and regional variations, and (iii) pigment degradation during storage, extraction and purification. Anthocyanins can also be produced through chemical synthesis and first few evidences of the chemical synthesis of cyanidin 3-*O*-β-d-glucoside from (+)-catechin were reported in 2006 (Kondo et al., 2006) and later in 2013 for the synthesis of cyanidin-4′-*O*-methyl-3-glucoside was also reported (Cruz et al., 2013). In general, the total chemical synthesis of often complex structures is commercially infeasible. Although many complex natural products have been synthesized and/or modified however, they have shown low production yields, the use of toxic catalysts, and extreme reaction conditions making them unsuitable for human consumption and large scale production (Chemler and Koffas, 2008).

Production of natural products using biotechnological methods, especially plant cell and tissue cultures and microbial cell factories is considered as a promising option since 1950s and has been widely studied. Depending on the cell type and culture conditions, cell suspension cultures enable more direct pigment production, preferably in amounts superior to those of the intact plants. Similarly, the genetic engineering of plants and microorganisms can also provide an efficient way to produce and modulate anthocyanin biosynthesis.

3.1. *Optimization of abiotic conditions for in vitro plant cell or tissue culture-based methods*

Biotechnological tools and techniques provide an opportunity to make use of cells, tissues or organs of economically important plants for growing them under *in vitro* conditions and/or to genetically manipulate them to obtain high-value compounds. Among others, the production of anthocyanins in *in vitro* plant cell or tissue culture has been reported from various plant species (Table 1) and *Vitis vinifera, Rosa hybrida* and *Daucas carota* were found to be most studied plants for the production of anthocyanins in *in vitro* cell or tissue culture (Fig. 2). Anthocyanin pigments are one of the most important constituents in grapes and wines, which play a dual role. Firstly, they form an integral part of their sensory attributes, since their levels, various forms and derivatives directly pertain to the coloration of the final product; secondly, they work as biologically active molecules with potential nutritional value (Stintzing and Carle, 2004; Belwal et al., 2017).

The production of anthocyanins and other compounds in *in vitro* conditions involved various systematic steps that includes, establishment of explant, formation of calli and cell suspensions and their induction for the biosynthesis of metabolites. Various abiotic factors (*i.e.*, medium composition, growth hormones, precursors, elicitors, pH, light and temperature) are tested to ensure the optimum production of anthocyanins. These abiotic conditions or factors influence the *in vitro* production of cell biomass and consequently that of anthocyanins that are critically been reviewed for developing current and future research strategies.

3.1.1. *Effect of medium composition*

Medium is an integral part of plant *in vitro* cell or tissue cultures and the subsequently bioactive compound production. Based on the culture goals and conditions, the level of micro and macro nutrients with a source of nitrogen, sugar and plant growth regulators (PGRs) varied. With the aim of increasing cell growth and anthocyanin production, various conditioned media (CM) have been tested in *in vitro* plant cell and tissue cultures. The CM is a cell free supernatant (used or spent) medium that worked as a special kind of additive, which comprises a range of simple sugars or carboxylic acids, proteins and polysaccharides secreted by cells into the culture medium. In addition, Mori et al. (2001) demonstrated that activities of phenylalanine ammonia lyase (PAL), chalcone synthase (CHS) and CHS transcript levels significantly increased in CM-cultured cells compared to that in the synthetic media-cultured cells. Recently, Ashokhan et al. (2020) observed that anthocyanin accumulation is dependent on the callus color derived from *Azadirachta indica*, and a significantly higher anthocyanin concentration is recorded in green calli (262.54 mg/g DW) than in brown (230.82 mg/g DW) and cream colored calli (115.62 mg/g DW).

Apart from metabolism and growth, secondary metabolites also play an important role in plant defenses and adaptation to varying biotic and abiotic stress conditions (Wink, 2008). Studies have been conducted on anthocyanins accumulation, considering nutritional stress by limiting the amounts of nutrients in the medium (Schiozer and Barata, 2007). Deficiency of nutrients especially nitrogen, phosphorus and sulfur, are accompanied by anthocyanin accumulation in plants as a strategy to avoid the over-accumulation of carbohydrates in tissues and to prevent physiological disorders (Baker and Braun, 2007). Apart from the overall concentration of total nitrogen, the ratio of ammonium (NH_4^+) to nitrate (NO_3^-) has also been shown to markedly affect the production of anthocyanin. For instance, changes in the ratio of NH_4^+ to NO_3^- in the MS (Murashige and Skoog) medium, significantly increased anthocyanin accumulation in callus cultures of *Cleome rosea* (Simões et al., 2009). Moreover, MS medium containing 60 mM of total nitrogen, shown to be the most suitable for anthocyanin production. Similar studies were also reported elsewhere (Table 1). The increasing concentrations of NO_3^- to NH_4^+were found to affect nitrogen metabolism (Meng et al., 2016) and consequently increased anthocyanin biosynthesis (Table 2). It was also reported that nitrogen supply affects anthocyanin biosynthesis and regulatory genes in *Vitis vinifera* (Soubeyrand et al., 2014). Modifying the ratio of ammonium nitrate (NH_4NO_3) and potassium nitrate (KNO_3) (20:37.6 mM) in cell suspension cultures of *Daucus carota* resulted in a 2.85-fold increase in the anthocyanin content (Saad et al., 2018). It was found that the ratio of NH_4NO_3 and KNO_3 affected the transcription level of anthocyanin biosynthesis genes (*PAL, 4CL, CHS, CHI, LDOX* and *UFGT*) and an increase in KNO_3 with respect to NH_4NO_3 favored the anthocyanin biosynthesis.

Sucrose, is another major factor influencing the accumulation of anthocyanins (Table 1). It was found that the sugar as a carbon source

Table 1
In vitro anthocyanin production by plant cell and tissue cultures

Species	Explant	Culture condition				Total anthocyanin content	Reference
		Medium	PGRs	Manipulation in nutrient medium	Elicitors		
Arabidopsis thaliana	*In vitro* germinated seed derived leaves	MS	NA	1/2 strength of NH_4NO_3 and KN03	5 0μmol/m^2/s of light intensity	1.5 mg/g FW	Shi and Xie (2010)
Arabidopsis thaliana	Red pap1-D cells	Modified MS	9 μM NAA	removed NH_4NO_3 and reduced concentration of KNO_3 to 9.4 mM/L	NA	3.3 mg/g FW	Liu et al. (2014)
Azadirachta indica	Leaves	MS	0.2 mg/L TDZ	NA	NA	262.54 mg/g DW	Ashokhan et al. (2020)
Cleome rosea	Stem	½ MS	0.9 μM 2,4-D, and 70 g/L sucrose	1:4 ratio of NH_4^+ to NO_3^-24 ± 2 °C, 2478 lux	NA	38.67 CV/g FW	Simões et al. (2009)
	Stem	½ MS	0.45 μM 2,4-D and 30 g/L sucrose	NA	NA	28.71 ± 2.20 CV/g FW	Simoes-Gurgel et al. (2011)
	Seedling	MS	2 mg/L IAA, 0.2 mg/L Kin	NA	0.5 μM Ionophore	0.46% DW	Sudha and Ravishankar (2003)
	Seeds	MS (solid)	2.5 mg/L IAA and 0.2 mg/L Kin	30° C	NA	2.8% DW	Narayan et al. (2005)
		MS (liquid)	2.5 mg/L IAA and 0.2 mg/L Kin	25°°C	NA	2.8% DW	
	Leaves	MS	11.41 μM IAA and 0.93 μM Kin	Lower concentration (0.45 mM) of KH_2PO_4	NA	20.90 mg/g FW	Saad et al. (2018)
Malussieversiif.niedzwetzkyana	Leaves	MS	0.6 mg/L NAA and 0.5 mg/LBA	NA	NA	1.6 A_{530}/g FW	Ji et al. (2015)
Melissa officinalis	Apical part	MS	0.5 mg/L BA	NA	O_3 fumigation (200 ppb, 3 h)	0.32 mg/g FW	Tonelli et al. (2015)
Melastomamalabathricum	Cell suspension culture	MS	0.25 mg/L BA and 0.5 mg/L NAA	45 g/L sucrose	3.5 mg/L MeJA	0.69 ± 0.22 CV/g FCM	Suan-See et al. (2011)
Melastomamalabathricum	Cell suspension culture	MS	0.25 mg/L BA and 0.5 mg/L NAA	pH 5.75	Light intensity (301–600 lux), temperature (20 ± 2°C)	1.62 ± 0.14 CV/g FCM	Chan et al. (2010)
Panax sikkimensis	Root	MS	4.5 μM 2,4-D and 1.2 μM kin with 3.0% sucrose 0.01% myoinositol	0.33 μM thiamine hydrochloride, 2.5 μM pyridoxine hydrochloride, 4.0 μM nicotinic acid	NA	2.76 mg/g FW	Mathur et al. (2010)
	Root	MS	5.4 μM NAA	NA	NA	14.29 mg/g DW	Biswas et al. (2015)
Prunussalicina × *Prunuspersica*	Shoot	MS	0.25 mg/L BA, 0.06 mg/L IBA and 0.03 mg/L GA3	NA	3 mg/L adenine sulphate and 50 μM MeJA	1.88 mg CA eq/g FW	Lucioli et al. (2017)
*Rosa hybrida*cv. 'PusaAjay'	Leaf discs	EM	4.0 mg/L 2,4-D	70 g/L sucrose	NH_4^+ and NO_3^- (low:high)	50.49 mg/kg FW	Ram et al. (2011)
Rosa hybrida	Leaves	Modified EM	2.45 μM IBA and 2.33 μM Kin	NA	0.5 μM MeJA	3.48 ± 0.07 CV/g FW	Ram et al. (2013)
Rosa gallica	Leaves	Modified MS	2 mg/L 2,4 D and 1 mg/L BAP	NA	NA	138 μmol/g FW	Tarrahi and Rezanejad (2013)
Rosa hybrida	Leaves	Modified MS	3 mg/L 2,4 D and 1 mg/L BAP	NA	NA	57 μmol/g FW	Tarrahi and Rezanejad (2013)
Raphanus sativus	Root tip	½MS	0.1 mg/L IBA and 0.5 mg/LNAA	NA	NA	0.15% DW	Betsui et al. (2004)
Raphanus sativus	Roots	½ MS	0.5 mg/L IBA	NA	25 °C under the 14 h/day light at 100 rpm	250 μg/100 mL	Betsui et al. (2004)
Vitis vinifera	Cell suspension culture	B5/MS/Morel, 1970 Medium	0.54 μM NAA and 0.93 μM Kin	NA	150 mM sucrose added at 7th day	100 g/L	Vitrac et al. (2000)
	Cell suspension culture	B5	0.1 mg/L NAA and 0.2 mg/L Kin	30 g/l sucrose, 250 mg/l casein NA hydrolysate	20 μ MeJA and 8000 Lux light	22.62 CV/gFCW	Zhang et al. (2002)
	Berries	B5	0.1 mg/L NAA and 0.2 mg/L Kin	20 g/L sucrose, 250 mg/L casein hydrolysate	20 mM MeJA and 80 mM Sucrose	4 μmol/g FW	Belhadj et al. (2008)

(*continued on next page*)

Table 1 (*continued*)

Species	Explant	Culture condition				Total anthocyanin content	Reference
		Medium	PGRs	Manipulation in nutrient medium	Elicitors		
Vitis vinifera L. cv. Gamay Fréaux	Berries	B5	0.5 mg/L NAA, and 0.12 mg/ LBA	20 g/L sucrose, 250 mg/L casein hydrolysate	10–4 M ABA	400 µg/g FW	Gagné et al. (2011a)
	Cell suspension culture	B5	NA	NA	1.4 mL/L saliva from *Manduca sexta* larvae	5.25 mol/L	Cai et al. (2012)
	Berries	B5	0.1 mg/L NAA, 0.2 mg/L Kin	30 g/L sucrose, 250 mg/L casein hydrolysate	50 mg/L MeJAand 1 mg/L dextran	200 CV/gDCW	Qu et al. (2011)
	Berries	B5	0.1 mg/L NAA, 0.2 mg/L Kin	3% sucrose, 0.25 g/L casein hydrolysate	Ethephon and PEF	2.2 mg/g DW	Saw et al. (2012)
	Cell line VV05	B5	0.1 mg/L NAA and 0.2 mg/L Kin with 30 g/L sucrose	250 mg/L casein hydrolysate	5 mg/L phenylalanine and 50 mg/L MeJA	2.76 CV/g DCW	Qu et al. (2011)
	Cell line VV06	B5	0.1 mg/L NAA and 0.2 mg/L Kin with 30 g/L sucrose	250 mg/L casein hydrolysate	5 mg/L phenylalanine and 50 mg/LMeJA	6.22 CV/g DCW	Qu et al. (2011)
Malus sieversii f. niedzwetzkyana	Leaves	MS	4 µmol/L BA + and 2 µmol/L NAA	NA	MeJA (10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} mol/L)	1.8 – 2.4 (ABS/g FW)	Sun et al. (2017)
					ABA (0.4, 2, 4, and 6 µmol/L)	1.3 – 0.4 (ABS/g FW)	
					MeJA (10 − 4 mol/L) + ABA (4 µmol/L)	0.9 (ABS/g FW)	

B5 Medium-Gamborg B5 medium (1968);LS medium -Linsmaier and Skoog (1965) medium; MS medium- Murashige and Skoog (1962) medium; WPM-Woody Plant medium (1981); EM -Euphorbia Millii medium;C3Geq = Cyanidin 3-glucoside equivalent; CV-Colour value; CAeq-Chlorogenic acid equivalent; DCW -Dry cell weight; DW-Dry Weight; FW-Fresh weight; FCW-Fresh Cell concentration; PEF- Pulse Electric Field; NA -not applied.

increases the anthocyanin accumulation in *V. vinifera* cell cultures due to its direct effect on anthocyanin regulatory and structural genes expression (*CHS, CHI, F3'H, F3H, DFR, LAR, LDOX* and *ANR*), while it induces a decrease in phenylalanine content (Dai et al., 2014). The concentration of sucrose in the medium modulates anthocyanins production. In some studies, a higher amount of sucrose (70 g/L) was found efficient for anthocyanin production in callus cultures of *Cleome rosea* (Simões et al., 2009) and *Rosa hybrida* (Ram et al., 2011). Compared to the cell suspension, a high amount of sucrose in the callus culture medium positively regulates anthocyanin production. This could be justified by the fact that at higher sucrose concentration exerts osmotic pressure on the cells, which is higher in case of cell suspensions as compared to calli. In a cell suspension culture, the degree of cell aggregation plays a major role in anthocyanins production. It was highlighted that during the culture of cell suspensions of *Cleome rosea*, cells were mostly spherical in shape, and form small aggregates (3–15 cells), which were found to produce anthocyanins in significant amounts as compared to large cell aggregates (Simoes-Gurgel et al., 2011). Further, small cell aggregates support the production of anthocyanins by capturing light efficiently and thus activate key metabolic enzymes, such as PAC1 and CHS (Zhang et al., 2002).

In summary, various media composition affect the *in vitro* cell growth and accumulation of secondary metabolites and largely MS, ES and B5 media were found suitable for cell culture and subsequently anthocyanin production. Variations, mainly in the ratio of NH_4^+ to NO_3^- (low/high) and sucrose concentration (> 30 g/L) were found to positively modulate the anthocyanin biosynthesis.

3.1.2. Effect of plant growth regulators (PGRs)

Plant growth regulators (PGRs) or hormones are the vital components of *in vitro* cell or tissue cultures, especially for the production of plant secondary metabolites. Auxins and cytokinins are PGRs which play important roles in the production of anthocyanins in *in vitro* conditions (Table 1). The most common used PGRs include naphthaleneacetic acid (NAA), 2,4-dichlorophenoxyacetic acid (2,4 D), indole-3-butyric acid (IBA), indole-3-acetic acid (IAA), kinetin (Kin) and benzyl adenine (BAP). These PGRs work differently under culture conditions and showed marked differences in terms of anthocyanin production. For instance, in cell cultures of *Camptotheca acuminate*, the anthocyanin content was significantly increased in the presence of Kin, compared to BAP (Pasqua et al., 2005). Auxin as such also induces anthocyanin accumulation in cells or tissue cultures. For instance, adventitious root cultures of *Raphanus sativus* cultivated in MS liquid medium supplemented with 0.5 mg/L IBA showed a significant increase in pigment accumulation (Betsui et al., 2004). Similarly, in *Panax sikkimensis,* a concentration of 5.4 µM of NAA increased the anthocyanin content up to a maximum of 199 mg/L (Mathur et al., 2010). Similar results on using auxins or cytokinins were recorded elsewhere (Table 1).

The molecular mechanisms at the basis of the effect of these PGRs revealed that exogenous cytokinin application increases anthocyanin biosynthesis by increasing the transcription level of key regulatory genes (*PAL1, CHS, CHI, DFR*) (Deikman and Hammer, 1995) (Table 2). It was also reported that cytokinins promote sugar-induced anthocyanin biosynthesis by increasing the transcription level of structural (*UF3GT*) and regulatory gene (*PAP1*) (Das et al., 2012). Auxin is generally known for cell elongation; however, it was also found to regulate anthocyanin biosynthesis via Aux/IAA–ARF (auxin response factor) signaling (Wang et al., 2018). Also, auxin was found to regulate the phenylpropanoid, flavonoid, and anthocyanin metabolism (Murthy et al., 2004; Zhou et al., 2008) through the regulation of transcription factors such as TT8, GL3 and PAP1, while also up regulating DFR and ANS of the anthocyanin biosynthetic pathway (Liu et al., 2014).

Moreover, the synergistic effect of combining auxin and cytokinin was also reported (Table 1). As such, maximum anthocyanin concentration (350 µg/g FW) was obtained in a medium supplemented with 2 µM Kin along with 2 µM of 2,4D and 292 mM sucrose (Pasqua

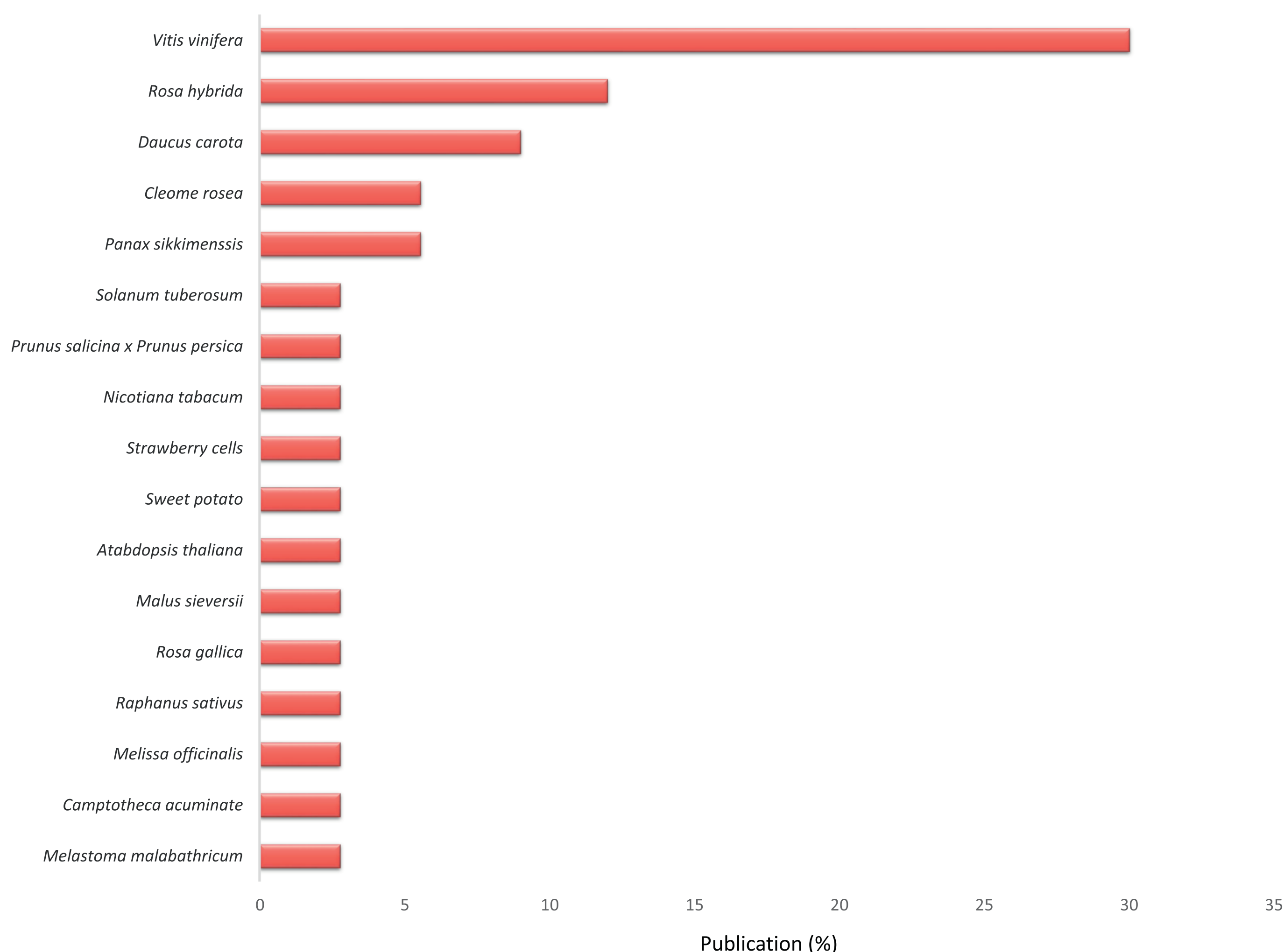


Fig. 2. List of species investigated for anthocyanin production in *in vitro* cell/callus cultures investigated during the last two decades.

et al., 2005). In some experiments, auxin treatment alone in red-fleshed apple (*Malus sieversii*) was found to inhibit anthocyanin production in cultures, more by 2,4 D as compared to NAA (Ji et al., 2015). It was recorded that 2,4 D alone was found to downregulate anthocyanin biosynthesis regulatory genes (*MdMYB10* and *MdbHLH3*) as well as structural genes. However, when the cultures were co-treated with cytokinin, an increase in the anthocyanin content was recorded, which dramatically reduced at high auxin concentrations. It was also recorded that at lower nitrogen (NH_4NO_3 and KNO_3) concentration in the medium, the auxin-induced inhibition of anthocyanin production could

Table 2
Effect of abiotic factors on anthocyanin biosynthesis and regulatory genes in *in vitro* culture conditions.

Factors/conditions	Effect on anthocyanin biosynthesis	Effect on biosynthetic pathway gene(s)	Effect on regulatory gene(s)	Reference(s)
NH_4^+ to NO_3^- (low:high)	Increased	↑ (PAL, 4CL, CHS, CHI, LDOX and UFGT)	NA	Saad et al. (2018)
High sucrose	Increased	↑ (CHS, CHI, F3′H, F3H, DFR, LAR, LDOX and ANR)	NA	Simoes-Gurgel et al. (2011), Dai et al. (2014)0
Cytokinin	Increased	↑ (PAL1, CHS, CHI, DFR and UF3GT)	↑ (PAP1)	Deikman and Hammer (1995), Das et al. (2012)
Auxin	Increased	↑ (DFR and ANS)	↑ (TT8, GL3 and PAP1) ↓ (*Md*MYB10 and *Md*bHLH3)	Zhou et al. (2008), Liu et al. (2014), Ji et al. (2015)
ABA	Increased	↑ (*Vv*PAL, *Vv*C4H, *Vv*CHI1 and *Vv*CHI2)	↑ (*Vv*MYBA1)	Gagné et al. (2011b)
MeJA	Increased	↑ (*Md*CHS, *Md*DFR, *Md*F3H and *Md*UFGT)	↑ (*Md*MYB3, *Md*MYB9 and *Md*MYB10 *Md*MYB24L)	Sun et al. (2017), Wang et al. (2019)
JA	Increased	↑ (DFR, LDOX, and UF3GT)	↑ (PAP1, PAP2 and GL3)	Shan et al. (2009)
Cerium	Increased	↑ (CHS, F3H, F3’5’H, DFR and 3GT)	NA	Lu et al. (2006)
Ethephon		↑ (*Md*ACS1, *Md*ACS3a, *Md*ACS4, *Md*ACS5a, *Md*ACS5b, and *Md*ACS6)	↑ (*Md*MYB1)	An et al. (2018)
Light induction	Increased	↑ (PAL and CHS)	NA	Zhang et al. (2002)
High temperature	Decreased	↓ (*Mp*CHS, *Mp*DFR, *Mp*LDOX and *Mp*UFGT)	↑ (*Mp*MYB15) ↓ (*Mp*MYB10)	Rehman et al. (2017)

↑: Upregulation, ↓: Downregulation, ABA: Abscisic acid, MeJA: Methyl jasmonate; JA: Jasmonic acid; NA: Not available

be reversed effectively. The level and combination of PGRs should thus be selected precisely, to achieve optimum growth and anthocyanin production.

While selecting the appropriate hormone combination and concentration, one must also take into account the production of cell biomass, anthocyanin and the optimum time period. For instance, in *Panax sikkimensis* root-derived calli, the maximum callus growth index (261.77) was recorded when the culture was induced by 4.5 µM 2,4 D and 1.2 µM Kin, while the maximum anthocyanin content was found to be 3.81 mg/g at 2.5 µM NAA and 1.2 µM Kin, with an exceptionally slow callus growth (Mathur et al., 2010). Also, selection of initial explants for culture preparation and for the production of anthocyanin is of prime importance. In *Rosa* spp. among different explants, leaves and stems showed increased callogenesis in combined treatment of 2,4 D (3 mg/L) and 6-BAP (1 mg/L), as compared to others. Moreover, anthocyanin and chlorophyll were also recorded in higher concentration in calli derived from leaves (Tarrahi and Rezanejad, 2013).

Besides auxins and cytokinins, abscisic acid (ABA) was also tested for *in vitro* anthocyanin production. The effective role of ABA as PGR in anthocyanin production was mainly exerted by regulating the expression of biosynthesis genes and endogenous ABA levels. It was found that an exogenous treatment with ABA on cell cultures of *V. vinifera* could induce the expression of upstream genes of the anthocyanin biosynthetic pathway (*VvPAL, VvC4H, VvCHI1* and *VvCHI2*) and that of the *VvMYBA1* transcription regulator gene resulting in a higher anthocyanin production (400 mg/g FW) (Gagné et al., 2011b).

In summary, PGRs play a central role in cell biomass production and subsequently anthocyanin production. The combined effect of auxin and cytokinin was profound and should be optimized for increasing the anthocyanin biosynthesis in the culture conditions.

3.1.3. Effect of precursors and elicitors

Anthocyanins biosynthesis pathway involves various enzymes and substrates, which are known to be affected by external conditions including precursors and elicitors. Different elicitors including abiotic (metal ions, inorganic compounds and UV irradiation) and biotic compounds obtained from various microbial sources are used to stimulate biosynthesis of secondary metabolites in *in vitro* culture conditions (Zhao et al., 2005; Vasconsuelo and Boland, 2007). Elicitors such as methyl jasmonate (Lucioli et al., 2017; Plata et al., 2003; Ram et al., 2013; Sudha and Ravishankar, 2003), jasmonic acid (Zhang et al., 2002; Curtin et al., 2003; Blando et al., 2005), and salicylic acid (Sudha and Ravishankar, 2003; Ram et al., 2013) have been efficiently used for the production of anthocyanins in *in vitro* cell or tissue culture of various plant species (Table 1). Moreover, the production of anthocyanins was also reported with some other elicitors such as, fungal endophyte *Sphaeropsis* sp B301 (Hao et al., 2010), ionophores (Sudha and Ravishankar, 2003), and pectins (Cai et al., 2012). Some gases were also used for increasing production of anthocyanins. For instance, Tonelli et al. (2015) applied ozone (O_3) treatment (200 ppb, 3 h) to *Melissa officinalis* callus cultures, which resulted in a two-fold increase in anthocyanins production, compared to the untreated control cells.

Some studies have indicated that methyl jasmonate (MeJA) works well for *V. vinifera* cell suspension cultures (Belhadj et al., 2008; Qu et al., 2011), which increased the production of anthocyanin by 2.8–4.1 folds. Moreover, MeJA was also reported to have a positive effect on anthocyanin production in *Rosa hybrida* cell cultures (Ram et al., 2013). Efficiency of the elicitation process depends on the plant material, contact time, culture conditions and elicitor concentration. The MeJA was found to increase anthocyanin biosynthesis by increasing the expression of anthocyanin regulatory (*MdMYB3, MdMYB9 and MdMYB10*) and structural genes (*MdCHS, MdDFR, MdF3H and MdUFGT*) (Sun et al., 2017) (Table 2). It was also found that MeJA upregulates the gene expression of *MdMYB24L,* which increased the transcription level of *MdDFR* and *MdUFGT* (Wang et al., 2019). The interaction of *MdMYB24L* and jasmonic acid (JA) signaling factors (*MdJAZ8*, *MdJAZ11*, and *MdMYC2*) were also recorded, with a positive interaction effect with *MdMYC2*, whereas *MdJAZ8* and *MdJAZ11* displayed a negative effect on the transcription of *MdUFGT*.

The simultaneous use of an elicitor, such as JA along with light irradiation in *V. vinifera* cell suspension cultures resulted in a significant increase in anthocyanin accumulation, showing the synergistic potential of integrated processes under *in vitro* conditions (Zhang et al., 2002). The molecular mechanisms underlying the effect of JA on anthocyanin accumulation revealed the role of F-box protein COI1 in regulating the transcription factors *PAP1*, *PAP2*, and *GL3*, which induced expression of anthocyanin biosynthetic genes *(DFR, LDOX, and UF3GT)* (Shan et al., 2009).

Some metal compounds, such as cerium were also tested for their capacity to induce anthocyanin production in *in vitro* cell suspension cultures of *Solanum tuberosum* (Lu et al., 2006). Cerium enhanced expression of five anthocyanin biosynthetic genes, namely chalcone synthase (*CHS*), flavanone 3-hydroxylase (*F3H*), flavonoid 3', 5'-hydroxylase (*F3'5'H*), dihydroflavonol 4-reductase (*DFR*) and flavonoid 3-O-glucosyltransferase (*3GT*), resulting in an increased anthocyanin production as well as promoting culture growth. Similarly, application of magnesium was found to increase anthocyanin production in cell cultures of *V. vinifera* (Sinilal et al., 2011).

Using precursors and elicitors in combination is an effective strategy for achieving better production of anthocyanins. Phenylalanine (5 mg/L) and MeJA (50 mg/L) were found to increase the anthocyanin content and yield by about 4.6- and 3.4-folds, respectively in *V. vinifera* low-producing cell line (Qu et al., 2011). This study also reported that using MeJA and phenylalanine at the same concentration along with 1mg/L dextran could further increase the anthocyanin content by 6.1-fold and yield by 4.6-fold in *V. vinifera* cell lines (Qu et al., 2011). The exogenously applied phenylalanine serves as a precursor for anthocyanin production, and further MeJA treatment increased the activity of PAL, beside its potential role in regulating anthocyanin biosynthesis genes, that further uplifted anthocyanin production. The combined effect of MeJA with sucrose was also found to modulate anthocyanin biosynthesis in cell cultures of *Melastoma malabathricum* (Suan-See et al., 2011), with higher sugar concentration positively regulating anthocyanin biosynthesis. It was interesting to note that sugar-induced DFR expression and further the anthocyanin biosynthesis by JA is sugar-dependent (Shan et al., 2009) and thus both works synergistically.

In another interesting study, the combination of ethephon and a mechanical stress induced by pulsed electric field (PEF) on the cell suspension cultures of *V. vinifera* exerted synergistic effects resulting in an increase in the anthocyanin content by 2.5-folds (Saw et al., 2012). Ethephon, a precursor of the gaseous elicitor ethylene was found to induce the ethylene biosynthesis genes (*MdACS1*, *MdACS3a*, *MdACS4*, *MdACS5a*, *MdACS5b*, and *MdACS6*), along with anthocyanin regulatory (*MdMYB1)* and biosynthesis genes (*MdDFR* and *MdUFGT)* (An et al., 2018). Also, ethephon induces flavonoid biosynthesis pathway genes (Ft4CL, FtCHS, *FtCHI, FtF3H, FtF3'H1, FtF3'H2, FtFLS1, FtFLS2, FtDFR and FtANS*) (Li et al., 2017) and increased anthocyanin biosynthesis was achieved.

In summary, these results suggested that biosynthesis of anthocyanin using elicitors and precursors is regulated by the interplay of multiple regulatory and biosynthesis genes, transcription factors, and enzymes of the anthocyanin pathway. Hence these must be chosen very precisely in terms of optimum concentration and combination for gaining maximum anthocyanin content and yield.

3.1.4. Effect of light, temperature and pH

The significant role of light/irradiation in the biosynthesis of anthocyanins in PCTC has been well identified (Antognoni et al., 2007; Guo et al., 2008). In general, anthocyanins accumulation is induced as a response to a prolonged exposure to red and far-red light, blue and UV-lights, which are mediated by phytochrome, cryptochrome and/or UV-B photoreceptors, respectively. In response to a continuous exposure to

UV irradiation, the catalytic activity of the anthocyanin biosynthesis enzymes and the gene expression increased that increased production of anthocyanin and thereby protection of cells against the action of UV light was also achieved (Xu et al., 2017a). As such, a significant increase in the accumulation of anthocyanins was achieved in carrot cell cultures under continuous UV-A light exposure (Hirner et al., 2001). Similarly, in the callus cultures of *Cleome rosea,* light-induced anthocyanin production was also observed (Simões et al., 2009). The enzymatic activity of phenylalanine ammonia-lyase (PAL) and chalcone synthase (CHS) was found to be affected by light, resulting in modulation of anthocyanin biosynthesis (Zhang et al., 2002).Various studies on cell culture systems used illumination for anthocyanin production (Blando et al., 2005; Pasqua et al., 2005), however anthocyanin production has also been reported in dark (Konczak-Islam et al., 2000). Studies indicated that intensity of light used to maximize anthocyanin production may vary from one species to another. For instance, maximum anthocyanin production was achieved with providing 8000 lux in strawberry cells (Mori et al., 2001), whereas 3478 lux light was required in *Cleome rosea* cells (Simões et al., 2009). The effect of light exposure time was also recorded in modulating anthocyanin biosynthesis. As such, in cell cultures of *P. sikkimensis*, a continuous light exposure resulted in a higher production of anthocyanins (2.19 mg/g) as compared to a 16 h light/8 h dark photoperiod (1.54 mg/g), while in case of complete dark conditions, a 5/12-folds lower anthocyanin production was recorded (Mathur et al., 2010). The light induced the expression level of regulatory factors and structural genes related to anthocyanin biosynthesis (Zhang et al., 2018).

Temperature also influences anthocyanin production in *in vitro* cell or tissue culture depending on the species studied. Simões et al. (2009) obtained maximum anthocyanins production rate while maintaining the temperature of *Cleome rosea* callus cultures at 24 ± 2 °C, whereas at higher temperatures callus browning was observed. This was linked with the formation of brown condensed products by the action of glucosidases, resulting in the hydrolysis of glycosidic bonds of anthocyanins (Schiozer and Barata, 2007). However, in another study including callus cultures of *Daucus carota*, a temperature of 30 °C (in solid medium) and 25 °C (in liquid medium) was found most suitable for anthocyanins biosynthesis (Narayan et al., 2005). The effect of abiotic factors, such as light intensity, irradiation, temperature and medium pH on biomass yield and anthocyanin production in *in vitro* cell cultures of *Melastoma malabathricum* was also studied. Optimum conditions for higher cell biomass and anthocyanin content induced by continuous light irradiation for 10 days was light intensity (301–600 lux), a low temperature of 20 °C and an average pH (5.25–6.25) (Chan et al., 2010). The results indicated that lower temperatures seem to be more suitable for anthocyanin production, while higher temperatures can reduce cell growth as well as induce browning of cell/callus cultures. At lower temperatures, related gene expression and enzymatic activity of the anthocyanin biosynthesis pathway increased along with the concentration of ABA, which facilitates the anthocyanin biosynthesis (Yamane et al., 2006). The interaction between light and temperature was also seen in callus cultures of apple (Wang et al., 2016). Under the exposure to light, a low temperature (16 °C) induced the expression of *MYB10* and *bHLH3/33* and other regulatory and structural genes, whereas at high temperature (32 °C), it induced *MYB16* expression, which shows negative effect on anthocyanin biosynthesis (Wang et al., 2016). Moreover, under higher temperatures, the expression of anthocyanin biosynthesis genes (*MpCHS, MpDFR, MpLDOX, MpUFGT*, and *MpMYB10)* was downregulated with increased expression of the *MpMYB15* repressor gene (Rehman et al., 2017) (Table 2). Also, at higher temperatures, the concentration of H_2O_2 increased due to enhanced activities of the superoxide dismutase (SOD) and the malondialdehyde (MDA) content, resulting in the degradation of anthocyanins (Rehman et al., 2017).

Generally, for anthocyanins production, a two-stage culture method should be adopted such as providing higher temperature for cell biomass growth followed by a lower temperature for anthocyanin production. Also, light conditions need to be optimized for its intensity, exposure time and types.

3.2. Metabolic engineering in plants

The continuously increasing commercial demands for naturally-derived colorants (anthocyanins), has raised the need for harnessing these chemicals from naturally available resources, specifically from terrestrial plants. With rapid expansion in urbanization, climate change, anthropogenic activities and loss of biodiversity, it is difficult to fulfil the industrial demands for anthocyanins from their natural sources. In addition, the already existing conventional extraction and purification methods for these compounds lead to a heterogeneous mixture of multiple compounds with identical chemistry, which are sometimes difficult to separate (Ryan and Revilla, 2003; Zhu et al., 2017; Zha and Koffas, 2017). Furthermore, another major issue encountered upon the direct extraction of anthocyanins is “browning effect” of plant’s anthocyanins extracts due to the polyphenol oxidase (PPO) mediated-oxidation, followed by condensation of the resulting compounds into undesirable brown pigments (Oszmianski and Lee, 1990). With the advent of recombinant DNA technologies, plant metabolic engineering has emerged as a potential biotechnological tool for improving anthocyanin biosynthesis in native or a model plant system (Shi and Xie, 2014) with a view to provide competitive strategies for their sustainable production.

3.2.1. Biotechnological interventions for engineering of anthocyanin biosynthesis in agri/horticultural crops

Metabolic engineering of the anthocyanins pathway was firstly initiated during the late 90’s when some researchers introduced brick coloured pelargonidin in petunia after transformation with the maize *DFR* gene (Meyer et al., 1987). Since then, a great progress has been achieved in terms of (1) selection of potential gene(s) sources, (2) improved genetic transformation protocols for many targeted species, (3) promoter identification, and (4) introduction of regulatory genes and silencing systems such as RNA interference (RNAi) and gene knockout strategies (Tanaka and Ohmiya, 2008; Zhang et al., 2014) (Table 3). In one such study, the RNAi-mediated suppression of anthocyanidin synthase (*ANS*) in *Torenia hybrida* has resulted in the reduction of the anthocyanin content consequently producing white flowers instead of blue ones (Nakamura et al., 2006). Efforts have thus been made to use the RNAi-mediating gene silencing technique to generate red-flower transgenic tobacco by suppressing two endogenous genes (*FLS*, *F3’H*) along with overexpression of a foreign *DFR* gene from gerbera, which shifts the flux towards pelargonidin synthesis (Nakatsuka et al., 2007). In contrast, overexpression of two Japanese gentians *MYB TF* genes *(GtMYB1R* and GtMYB1R9) in tobacco caused a reduction in the anthocyanin content leading to pink-to-white flower phenotypic changes (Nakatsuka et al., 2013). Such changes in flower/fruit colors (brick red/scarlet, red/magenta, and violet/blue) also appeared as a consequence of the synthesis of new anthocyanins (pelargonidin, cyanidin, and delphinidin) after modifying the B-ring hydroxylation pattern of anthocyanins by altering *F3’H* and *F3’5’*H expression (Tanaka and Ohmiya, 2008). Likewise, reduction in the diacylated delphinidin level by inhibiting 5,3’-acyl transferase in gentian was introduced as a new red-purple colored flowering phenotype (Nakatsuka et al., 2010). In one of the studies, *Pyrus communis* PAL gene was cloned and its expression was investigated in two pear fruit tissue (Ban et al., 2017). In Red d'Anjou variety the anthocyanin accumulation is well correlated with increased PAL expression, while in Red Bartlett no correlation was observed.

Complementing a specific anthocyanins compound absent in an ornamental plant by introducing anthocyanin biosynthetic genes from a novel plant source could be a very useful strategy to introduce new commercial traits (Fig. 3). In that sense, efforts have been placed to

introduce the *F3'5'H* gene from *Petunia* and *Viola* to *Rosa hybrida*, resulting in the accumulation of delphinidin and consequently a bluish-colored floral phenotype (Katsumoto et al., 2007). The lack of the chalcone isomerase (*CHI*) gene in tomato is the main cause of unavailability of naturally occurring anthocyanin-rich "purple tomatoes". Similar efforts have been made to introduce the *CHI* gene in tomato *via* interspecific crosses with wild species or *via* the heterologous expression of *CHI* from *Petunia* along with other regulatory genes (*bHLH* and *MYB* TFs) to produce an altered phenotype (purple tomato) with longer shelf-life (Butelli et al., 2008; Gonzali et al., 2009). For generation of blue-colored chrysanthemums, researchers have introduced flavonoid-3′,5-hydroxylase (*F3'5'H*) and UDP-glucose:anthocyanin 3′,5′-*O*-glucosyltransferase (*A3'5'GT*) genes from Canterbury bells and butterfly pea, thus enhancing the delphinidin-based anthocyanin content by giving the desired floral trait (Noda et al., 2017).

Although biosynthetic pathway genes have been well explored for improving anthocyanins content in targeted organisms, understanding of their cellular transportation networks are still poorly investigated. Transparent Testa (*AtTT19*), a transporter gene from *Arabidopsis* has been studied in relation to its role in the vacuolar transport of anthocyanins (Sun et al., 2012). GUS-mediated localization confirmed role of *TT19* in the tonoplast transport of anthocyanins, though mutating this gene turned down anthocyanin accumulation. Other than this, *TT12* (a MATE family antiporter), *AHA10* (a plasma membrane H^+-ATPase), *MRP3* (an ABC transporter-type) were also suggested to be associated with anthocyanin biosynthesis (Shi and Xie, 2014).

3.2.2. Transcription factors as key targets

Transcription factors (TFs) play a major role in gene expression and a vast number of targeted TFs have already been identified in the biosynthesis of anthocyanins. Manipulation of expression of these TFs, mainly that of the R2R3 MYB, bHLH and WD40-type families (conserved MBW complex) to modulate anthocyanin biosynthesis and change phenotypes have been performed in many plant species (Mathews et al., 2003; Tanaka and Ohmiya, 2008; Outchkourov et al., 2018). Overall, five MYBs (PAP1, PAP1/MYB75; PAP2/MYB90; MYB113, MYB12L and MYB114L), three bHLHs (Glabra 3, GL3; Enhancer of Glabra, EGL3 and TT8) and one WD40 protein (TTG1) regulate anthocyanin biosynthesis in *A. thaliana* (Shi and Xie, 2014). Furthermore, in a T-DNA insertional mutagenesis experiment in tomato, a MYB TF gene (*ANT1*) responsible for purple pigmentation was identified, which was further confirmed by its heterologous expression in *Arabidopsis* leading to upregulation of genes related to anthocyanin biosynthesis and vacuolar transport (Mathews et al., 2003). In another study, overexpression of two TF genes from snapdragon altered the phenotype of tomato from red-colored to purple-colored fruits with a high anthocyanin content equivalent to that of blackberries and blueberries (Butelli et al., 2008). These engineered tomatoes not only displayed a high anthocyanin content but also showed extended shelf-life and reduced susceptibility to fruit decaying fungus, *Botrytis cinerea* (Butelli et al., 2008).

Overexpression of *IbMYB1* TF gene in *Ipomoea batatas* calli and its expression in *Arabidopsis* resulted into an ectopic pigmentation in different tissues of heterologous host, while increasing total anthocyanin content by 200 folds in transformed *I. batatas* calli (Mano et al., 2007). However, expressing this TF gene under the control of three different promoters (SPO-M, SPA-M, and 35S-M) in *Nicotiana tabacum* SR1 led to different anthocyanin content with maximum found with SPO-M promoter, while causing some undesirable phenotypes (An et al., 2015). Similarly, the heterologous expression of *AtMYB90/PAP2* (production of anthocyanin pigment 2) in tomato resulted in a greater accumulation of anthocyanins in all plant parts as a consequence of up-regulated expression of *SlAN1*(bHLH) gene (Li et al., 2018). The heterologous introduction of *AtPAP1* (MYB TF) led to an anthocyanin production of 0.4–0.8 mg/g, with a high content of cyanidin 3-*O*-rutinoside and an increased red/purple pigmentation (He et al., 2017). Likewise, overexpression of an R2R3-type MYB gene form purple carrot (*DcMYB6*) resulted into a 66–228-fold increase in anthocyanin content with appearance of dark-purple pigments (Xu et al., 2017b).

Co-expression of two MYB TFs genes (*AmRos1* and *AmDel*) from *A. majus* in *N. tabacum* produced dark red colored anthocyanins *i.e.*, cyanidin 3-*O*-rutinoside (C3R) (Appelhagen et al., 2018). Further, addition of flavonoid 3′,5′-hydroxylase gene from *P. hybrida* (*PhF3'5'H*) into *N. tabacum* cell culture initiated production of delphinidin 3-*O*-rutinoside (D3R) along with C3R, resulting in pale-red colored cell cultures (Appelhagen et al., 2018). In another study, introduction of these two TF genes under the control of an inducible promoter in tomato led to an accelerated biosynthesis of anthocyanins in transformed calli and vegetative tissues and also enhanced root branching, leaf conductance and seed germination within 24 h of dexamethasone exposure (Outchkourov et al., 2018). Recently, two R2R3-MYB TFs (*PsMYB114L* and *PsMYB12L*) were identified from *Paeonia suffruticosa* petals, the heterologous expression of which in *A. thaliana* and apple calli consequently elevated anthocyanin biosynthesis which was noticed by the appearance of purple-red leaves and red-colored calli, respectively (Zhang et al., 2019). Along with this, efforts were made to produce anthocyanins in *Nicotiana* species (*N. benthamiana* and *N. tabacum*) after introducing two TF genes from both *Antirrhinum majus* (*AmRos1* and *AmDel*) and *Medicago truncatula* (*MtLAR* and *MtANR*). The transient expression of this multi-genic construct led to purple color in agro-infiltrated tobacco leaves while stable transgenic tobacco plants produced proanthocyanidins (PAs) up to 3.48 mg/g dry weight (Fresquet-corrales et al., 2017).

In another case, DELLA protein, a major component of the gibberellin signalling pathway was also explored as a potential candidate for enhancing anthocyanins biosynthesis in *A. thaliana* (Xie et al., 2016). These DELLA proteins act as sequesters of the *MYBL2* and *JAZ* inhibitory genes and remove their inhibition on MBW complex, leading to an elevated anthocyanin biosynthesis. Likewise, overexpression of *IbMADS10* enhanced the accumulation of anthocyanins leading to high pigmentation in sweet potato, affirming to its potential for heterologous anthocyanin production (Lalusin et al., 2006).

3.3. Metabolic engineering in microbes

Anthocyanin biosynthetic pathways have been well explored (Fig. 1 and 3) and characterized in terms of genetics as well as enzymatic dynamics (Levisson et al., 2018). For example, the production of pelargonidin-3-*O*-glucoside from phenylalanine, theoretically requires the expression of a total of 11 transgenes in suitable heterologous systems. However, due to high genomic complicities and various post-transcriptional and post-translational modifications, efficient engineering of the entire biosynthetic pathway of anthocyanins in a typical heterologous plant system is a very challenging task (Desai et al., 2010). On the other hand, due to several intrinsic characteristics of microorganisms such as rapid growth, well-characterized metabolisms, standardized up-scaling culture practices, easy genetic modifications and gene editing coupled with advanced genome sequencing, enzyme/ metabolic networking and other bioinformatics supports, the heterologous production of many natural products including anthocyanins has successfully been achieved as a sustainable and cost-effective approach (Pandey et al., 2016; Zha and Koffas, 2017). Engineered bacteria, especially *Escherichia coli*, have extensively been genetically modified for the heterologous production of different kinds of natural products, and were found to be able to convert the flavonoids, naringenin or eriodictyol, to the corresponding anthocyanins, P3G or C3G, respectively. With continuous scientific progress, the yeast cells (*Saccharomyces cerevisiae*) were also explored as potential hosts for anthocyanins production (Table 4). In addition to the metabolic engineering of anthocyanins biosynthetic pathway genes, optimization of cofactors and co-substrates also have to be taken into consideration for heterologous production of anthocyanins. For example, proper availability of sodium

Table 3
Metabolic engineering of various gene(s) to improve/introduce anthocyanin biosynthesis in targeted crops.

Targeted plant	Transgenic/ endogenous	Gene(s) source	Gene(s)	Overexpressed/Silenced	Accumulated anthocyanins	Original phenotype	Changed phenotype	References
P. hybrida	Transgenic	*Z. mays*	*DFR*	Overexpressed	Pelargonidin	Pale pink flower	Brick red flower	Meyer et al. (1987)
S. lycopersicum	Transgenic	*P. hybrida*	*CHI*	Overexpressed	78-fold increase in fruit peel flavonols	WT phenotype	No phenotypic changes	Muir et al. (2001)
S. lycopersicum	Endogenous	*S. lycopersicum*	*ANT1* (R2R3 MYB)	Overexpressed	Increased total anthocyanin (500-fold)	Red coloured fruit	Purple coloured fruit	Mathews et al. (2003)
N. tabacum	Transgenic				ND	WT phenotype	Intensely purple plants	
N. tabacum	Transgenic	*Campanula medium*	*F3'5'H*	Overexpressed	Delphinidin	White flower	Violet to blue flower	Okinaka et al. (2003)
I. batatas	Endogenous	*I. batatas*	*MADS10*	Overexpressed	Increased TAC	Normal pigmentation	High pigmentation	Lalusin et al. (2006)
Torenia hybrida	Endogenous	*T. hybrida*	*CHS*	RNAi-silenced	Naringenin	Blue	White	Nakamura et al. (2006)
Rosa hybrida	Transgenic	*P. hybrida*	*F3'5'H*	Overexpressed	Delphinidin	Pink flower	Blue/violet flower	Katsumoto et al. (2007)
I. batatas	Endogenous	*I. batatas*	*MYB1*	Overexpressed	Increased TAC	WT phenotype	Highly pigmented leaf and tubers	Mano et al. (2007)
N. tabacum	Endogenous& transgenic	*Gerbera hybrida*	*ntfls, ntf3'h, GhDFR*	RNAi-silenced/ Overexpressed	Pelargonidin	White flower	Red flower	Nakatsuka et al. (2007)
Osteospermum hybrida	Transgenic	*G. hybrida, Fragaria × ananassa*	*DFR*	Overexpressed	Pelargonidin	White flower	Pale-pink colored flower	Seitz et al. (2007)
S. lycopersicum	Transgenic	*Antirrhinum majus*	*DEL, ROS1*	Overexpressed	Increased TAC	Red fruits	Purple fruits	Butelli et al. (2008)
T. hybrida	Endogenous	*T. hybrida*	*F3'H, F3'5'H*	RNAi-silenced	Delphinidin	Voilet	Pink	Tanaka and Ohmiya (2008)
Dianthus caryophyllus	Transgenic	*P. hybrida, Viola tricolor*	*F3'5'H, DFR*	Overexpressed	Delphinidin	White	Voilet	Tanaka and Ohmiya (2008)
Hybrid tea rose	Transgenic	*V. tricolor*	*F3'5'H*	Overexpressed	Delphinidin	Pink	Voilet	Tanaka and Ohmiya (2008)
Cyclamen persicum	Endogenous	*C. persicum*	*F3'5'H*	Antisense-silenced	ND	Purple flower	Red/pink flower	Nishihara and Nakatsuka (2011)
Gentiana sp.	Endogenous	*Gentiana sp.*	*CHS*	Antisense-silenced	ND	Blue	White	Nishihara and Nakatsuka (2011)
Gentiana sp.	Endogenous	*Gentiana sp.*	*F3'5'H*	RNAi-silenced	ND	Blue	Magenta	Nishihara and Nakatsuka (2011)
Nierembergia sp.	Endogenous	*Nierembergia sp.*	*F3'5'H*	Antisense-silenced	ND	Voilet	Pale blue	Nishihara and Nakatsuka (2011)
N. tabacum	Transgenic	*Gentiana triflora*	*GtMYB1R, GtMYB1R9*	Overexpressed	Reduced TAC	Pink flower	White flower	Nakatsuka et al. (2013)
S. lycopersicum	Transgenic	*A. thaliana*	*CPC (MYB), GL3 (bHLH)*	Overexpressed	Varied TAC	Green leaf veins	Reddish-purple leaf veins	Wada et al. (2014)
N. tabacum var. Dark tobacco	Transgenic	*A. thaliana*	*AtPAP1 (MYB)*	Overexpressed	High cyanidin 3-*O*-rutinoside	WT phenotype	Red/purple leaf	He et al. (2017)
Chrysan-themum morifolium	Transgenic	*Clitoria ternatea, C. medium*	*CtA3'5'GT,CamF3'5'H*	Overexpressed	Delphinidin	Red-purple flower	Blue/violet-blue flower	Noda et al. (2017)
A. thaliana	Transgenic	*Daucus carota*	*DcMYB6*	Overexpressed	Increased (66–228-fold)	Green tissues	Dark-purple pigments in the leaves, siliques, and seed coats	Xu et al. (2017a)
S. lycopersicum	Transgenic	*A. thaliana*	*MYB90/PAP2*	Overexpressed	Increased total anthocyanin content	Green vegetative tissue	Purple vegetative tissue	Li et al. (2018)
S. lycopersicum	Transgenic	*A. majus*	*ROS1 (MYB), DEL (bHLH)*	Overexpressed	Delphinidin, petunidin	WT phenotype	Purple-colored calli, root, stem and leaves	Outchkourov et al. (2018)
A. thaliana	Transgenic	*Paeonia suffruticosa*	*MYB114L, MYB12L*	Overexpressed	ND	Green leave	Purple-red leaves	Zhang et al. (2019)
Apple calli	Transgenic	*P. suffruticosa*	*MYB114L, MYB12L*	Overexpressed	ND	Normal callus	Red-coloured callus	Zhang et al. (2019)
Lycium ruthenicum, L. barbarum	Endogenous	*L. ruthenicum, L. barbarum*	*LrAN2, LbAN2 (MYB)*	Overexpressed	High anthocyanin content	Green fruits	Yellow, white, purple, and jujube red fruits	Zong et al. (2019)

DFR: dihydroflavonol reductase, CHI: chalcone isomerase, F3'H: flavanone 3'-hydroxylase, F3'5'H: flavanone 3'5'-hydroxylase, CHS: chalcone synthase, TAC: total anthocyanin content, WT: wild type, nm: nanometer, Petunia (cv. Mitchell): *Petunia axillaris* X (*P. axillaris* X *P. hybrida*), DW: dry weight, ND: not determined.

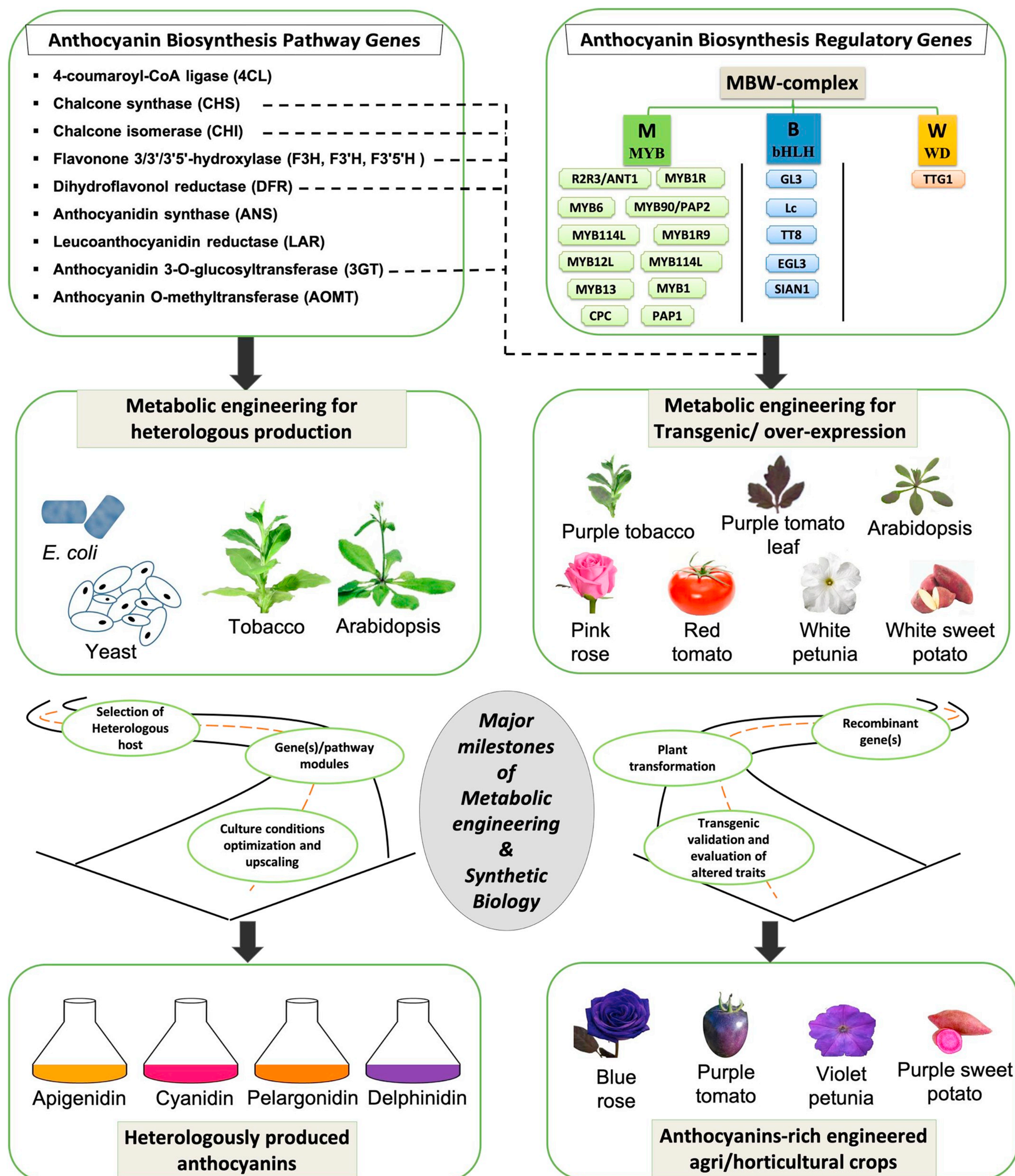


Fig. 3. Metabolic engineering strategies for anthocyanins biosynthesis. Typically, one or more than one anthocyanins biosynthetic pathway genes have been targeted to achieve anthocyanin production in heterologous hosts (bacteria, fungi and plants). On the other hand, efforts were also made to target regulatory genes of anthocyanin biosynthesis (MBW-complex) to introduce new traits in commercial crops and increase anthocyanins content in food crops. Dotted lines (—) represent biosynthetic pathway genes targeted for transgenic development to enhance anthocyanin contents in the targeted plants.

ascorbate and 2-oxoglutarate is required as cofactor and co-substrate, respectively for the oxidative activity of the anthocyanidins synthase (Turnbull et al., 2004). Like-wise, sufficient supply of UDP-glucose is a prerequisite for glycosylation steps of anthocyanins (Yan et al., 2008).

3.3.1. In prokaryotes

Having simplest cellular organization and enriched genomic resources, unicellular prokaryotic cells such as *E. coli* and *Streptomyces venezuelae* are considered to be the most suitable hosts for heterologous production of natural products, and thus, also often used for anthocyanins production (Zha and Koffas, 2017). Initially, anthocyanins biosynthetic genes, such as flavonoid 3'-hydroxylase (*F3'H*), dihydroflavonol 4-reductase (*DFR*), anthocyanidin synthase (*ANS*) and flavonoid 3'-glucosyltransferase (*F3'GT*) were co-expressed successfully using a pET vector transformed in modified *E. coli* (Yan et al., 2005). The engineered *E. coli* produced 6.0 µg/L of cyanidin 3-O-glucoside (C3G) and 5.6 µg/L of pelargonidin 3-*O*-glucoside (P3G) under supply of naringenin and eriodictyol as substrates, respectively. Parallelly, a 3-glucoside-specific malonyltransferase i.e. the malonyl-coenzyme A: anthocyanidin 3-*O*-glucoside-6”-*O*-malonyltransferase (3MaT) from *Dahelia* flowers was introduced into *E. coli* to catalyze the region pecific transfer of the malonyl group from malonyl-coenzyme A into P3G for the production of the pelargonidin 3-*O*-6”-*O*-malonylglucoside (Suzuki et al., 2002).

Codon optimization is a crucial step for the efficient heterologous expression of the target gene(s) from eukaryotic origin into a prokaryotic system. Considering this, four codons of the flavonoid 3', 5'-hydroxylase (*F3'5'H*) from *Catharanthus roseus* were removed from 5'end along with replacement of fifth and sixth residues as methionine and leucine. These two amino-acids required for *N*-terminal membrane anchoring and suitable for bacterial and fungal hosts (Leonard et al., 2006, 2008). Multi-enzyme fusion is also a potential approach to enhance anthocyanin production, as observed by fusion of F3GT from *Arabidopsis thaliana* to the *N*-terminus of ANS from *Petunia hybrida* in *E. coli*, which led to higher production of C3G (78.9 mg/L) compared to their native form (4.0 mg/L; Yan et al., 2008). This improvement in anthocyanin production may be linked to the extended activity of the chimeric enzymatic complex (multi-enzyme fusion) and a higher availability of unstable intermediates which are used for successive enzymatic biochemical conversions. In order to produce red wine pyranoanthocyanins through synthetic biology, an *E. coli* cell was first engineered with TAT and phenolic acid decarboxylase (PDC) to produce 4-vinylphenol, and then co-cultured with another cyanadin 3-glucoside producing *E. coli* cells to obtain a titer of 19.5 mg/L of pyranocyanidin 3-glucoside-4-phenol. Further, incorporation of HpaBC in 4-vinylphenol-producing *E. coli* resulted in the production of pyranocyanidin 3-*O*-glucoside-4-catechol upto 13 mg/L (Akdemir et al., 2019; Zha et al., 2020).

Manipulating intracellular UDP-glucose levels was also targeted for the metabolic engineering of anthocyanin production. Higher levels of intracellular UDP-glucose can be achieved by overexpressing bacterial UDP-glucose biosynthetic pathway genes (*pyrE*, *pyrR*, *cmk*, *ndk*, *pgm*, *galU*) and exogenous *ANS* and *3GT* genes using orotic acid as a substrate and resulting in an accumulation of C3G upto 97 mg/L (Leonard et al., 2008). In another effort, the polyculture of four *E. coli* strains expressing 15 exogenous or modified bacterial pathway enzymes from various plant sources and other microbes achieved the production of Callistephin (pelargonidin 3-*O*-glucoside) up to 9.5 mg/L, exclusively from 20 g/L glucose as a single-carbon source (Jones et al., 2017). Likewise, co-expression of anthocyanin synthase of *P. hybrida* (*PhANS*), *anthocyanidin 3-O-glucosyltransferase of A. thaliana (*At*3GT),* anthocyanin *O*-methyltransferase of *Vitis vinifera (*Vv*AOMT1) and the* fragrant cyclamen ‘Kaori-no-mai’ (*Ckm*OMT2) in *E. coli*, were achieved with production of peonidin 3-*O*-glucoside (P3G) up to 2.7 mg/L using (+)-catechin as well as endogenous UDP-glucose and *S*-adenosyl-L-methionine (SAM) as sugar and methyl group donors, respectively (Cress et al., 2017).

The CRISPR-Cas9, a recently discovered guide-RNA (Clustered Regularly Interspaced Short Palindromic Repeats) and nuclease (Cas9 protein) based bacterial defense system against their invading bacteriophages, became a powerful genetic tool for gene/genome editing (Doudna and Charpentier, 2014; Belhaj et al., 2015). Further, to increase the production of peonidin-3-*O*-glucoside (P3G) in an engineered *E. coli* strain *(*Cress et al., 2017*),* CRISPRi-mediated suppression of methionine biosynthesis was conducted to improve the bioavailability of SAM for the *O*-methylation of C3G, enhancing the P3G production by 21-fold (51 mg/L; Cress et al., 2017). Collectively, selection and co-expression of the most efficient enzyme sources, optimum supply of the desired substrates, culture conditions and UDP-glucose availability have greatly influenced the heterologous production of anthocyanins reaching maximum level of 350 mg/L for C3G and 113 mg/L of pelargonidin 3-O-glucoside, respectively in *E. coli* (Yan et al., 2008; Leonard et al., 2008). Similarly, the work of Lim et al. (2015) reported a promising cost-effective process for large-scale production of C3G (a titer of 350 mg/L) using the comparatively cheap (+)-catechin as a substrate in recombinant *E. coli* under optimized cultured conditions. Most recently, metabolic engineering of *Corynebacterium glutamicum*co-expressing anthocyanidin synthase (*ANS)* from *P. hybrid* and 3-*O*-glucosyltransferase (*3GT)* from *A. thaliana* achieved a 15-fold (40 mg/L) C3G titer increment which could be up-scaled > 100 fold by increasing exogenous UDP-glucose supply (Zha et al., 2018). Continuingly, researches have also focused on an engineered lactic acid bacterium strain *Lactococcus lactis*, harboring *ANS* and *3GT* genes for production of red-purple, orange and yellow anthocyanins using green tea as a substrate. This engineered *L. lactis* was also used to produce two pyranoanthocyanins (methylpyranodelphinidin and methylpyranopetunidin) utilizing gallocatechin as a substrate (Solopova et al., 2019; Zha et al., 2020).

3.3.2. In eukaryotes

Yeast cells, as *S. cerevisiae, Pichia pastoris, Yarrowia lipolytica* and *Kluyveromyces marxianus* have been commonly used as cell factories for various synthetic biology applications, including biofuels, recombinant proteins and pharmaceutically-important natural compound production (Ruta and Farcasanu, 2019). Being eukaryotic cellular organization, yeast cells offer many advantages over their prokaryotic counterparts in terms of well-established genetic tools, availability of post-transcriptional and post-translational modifications and suitability of incorporating membrane proteins for a synthetic biology application (Jensen and Keasling, 2015; Walker and Pretorius, 2018; Liu et al., 2019b). Recently, some works were also conducted to engineer *S. cerevisiae* to produce pelargonidin 3-*O*-glucoside after incorporating the anthocyanin biosynthetic specific genes (*F3H/ANS/3GT/DFR*) from *A. thaliana and Gerbera hybrid* (Levisson et al., 2018). It was also reported that in order to optimize anthocyanins production, biosynthetic genes were integrated into the yeast genome and the formation of phloretic acid (side product) was prevented by engineering the yeast chassis. Further, glycosidase activity was reduced by engineering to prevent degradation of pelargonidin 3-*O* glucoside. These results showed potential of engineered cells for the production of anthocyanins (Levisson et al., 2018). In another study, formation of pyranoanthocyanins in red wine was compared using four different yeast strains (*S. cerevisiae*, *Schizosaccharomyces pombe*, *Saccharomycodes ludwigii* and *Torulaspora delbrueckii*) wherein maximum yield (5 mg/L) was observed in case of fermentation with *S. pombe* (Escott et al., 2016; Morata et al., 2019). Earlier, Morata et al. (2012) also compared the pyranoanthocyanin production using *S. pombe*, *S. cerevisiae* and *S. uvarum* during grape wine fermentation and observed yields in the range of 11.9 to 19.4 mg/L. In addition, formation of pyranoanthocyanins was also observed in blood orange and black carrot juice through condensation of anthocyanins with other free hydroxycinnamic acids (coumaric, caffeic, ferulic, and sinapic acid) (Hillebrand et al., 2004). Furthermore,

Table 4
List of targeted genes, host organisms, culture conditions and yields of the heterologous production of anthocyanins.

Gene constructs	No. of genes	Host organism	Host strain	Culture conditions	Temp. (°C)/ pH	Sugar source	Substrate	Products and Yield	Reference
*Md*F3'H/*Aa*DFR/*Md*ANS/*Ph*F3GT	4	*E. coli*	JM109	IPTG induced M9 minimal media	RT/7	UDP-glucose	Naringenin Eriodictyol	Pelargonidin (6.0 μg/L) Cyanidin (5.6 μg/L)	Yan et al. (2005)
*Md*F3'H/*Aa*DFR/*At*3GT/*Ph*ANS	4	*E. coli*	BL21 (DE3)	IPTG induced M9 minimal media with 2-oxogltarate & Sodium acetate	30/7	UDP-glucose	Naringenin Eriodictyol	Pelargonidin (0.65 mg/L) Cyanidin (2.01 mg/L)	Yan et al. (2008)
*Md*F3'H/*Aa*DFR/At3GT/*Ph*ANS/*Du*LAR	5						Naringenin Eriodictyol	Pelargonidin (1.06 mg/L) Cyanidin (2.22 mg/L)	
*Md*F3'H/*Aa*DFR/*At*3GT/*Ph*ANS/*Du*LAR/*Dv*3MaT	6						Naringenin Eriodictyol	Pelargonidin (0.82 mg/L) Cyanidin (1.75 mg/L)	
*At*3GT/*Ph*ANS	2	*E. coli*	BL21 (DE3)	IPTG induced M9 minimal media	30/5	UDP-glucose	Catechin	Cyanidin (38.9 mg/L)	Yan et al. (2008)
*At*3GT/*Ph*ANS/(*E. coli* gene: galU/pgm)	2						Catechin	Cyanidin (61.4 mg/L)	
Fusion of *At*3GT/*Ph*ANS/(*E. coli* gene: galU/pgm)	2						Catechin Afzelechin	Cyanidin (70.7 mg/L) Pelargonidin (78.9 mg/L)	
Fusion of 3GT/ANS/(*E. coli* gene: galU/pgm/ndk/Δudg/gale)	2	*E. coli*	BL21 (DE3)	M9 minimal media with orotic acid	NR/5	ND	Catechin	Cyanidin (97 mg/L)	Leonard et al. (2008)
3GT + ANS/(*E. coli* gene: pgm/galU/ndk/cmk)	2	*E. coli*	BL21 (DE3)	IPTG induced M9 media with 2-oxogltarate, sodium ascorbate and orotic acid	30/5	1% glucose	Catechin	Cyanidin (107.2 mg/ L)	Lim et al. (2015)
3GT + ANS/(*E. coli* gene: galU/pgm/ndk/cmk/ycjU)	2							Cyanidin (122.3 mg/L)	
3GT/ANS/ (*E. coli* gene: galU/pgm/ndk/YadH/ΔtolC)	2							Cyanidin (350 mg/L)	
*At*F'3GT/*Ph*ANS/*Vv*AOMT/↓CRISPRiMeJ	3	*E. coli*	BL21 (DE3)	1 mM IPTG induced AMM media	30/NR	1% glucose	Catechin	Peonidin (56 mg/L)	Cress et al. (2017)
*Ph*ANS/*At*3GT/*Vv*AOMT1/*Ckm*AOMT	4	*E. coli*	BL21 Star™ (DE3)	1 mM IPTG induced AMM media & SAM	30/NR	UDP-glucose	(+)-catechin	Peonidin (2.7 mg/L)	Cress et al. (2017)
*Am*Ros1/*Am*Del/MtLAR/*Mt*ANR	4	*N. benthamiana*	Transgenic	Hygromycin resistant transgenic media under 16 h light/ 8 h dark photoperiod	25/7	Host carbon pool	ND	Proanthocyanidins (3.48 mg/g DW)	Fresquet-corrales et al. (2017)
PAL/4CL/CHS/CHI/F3H/DFR/LAR/ANS/3GT/Other *E. coli* genes	15	*E. coli*	rpoA14(DE3) BL21star™	Four-strain Polycultured AMM media + 1mM IPTG	30/NR	20 g/L Glucose	Glucose	Callistephin (9.5 mg/L)	Jones et al. (2017)
*Am*Del/*Am*Ros1/*Ph*F3'5'H	3	*N. tabacum*	Cell culture	LS media with 2,4-D& 100 mg/L Kanamycin	37/7	Host carbon pool	ND	Cyanidin (30 mg/L)	Appelhagen et al. (2018)
F3H/ANS/3GT/DFR	4	*S. cerevisiae*	PATW076	SMA medium	30/5	Glucose	Naringenin	Pelargonidin (0.001 μmol/g)	Levisson et al. (2018)
ANS/3GT	2	*C. glutamicum*	ATCC 13032	AMM media₊ yeast extract + casaminoacids + IPTG	30/NR	UDP-glucose	Catechin	Cyaniding (40 mg/L)	Zha et al. (2018)
ANZ/3GT	2	*L. lactis*	NZLdh-3gt	GM17 media with 2-oxoglutarate& 2.5 mM ascorbic acid	30/3	1% glucose	Green tea	Cyaniding + Delphinidin + Pronoanthocyanidins (1.5 mg/L)	Solopova et al. (2019)

Md: Malus domestica, Aa:Anthurium andraeanum, Ph: Petunia hybrida, At: Arabidopsis thaliana, Vv: Vitis vinifera, Ec: Escherichia coli, Du: Desmodium uncinatum, *Dv: Dahlia variabilis, Am: Antirrhinum majus, Mt: Medicago truncatula, N. benthamiana: Nicotiana benthamiana, N. tabacum: Nicotiana tabacum, S. cerevisiae: Saccharomyces cerevisiae, C. glutamicum: Corynebacterium glutamicum, L. lactis: Lactococcus lactis*, ANS: anthocyanidin synthase, 3GT:anthocyanidin 3-*O*-glucosyltransferase, AOMT:anthocyanin *O*-methyltransferase, PAL: phenylalanine ammonia-lyase, 4CL: para-coumaroyl-CoA ligase, CHS: chalcone synthase, CHI: chalcone isomerase, F3'H: flavonoid 3'-hydroxylase, F3'5'H: Flavonoid-3',5'-hydroxylase, F3H, flavanone 3-hydroxylase, DFR: dihydroflavonol reductase, FLS: flavonol synthase, LAR: leucoanthocyanidin reductase, SAM:S-adenosyl-L-methionine, 3MaT: 3-O-glucoside-6"-O-malonyltransferase, galU: UTP–glucose-1-phosphate uridylyltransferase, pgm: Phosphoglycerate mutase, ndk: Nucleoside diphosphate kinase, Δudg: Uracil-DNA glycosylase, gale: UDP-glucose 4-epimerase, cmk: cytidine monophosphate kinase, ycjU: Beta-phosphoglucomutase, yadH: Inner membrane transport permease, ΔtolC: Efflux pump, CkmAOMT: 'Kaori-no-mai' AOMT, Del: bHLH transcription factor, Ros1: R2R3 MYB transcription factor, DW: dry weight, RT: room temperature, IPTG: Isopropyl β- D-L-thiogalactopyranoside, M9 media: a minimal media for bacterial culturing, AMM media: antimitotic medium, LS media: Linsmaier and Skoog media, SMA media: Standard Methods Agar media, GM17 media: a minimal media for bacterial culturing, RT: room temperature, NR: not recorded, ND: not define.

formation of quinonoid bases during this condensation reaction to reduce the formation of non-colored carbinol bases is majorly responsible for higher stability of these pyranoanthocyanins (Ruta and Farcasanu, 2019).

At present, there is no sufficient literature available describing *P. pastoris* as a heterologous host for anthocyanin production, although it has successfully been used to produce xantophylls (β-carotene) (Araya-Garay et al., 2012), and hence, could also be engineered for anthocyanin production. Like-wise, evidences are also lacking for the use of *Y. lipolytica* and *K. marxianus* for synthetic biology of anthocyanins, and this requires further scientific interventions. Additionally, despite the role of yeast cells is well documented for the formation of pyranoanthocyanins during red wine aging, there are no solid evidence available in favour of engineering fermenting yeast for the up-scaled production of these more complex, but nutraceutically more relevant metabolites.

4. Challenges and future perspectives

The demand for the commercial production of anthocyanins has steadily increased in recent years, mainly due to increasing consumer preferences towards natural food additives, nutraceuticals and healthy foods. Anthocyanins and their aglycones (anthocyanidins) constitute a large group of natural pigments, which are approved as food additives colorant in many countries, in the EU, for example, they are assigned E163 numbers. Further, anthocyanins are popular due to their safety and potential applications in medicine, cosmetics and nutraceutical products. Apart from the direct extraction of anthocyanins from natural resources, *in vitro* plant cell or tissue cultures and genetic modifications in microorganisms and plants (as discussed in above sections) have proven potential role of biotechnology in driving other sustainable and effective ways of commercial production of anthocyanins. However, various challenges and limitations of these technologies are noticed, which are enumerated below along with possible future recommendations.

- Synthetic biology (both microbial and plant) offers an excellent tool to engineer heterologous hosts for production of commercially important anthocyanins in sustainable way to reduce our dependency on natural resources. However, despite having shorter growth time, well-standardized genetic manipulation protocols and easily up-scaling culture conditions, low titers with higher cost is one of the major challenges of anthocyanins production from engineered bacteria compared to conventional plant extraction methods.
- Lack of proper eukaryotic cell environment (nuclear-organelle compartmentalization and post-transcriptional/ translational modifications) and formation of protein inclusion bodies, heterologous expression of eukaryotic genes in prokaryotic host, especially membrane proteins could be a challenging task. However, several technological advancements such as codon optimization, amino acid substitution and site-directed mutagenesis with other bioinformatics tools could be utilized to overcome these difficulties.
- Comparatively, plant cell or tissue cultures have shown effective results in the production of anthocyanins though with relatively low yields. However, inconsistent culture conditions, higher production cost and scale-up difficulties would impose challenges. Optimization of abiotic culture conditions (medium, PGRs, elicitors and precursors, light, temperature and pH) and regulating the biosynthesis pathway both at the enzyme and the gene levels could be an advantage for obtaining better yield and scale-up of culture conditions. The morphologically and anatomically different plant tissues also provide an excellent advantage for tissue-specific expression and production of anthocyanins and can even be introduced in main-stream food products (bio-fortification) instead of going for extraction and formulation.
- Despite availability of various types of bioreactors, the economic feasibility of the large-scale production of anthocyanins remains a challenging issue. Although not much efforts have been made on scale-up of the anthocyanin production, owing to the complexity of the biosynthesis of natural products, the selection and modifications of bioreactors in terms of light illumination, impeller design, automatic withdraw of the sample, are some of the areas to be worked upon. Also media composition, air flow rate, agitation rate, light and temperature need to be optimized to increase the anthocyanin biosynthesis.
- With rapidly decline DNA synthesis cost along with emerging advanced gene/genome editing tools (*i.e.* CRISPR-Cas, nanocarriers for gene transfer) and directed evolution (DE), a remarkable progress could be expected to engineer model plants or even native sources of commercially important anthocyanins. Similarly, several other simpler autotrophic heterologous hosts such as green algae (e.g. *Chlamydomonas reinhardtii* and *Phaeodactylum tricornutum*) and mosses (e.g. *Physcomitrella patens*) are being introduced in plant synthetic biology to engineer anthocyanin biosynthetic pathway before going to complex multicellular plant systems.
- Identification and isolation of novel anthocyanin related P450 enzymes along with their associated reductases and UGTs is a challenging task. However, with the advancement in sequencing technologies (Next-Generation Sequencing) and genome assembly of many plant species, it could be possible to identify novel gene variants of anthocyanin biosynthetic pathway, which can be used for metabolic engineering and to modulate the anthocyanin biosynthesis.
- Even though, metabolic engineering of plants and microorganisms showed some promising success for the production of anthocyanins; however, the accumulation and transportation of anthocyanins from these engineered cells is a challenging task to get purified final products with higher yield. Several efflux pumps (YadH and TolC) have been incorporated in engineered *E. coli* cells for enhancing cyanidin 3-*O*-glucoside accumulation and transportation. Further, discovery and introduction of novel anthocyanins-specific plant transporters *i.e.*, MRP3 (ABC family), Bronze-2 and Testa12 (MATE family) could be searched from rapidly increasing plant genomic resources.
- Identification of highly efficient promoters from novel sources could also be a very fruitful strategy to replenish the biological hurdles between eukaryotes (sources for anthocyanin-related genes/pathways) and prokaryotes (common hosts for heterologous production) (Fig. 3). There is a substantial scientific consideration required for characterization of negative regulators of anthocyanin biosynthesis that can futuristically be targeted for gene-editing and RNAi-mediated gene silencing approaches to manipulate the anthocyanin content.
- The stability issue of anthocyanins appears as one of the major limiting factors for heterologous production of these metabolites. Usually, pH stability of anthocyanins in their native plant source is facilitated by cytosolic and vacuolar pH adjustments, which are lacking in the bacterial host. One of the currently adopted strategies is culturing engineered bacteria in initial medium maintained at pH 7 to support proper growth and gene(s) expression, followed by culture transfer to a fresh medium with lowered pH (5.0) to facilitate anthocyanin biosynthesis and stabilization. However, such a shifting procedure enhances the complexity of the overall process.
- Due to enhanced pharmaceutical properties and chemical stability compared to their parent anthocyanins, scientific efforts are also required to explore the novel sources of pyranoanthocyanins along with improvement in their extraction, processing and synthetic biology for sustainable production.
- Secretion of metabolic intermediates, metabolic toxicity, precursors and cofactors imbalance, and reduced enzyme activity in heterologous system also leads to a low yield of desirable metabolites. Despite these challenges, continuous innovation in culture

optimization and biotechnological advances in engineering recombinant microorganisms (microbial cell factories) during the past two decade, may have become competitive sources of commercially produced anthocyanins.

Consequently, the biotechnological production of anthocyanins possesses several competitors; however, considering advantages and limitations of each strategy, all above-listed ways may find relevant niches in the area of anthocyanin biosynthesis and its production through biotechnological approaches.

Acknowledgements

TB and ZL acknowledges funding from the National Key Research and Development Program of China (2019YFC1604504, 2019YFD1002301) and Technology Innovation Progamme of Zhejiang Province (ZJWR0102001), China. MIG acknowledges funding from the European Union's Horizon 2020 research and innovation programme, project PlantaSYST (SGA No 739582 under FPA No. 664620), and the BG05M2OP001-1.003-001-C01 project, financed by the European Regional Development Fund through the "Science and Education for Smart Growth" Operational Programme.

References

Akdemir, H., Silva, A., Zha, J., Zagorevski, D.V., Koffas, M.A.G., 2019. Production of pyranoanthocyanins using *Escherichia coli* cocultures. Metab. Eng. 55, 290–298.
Alfenito, M.R., Souer, E., Goodman, C.D., Buell, R., Mol, J., Koes, R., Walbot, V., 1998. Functional complementation of anthocyanin sequestration in the vacuole by widely divergent glutathione S-transferases. Plant Cell 10, 1135–1150.
An, C.H., Lee, K., Lee, S., Jeong, Y.J., Woo, S.G., Chun, H., Park, Y.I., Kwak, S.S., Kim, C.Y., 2015. Heterologous expression of IbMYB1a by different promoters exhibits different patterns of anthocyanin accumulation in tobacco. Plant Physiol. Biochem. 89, 1–10.
An, J.P., Wang, X.F., Li, Y.Y., Song, L.Q., Zhao, L.L., You, C.X., Hao, Y.J., 2018. EIN3-LIKE1, MYB1, and ETHYLENE RESPONSE FACTOR3 act in a regulatory loop that synergistically modulates ethylene biosynthesis and anthocyanin accumulation. Plant Physiol. 178 (2), 808–823.
Antognoni, F., Zheng, S., Pagnucco, C., Baraldi, R., Poli, F., Biondi, S., 2007. Induction of flavonoid production by UV-B radiation in *Passiflora quadrangularis* callus cultures. Fitoterapia 78 (5), 345–352.
Appelhagen, I., Keim, A., Wendell, M., Hvoslef-eide, A., Russell, J., Oertel, A., Martens, S., Mock, H.P., Martin, C., Matros, A., 2018. Colour bio-factories: towards scale-up production of anthocyanins in plant cell cultures. Metab. Eng. 48, 218–232.
Araya-Garay, J.M., Ageitos, J.M., Vallejo, J.A., Veiga-Crespo, P., Sanchez-Perez, A., 2012. Construction of a novel *Pichia pastoris* strain for production of xanthophylls. AMB Express 2, 24.
Ashokhan, S., Othman, R., Abd Rahim, M.H., Karsani, S.A., Yaacob, J.S., 2020. Effect of plant growth regulators on coloured callus formation and accumulation of azadirachtin, an essential biopesticide in *Azadirachta indica*. Plants 9 (3), e352.
Baker, R.F., Braun, D.M., 2007. tie-dyed1 functions non-cell autonomously to control carbohydrate accumulation in maize leaves. Plant Physiol. 144 (2), 867–878.
Ban, Z., Luo, Z., Chen, C., Gong, J., Yuan, Q., Yu, L., Yu, W., Li, L., 2017. Cloning of PcPAL gene from pyrus communis and characterization of its expression in two cultivars with different anthocyanin accumulation levels. J. Biobased Mater. Bio. 11 (4), 343–348.
Baudry, A., Heim, M.A., Dubreucq, B., Caboche, M., Weisshaar, B., Lepiniec, L., 2004. TT2, TT8, and TTG1 synergistically specify the expression of BANYULS and proanthocyanidin biosynthesis in *Arabidopsis thaliana*. Plant J. 39, 366–380.
Belhadj, A., Telef, N., Saigne, C., Cluzet, S., Barrieu, F., Hamdi, S., Mérillon, J.M., 2008. Effect of methyl jasmonate in combination with carbohydrates on gene expression of PR proteins, stilbene and anthocyanin accumulation in grapevine cell cultures. Plant Physiol. Biochem. 46 (4), 493–499.
Belhaj, K., Chaparro-Garcia, A., Kamoun, S., Patron, N.J., Nekrasov, V., 2015. Editing plant genomes with CRISPR/Cas9. Curr. Opin. Biotechnol. 32, 76–84.
Belwal, T., Nabavi, S.F., Nabavi, S.M., Habtemariam, S., 2017. Dietary anthocyanins and insulin resistance: When food becomes a medicine. Nutrients 9 (10), 1111.
Belwal, T., Huang, H., Li, L., Duan, Z., Zhang, X., Aalim, H., Luo, Z., 2019. Optimization model for ultrasonic-assisted and scale-up extraction of anthocyanins from Pyrus communis 'Starkrimson'fruit peel. Food Chem. 297, 124993.
Betsui, F., Tanaka-Nishikawa, N., Shimomura, K., 2004. Anthocyanin production in adventitious root cultures of *Raphanus sativus* L. cv. Peking Koushin. Plant Biotechnol. 21 (5), 387–391.
Biswas, T., Mathur, A., 2017. Plant anthocyanins: biosynthesis, bioactivity and in vitro production from tissue cultures. Adv. Biotechnol. Microbiol. 5 (5), 555672.
Biswas, T., Singh, M., Mathur, A.K., Mathur, A., 2015. A dual purpose cell line of an Indian congener of ginseng-*Panax sikkimensis* with distinct ginsenoside and anthocyanin production profiles. Protoplasma 252 (2), 697–703.
Blando, F., Scardino, A.P., De Bellis, L., Nicoletti, I., Giovinazzo, G., 2005. Characterization of in vitro anthocyanin-producing sour cherry (*Prunus cerasus* L.) callus cultures. Food Res. Int. 38 (8-9), 937–942.
Borochov-Neori, H., Judeinstein, S., Harari, M., Bar-Ya'akov, I., Patil, B.S., Lurie, S., Holland, D., 2011. Climate effects on anthocyanin accumulation and composition in the pomegranate (*Punica granatum* L.) fruit arils. J. Agric. Food Chem. 59 (10), 5325–5334.
Butelli, E., Titta, L., Giorgio, M., Mock, H.P., Matros, A., Peterek, S., Schijlen, E.G.W.M., Hall, R.D., Bovy, A.G., Luo, J., Martin, C., 2008. Enrichment of tomato fruit with health-promoting anthocyanins by expression of select transcription factors. Nat. Biotechnol. 26, 1301–1308.
Cai, Z., Kastell, A., Mewis, I., Knorr, D., Smetanska, I., 2012. Polysaccharide elicitors enhance anthocyanin and phenolic acid accumulation in cell suspension cultures of *Vitis vinifera*. Plant Cell Tiss. Organ Cult. 108 (3), 401–409.
Carey, C.C., Strahle, J.T., Selinger, D.A., Chandler, V.L., 2004. Mutations in the pale-aleurone color1 regulatory gene of the *Zea mays* anthocyanin pathway have distinct phenotypes relative to the functionally similar TRANSPARENT TESTA GLABRA1 gene in *Arabidopsis thaliana*. Plant Cell 16, 450–464.
Chan, L.K., Koay, S.S., Boey, P.L., Bhatt, A., 2010. Effects of abiotic stress on biomass and anthocyanin production in cell cultures of *Melastoma malabathricum*. Biol. Res. 43 (1), 127–135.
Chandler, S., Tanaka, Y., 2007. Genetic modification in floriculture. Crit. Rev. Plant Sci. 26, 169–197.
Chemler, J.A., Koffas, M.A., 2008. Metabolic engineering for plant natural product biosynthesis in microbes. Curr. Opin. Biotechnol. 19 (6), 597–605.
Chouhan, S., Sharma, K., Zha, J., Guleria, S., 2017. Recent advances in the recombinant biosynthesis of polyphenols. Front. Microbiol. 8, 2259.
Cress, B.F., Leitz, Q.D., Kim, D.C., Amore, T.D., Suzuki, J.Y., Linhardt, R.J., Koffas, M.A., 2017. CRISPRi- mediated metabolic engineering of *E. coli* for O-methylated anthocyanin production. Microb. Cell. Fact. 16, 1–14.
Cruz, L., Mateus, N., de Freitas, V., 2013. First chemical synthesis report of an anthocyanin metabolite with in vivo occurrence: cyanidin-4′-O-methyl-3-glucoside. Tetrahedron Lett. 54 (22), 2865–2869.
Curtin, C., Zhang, W., Franco, C., 2003. Manipulating anthocyanin composition in *Vitis vinifera* suspension cultures by elicitation with jasmonic acid and light irradiation. Biotechnol. Lett. 25 (14), 1131–1135.
Cutanda-Perez, M.C., Ageorges, A., Gomez, C., Vialet, S., Terrier, N., Romieu, C., Torregrosa, L., 2009. Ectopic expression of *VlmybA1* in grapevine activates a narrow set of genes involved in anthocyanin synthesis and transport. Plant Mol. Biol. 69, 633–648.
Dai, Z.W., Meddar, M., Renaud, C., Merlin, I., Hilbert, G., Delrot, S., Gomès, E., 2014. Long-term in vitro culture of grape berries and its application to assess the effects of sugar supply on anthocyanin accumulation. J. Exp. Bot. 65 (16), 4665–4677.
Das, P.K., Shin, D.H., Choi, S.B., Yoo, S.D., Choi, G., Park, Y.I., 2012. Cytokinins enhance sugar-induced anthocyanin biosynthesis in Arabidopsis. Mol. Cells 34 (1), 93–101.
Deikman, J., Hammer, P.E., 1995. Induction of anthocyanin accumulation by cytokinins in *Arabidopsis thaliana*. Plant Physiol. 108 (1), 47–57.
Desai, P.N., Shrivastava, N., Padh, H., 2010. Production of heterologous proteins in plants: Strategies for optimal expression. Biotechnol. Adv. 28, 427–435.
Doudna, J.A., Charpentier, E., 2014. The new frontier of genome engineering with CRISPR-Cas9. Science 346 (6213), 1258096.
El-Kereamy, A., Chervin, C., Roustan, J.-P., Cheynier, V., Souquet, J.-M., Moutounet, M., Raynal, J., Ford, C., Latché, A., Pech, J.-C., Bouzayen, M., 2003. Exogenous ethylene stimulates the long-term expression of genes related to anthocyanin biosynthesis in grape berries. Physiol. Plant. 119, 175–182.
Escott, C., Morata, A., Loira, I., Tesfaye, W., Suarez-Lepe, J.A., 2016. Characterization of polymeric pigments andpyranoanthocyanins formed in microfermentations ofnon-*Saccharomyces* yeasts. J. Appl. Microbiol. 121, 1346–1355.
Feller, A., Machemer, K., Braun, E.L., Grotewold, E., 2011. Evolutionary and comparative analysis of MYB and bHLH plant transcription factors. Plant J. 66, 94–116.
Ferreyra, F.M.L., Rius, S., Casati, P., 2012. Flavonoids: biosynthesis, biological functions, and biotechnological applications. Front. Plant Sci. 28 (3), 222.
Fresquet-corrales, S., Roque, E., Sarrion-Perdigones, A., Rochina, M., Lopez-Gresa, M.P., Diaz-Mula, H.M., Bellés, J.M., Tomás-Barberán, F., Beltrán, J.P., Cañas, L.A., 2017. Metabolic engineering to simultaneously activate anthocyanin and proanthocyanidin biosynthetic pathways in *Nicotiana* spp. PLoS One. 12, e0184839.
Fukada-Tanaka, S., Inagaki, Y., Yamaguchi, T., Saito, N., Iida, S., 2000. Colouring enhancing protein in blue petals. Nature 407, 581.
Gagné, S., Cluzet, S., Mérillon, J.M., Gény, L., 2011a. ABA initiates anthocyanin production in grape cell cultures. J. Plant Growth Regul. 30, 1), 1–10.
Gagné, S., Cluzet, S., Merillon, J.M., Gény, L., 2011b. ABA initiates anthocyanin production in grape cell cultures. Plant Growth Regul. 30, 1–10.
Global Anthocyanin Market report, 2020. 2025. https://www.mordorintelligence.com/industry-reports/anthocyanin-market.
Gonzali, S., Mazzucato, A., Perata, P., 2009. Purple as a tomato: Towards high anthocyanin tomatoes. Trends Plant Sci. 14, 6–10.
Goodman, C.D., Casati, P., Walbot, V., 2004. A multidrug resistanceeassociated protein involved in anthocyanin transport in *Zea mays*. Plant Cell 16, 1812–1826.
Grotewold, E., 2005. Plant metabolic diversity: A regulatory perspective. Trends Plant Sci. 10, 57–62.
Grotewold, E., Davis, K., 2008. Trafficking and sequestration of anthocyanins. Nat. Prod. Comm. 3, 1251–1258.
Guo, J., Han, W., Wang, M., 2008. Ultraviolet and environmental stresses involved in the induction and regulation of anthocyanin biosynthesis: A review. Afr. J. Biotechnol. 7 (25), 4966–4972.
Hao, G., Du, X., Zhao, F., Ji, H., 2010. Fungal endophytes-induced abscisic acid is

required for flavonoid accumulation in suspension cells of *Ginkgo biloba*. Biotechnol. Lett. 32 (2), 305–314.
He, F., Mu, L., Yan, G.-L., Liang, N.-N., Pan, Q.-H., Wang, J., Reeves, M.J., Duan, C.-Q., 2010. Biosynthesis of anthocyanin and their regulation in colored grapes. Molecules 15, 9057–9091.
He, X., Li, Y., Lawson, D., Xie, D., 2017. Metabolic engineering of anthocyanins in dark tobacco. Physiol. Plant 159, 2–12.
Hernandez, J.M., Feller, A., Morohashi, K., Frame, K., Grotewold, E., 2007. The basic helix-loop-helix domain of maize R links transcriptional regulation and histone modifications by recruitment of an EMSY-related factor. Proc. Natl. Acad. Sci. 104, 17222–17227.
Hillebrand, S., Schwarz, M., Winterhalter, P., 2004. Characterization of anthocyanins andpyranoanthocyanins from blood orange [*Citrus sinensis* (L.) Osbeck] juice. J. Agric. FoodChem. 52, 7331–7338.
Hirner, A.A., Veit, S., Seitz, H.U., 2001. Regulation of anthocyanin biosynthesis in UV-A-irradiated cell cultures of carrot and in organs of intact carrot plants. Plant Sci. 161 (2), 315–322.
Iorizzo, M., Cavagnaro, P.F., Bostan, H., Zhao, Y., Zhang, J., Simon, P.W., 2019. A cluster of MYB transcription factors regulates anthocyanin biosynthesis in carrot (*Daucus carota* L.) root and petiole. Front. Plant Sci. 9, 1927.
Jeandet, P., Sobarzo-Sánchez, E., Clément, C., Nabavi, S.F., Habtemariam, S., Nabavi, S.M., Cordelier, S., 2018. Engineering stilbene metabolic pathways in microbial cells. Biotechnol. Adv. 36 (8), 2264–2283.
Jensen, M.K., Keasling, J.D., 2015. Recent applications of synthetic biology tools for yeast metabolic engineering. FEMS Yeast Res. 15, 1–10.
Jeong, S.T., Goto-Yamamoto, N., Kobayashi, S., Esaka, M., 2004. Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins. Plant Sci. 167, 247–252.
Ji, X.H., Wang, Y.T., Zhang, R., Wu, S.J., An, M.M., Li, M., Wang, C.Z., Chen, X.L., Zhang, Y.M., Chen, X.S., 2015. Effect of auxin, cytokinin and nitrogen on anthocyanin biosynthesis in callus cultures of red-fleshed apple (*Malus sieversii* f. niedzwetzkyana). Plant Cell Tiss. Organ Cult. 120 (1), 325–337.
Jones, J.A., Vernacchio, V.R., Collins, S.M., Shirke, A.N., Xiu, Y., Englaender, J.A., Cress, B.F., McCutcheon, C.C., Linhardt, R.J., Gross, R.A., Koffas, M.A.G., 2017. Complete biosynthesis of anthocyanins using *E. coli* polycultures. mBio 8 (3) e00621-27.
Katsumoto, Y., Fukuchi-Mizutani, M., Fukui, Y., Brugliera, F., Holton, T.A., Karan, M., Nakamura, N., Yonekura-Sakakibara, K., Togami, J., Pigeaire, A., Tao, G.Q., Nehra, N.S., Lu, C.Y., Dyson, B.K., Tsuda, S., Ashikari, T., Kusumi, T., Mason, J.G., Tanaka, Y., 2007. Engineering of the rose flavonoid biosynthetic pathway successfully generated blue-hued flowers accumulating delphinidin. Plant Cell Physiol. 48, 1589–1600.
Khoo, H.E., Azlan, A., Tang, S.T., Lim, S.M., 2017. Anthocyanidins and anthocyanins: Colored pigments as food, pharmaceutical ingredients, and the potential health benefits. Food Nutr. Res. 61 (1), 1361779.
Kobayashi, S., Goto-Yamamoto, N., Hirochika, H., 2005. Association of *VvmybA1* gene expression with anthocyanin production in grape (*Vitis vinifera*) skin -color mutants. J. Jpn. Soc. Hort. Sci. 74, 196–203.
Konczak-Islam, I., Yoshinaga, M., Nakatani, M., Terahara, N., Yamakawa, O., 2000. Establishment and characteristics of an anthocyanin-producing cell line from sweet potato storage root. Plant Cell Rep. 19 (5), 472–477.
Kondo, T., Oyama, K.I., Nakamura, S., Yamakawa, D., Tokuno, K., Yoshida, K., 2006. Novel and efficient synthesis of cyanidin 3-O-β-D-glucoside from (+)-catechin via a flav-3-en-3-ol as a key intermediate. Org. Lett. 8 (16), 3609–3612.
Kong, J.M., Chia, L.S., Goh, N.K., Chia, T.F., Brouillard, R., 2003. Analysis and biological activities of anthocyanins. Phytochemistry 64, 923–933.
Kutchan, T.M., 2005. A role for intra and inter cellular translocation in natural product biosynthesis. Curr. Opin. Plant Biol. 8, 292–300.
Lalusin, A.G., Nishita, K., Kim, S.H., Ohta, M., Fijimura, T., 2006. A new MADS-box gene (IbMADS10) from sweet potato (*Ipomoea batatas* (L.) Lam) is involved in the accumulation of anthocyanin. Mol. Genet. Genomics 275, 44–54.
Leonard, E., Yan, Y., Koffas, M.A.G., 2006. Functional expression of a P450 flavonoid hydroxylase for the biosynthesis of plant-specific hydroxylatedflavonols in *Escherichia coli*. Metab. Eng. 8, 172–181.
Leonard, E., Yan, Y., Fowler, Z.L., Li, Z., Lim, C.G., Lim, K.H., Koffas, M.A.G., 2008. Strain improvement of recombinant *Escherichia coli* for efficient production of plant flavonoids. Mol. Pharmaceutics 5, 257–265.
Levisson, M., Patinios, C., Hein, S., Groot, P.A., de Daran, J.M., Hall, R.D., Martens, S., Beekwilder, J., 2018. Engineering *de novo* anthocyanin production in *Saccharomyces cerevisiae*. Microb. Cell. Fact. 17, 1–16.
Li, X., Thwe, A.A., Park, C.H., Kim, S.J., Arasu, M.V., Abdullah Al-Dhabi, N., Lee, S.Y., Park, S.U., 2017. Ethephon-induced phenylpropanoid accumulation and related gene expression in tartary buckwheat (*Fagopyrum tataricum* (L.) Gaertn.) hairy root. Biotechnol. Biotec. Eq. 31 (2), 304–311.
Li, N., Wu, H., Ding, Q., Li, H., Li, Z., Ding, J., Li, Y., 2018. The heterologous expression of *Arabidopsis* PAP2 induces anthocyanin accumulation and inhibits plant growth in tomato. Funct. Integr. Genomics 18, 341–353.
Li, Y., Cui, W., Wang, R., Lin, M., Zhong, Y., Sun, L., Qi, X., Fang, J., 2019. MicroRNA858-mediated regulation of anthocyanin biosynthesis in kiwifruit (*Actinidia arguta*) based on small RNA sequencing. PLoS One 14 (5), e0217480.
Lim, C.G., Wong, L., Bhan, N., Dvora, H., Xu, P., Venkiteswaran, S., Koffas, M.A.G., 2015. Development of a recombinant *Escherichia coli* strain for overproduction of plant pigment, anthocyanin. Appl. Environ. Microbiol. 81 (18), 6276–6284.
Liu, Z., Shi, M.Z., Xie, D.Y., 2014. Regulation of anthocyanin biosynthesis in *Arabidopsis thaliana* red pap1-D cells metabolically programmed by auxins. Planta 239 (4), 765–781.
Liu, Y., Tikunov, Y., Schouten, R.E., Marcelis, L.F.M., Visser, R.G.F., Bovy, A., 2018. Anthocyanin biosynthesis and degradation mechanisms in solanaceous vegetables: A review. Front. Chem. 6, 52.
Liu, Y., Lin-Wang, K., Espley, R.V., Wang, L., Li, Y., Liu, Z., Zhou, P., Zeng, L., Zhang, X., Zhang, J., Allan, A.C., 2019a. StMYB44 negatively regulates anthocyanin biosynthesis at high temperatures in tuber flesh of potato. J. Exp. Bot. 70 (15), 3809–3824.
Liu, Z., Zhang, Y., Nielsen, J., 2019b. Synthetic biology of yeast. Biochemistry 58, 1511–1520.
Lu, Q., Yang, Q., Zou, H., 2006. Effects of cerium on accumulation of anthocyanins and expression of anthocyanin biosynthetic genes in potato cell tissue cultures. J. Rare Earths 24 (4), 479–484.
Lucioli, S., Di Bari, C., Nota, P., Frattarelli, A., Forni, C., Caboni, E., 2017. Methyl jasmonate promotes anthocyanins' production in *Prunus salicina* × *Prunus persicain vitro* shoot cultures. Plant Biosyst. 151 (5), 788–791.
Mano, H., Ogasawara, F., Sato, K., Higo, H., Minobe, Y., 2007. Isolation of a regulatory gene of anthocyanin biosynthesis in tuberous roots of purple-fleshed sweet potato. Plant Physiol. 143, 1252–1268.
Marchev, A.S., Yordanova, Z.P., Georgiev, M.I., 2020. Green (cell) factories for advanced production of plant secondary metabolites. Critic. Rev. Biotechnol. https://doi.org/10.1080/07388551.2020.1731414.
Marienhagen, J., Bott, M., 2013. Metabolic engineering of microorganisms for the synthesis of plant natural products. J. Biotechnol. 163 (2), 166–178.
Marrs, K.A., Alfenito, M.R., Lloyd, A.M., Walbot, V., 1995. A glutathione S-transferase involved in vacuolar transfer encoded by the maize gene Bronze-2. Nature 375, 397–400.
Mathews, H., Clendennen, S.K., Caldwell, C.G., Liu, X.L., Connors, K., Matheis, N., 2003. Activation tagging in tomato identifies a transcriptional regulator of anthocyanin biosynthesis, modification, and transport. Plant Cell 15, 1689–1703.
Mathur, A., Mathur, A.K., Gangwar, A., Yadav, S., Verma, P., Sangwan, R.S., 2010. Anthocyanin production in a callus line of *Panax sikkimensis* Ban. In Vitro Cell. Dev. Biol.—Plant 46 (1), 13–21.
Meng, S., Su, L., Li, Y., Wang, Y., Zhang, C., Zhao, Z., 2016. Nitrate and ammonium contribute to the distinct nitrogen metabolism of *Populus simonii* during moderate salt stress. PloS one 11 (3).
Meyer, P., Heidemann, I., Forkmann, G., Saedler, H., 1987. A new petunia flower colour generated by transformation of a mutant with a maize gene. Nature 330, 677–678.
Morata, A., Calderon, F., Gonzalez, M.C., Suarez-Lepe, J.A., 2007. Formation of the highly stable pyranoanthocyanins (vitisins A and B) in red wines by the addition of pyruvic acid and acetaldehyde. Food Chem. 100, 1144–1152.
Morata, A., Benito, S., Loira, I., Palomero, F., Gonzalez, M.C., Suarez-Lepe, J.A., 2012. Formation of pyranoanthocyanins by *Schizosaccharomyces pombe* during the fermentation of red must. Int. J. Food Microbiol. 159, 47–53.
Morata, A., Escott, C., Loira, I., Fresno, J.M.D., Gonzalez, C., Suarez-Lepe, J.A., 2019. Influence of *Saccharomyces* and non-*Saccharomyces* yeasts in the formation of pyranoanthocyanins and polymeric pigments during red wine making. Molecules 24 (4490), 1–18.
Mori, T., Sakurai, M., Sakuta, M., 2001. Effects of conditioned medium on activities of PAL, CHS, DAHP synthase (DS-Co and DS-Mn) and anthocyanin production in suspension cultures of *Fragaria ananassa*. Plant Sci. 160 (2), 355–360.
Muir, S.R., Collins, G.J., Robinson, S., Hughes, S., Bovy, A., Ric De Vos, C.H., van Tunen, A.J., Verhoeyen, M.E., 2001. Overexpression of petunia chalcone isomerase in tomato results in fruit containing increased levels of flavonols. Nat. Biotechnol. 19, 470–474.
Murthy, H.N., Lee, E.J., Paek, K.Y., 2004. Production of secondary metabolites from cell and organ cultures: Strategies and approaches for biomass improvement and metabolite accumulation. Plant Cell Tiss. Org. Cult. 118, 1–16.
Nakamura, N., Fukuchi-Mizutani, M., Suzuki, K., Miyazaki, K., Tanaka, Y., 2006. RNAi suppression of the anthocyanidin synthase gene in *Torenia hybrida* yields white flowers with higher frequency and better stability than antisense and sense suppression. Plant Biotechnol. 23, 13–17.
Nakatsuka, T., Abe, Y., Kakizaki, Y., Yamamura, S., Nishihara, M., 2007. Production of red-flowered plants by genetic engineering of multiple flavonoid biosynthetic genes. Plant Cell Rep. 26, 1951–1959.
Nakatsuka, T., Mishiba, K., Kubota, A., Abe, Y., Yamamura, S., Nakamura, N., Tanaka, Y., Nishihara, M., 2010. Genetic engineering of novel flower colour by suppression of anthocyanin modification genes in gentian. J. Plant Physiol. 167, 231–237.
Nakatsuka, T., Yamada, E., Saito, M., 2013. Heterologous expression of gentian MYB1R transcription factors suppresses anthocyanin pigmentation in tobacco flowers. Plant Cell Rep. 32, 1925–1937.
Narayan, M.S., Thimmaraju, R., Bhagyalakshmi, N., 2005. Interplay of growth regulators during solid-state and liquid-state batch cultivation of anthocyanin producing cell line of *Daucus carota*. Process Biochem. 40 (1), 351–358.
Nesi, N., Debeaujon, I., Jond, C., Pelletier, G., Caboche, M., Lepiniec, L., 2000. The TT8 gene encodes a basic helix-loop-helix domain protein required for expression of DFR and BAN genes in *Arabidopsis siliques*. Plant Cell 12 (10), 1863–1878.
Nishihara, M., Nakatsuka, T., 2011. Genetic engineering of flavonoid pigments to modify flower color in floricultural plants. Biotechnol. Lett. 33, 433–441.
Noda, N., Yoshioka, S., Kishimoto, S., Nakayama, M., Douzono, M., Tanaka, Y., Aida, R., 2017. Generation of blue chrysanthemums by anthocyanin B-ring hydroxylation and glucosylation and its coloration mechanism. Sci. Adv. 3, 1–11.
Ochoa-Villarreal, M., Howat, S., Hong, S.M., Jang, M.O., Jin, Y.W., Lee, E.K., Loake, G.J., 2016. Plant cell culture strategies for the production of natural products. BMB Rep. 49 (3), 149–158.
Okinaka, Y., Shimada, Y., Nakano-Shimada, R., Ohbayashi, M., Kiyokawa, S., Kikuchi, Y., 2003. Selective accumulation of delphinidin derivatives in tobacco using a putative flavonoid 30,50-hydroxylase cDNA from *Campanula medium*. Biosci. Biotechnol. Biochem. 67, 161–165.
Oszmianski, J., Lee, C.Y., 1990. Inhibition of polyphenol oxidase activity and browning

by honey. J. Agr. Food. Chem. 38 (10), 1892–1895.
Outchkourov, N.S., Karlova, R., Hölscher, M., Schrama, X., Blilou, I., Jongedijk, E., Simon, C.D., van Dijk, A.D.J., Bosch, D., Hall, R.D., Beekwilder, J., 2018. Transcription factor-mediated control of anthocyanin biosynthesis in vegetative tissues. Plant Physiol. 176, 1862–1878.
Pandey, R.P., Parajuli, P., Koffas, M.A.G., Sohng, J.K., 2016. Microbial production of natural and non-natural flavonoids: Pathway engineering, directed evolution and systems/ synthetic biology. Biotechnol. Adv. 34, 634–662.
Pasqua, G., Monacelli, B., Mulinacci, N., Rinaldi, S., Giaccherini, C., Innocenti, M., Vinceri, F.F., 2005. The effect of growth regulators and sucrose on anthocyanin production in *Camptotheca acuminata* cell cultures. Plant Physiol. Biochem. 43 (3), 293–298.
Petroni, K., Tonelli, C., 2011. Recent advances on the regulation of anthocyanin synthesis in reproductive organs. Plant Sci. 181, 219–229.
Plata, N., Konczak-Islam, I., Jayram, S., McClelland, K., Woolford, T., Franks, P., 2003. Effect of methyl jasmonate and p-coumaric acid on anthocyanin composition in a sweet potato cell suspension culture. Biochem. Eng. J. 14 (3), 171–177.
Pourcel, L., Irani, N.G., Lu, Y., Riedl, K., Schwartz, S., Grotewold, E., 2010. The formation of anthocyanic vacuolar inclusions in *Arabidopsis thaliana* and implications for the sequestration of anthocyanin pigments. Mol. Plant 3, 78–90.
Qu, J., Zhang, W., Yu, X., 2011. A combination of elicitation and precursor feeding leads to increased anthocyanin synthesis in cell suspension cultures of *Vitis vinifera*. Plant Cell Tiss. Organ Cult. 107 (2), 261–269.
Quina, F.H., Bastos, E.L., 2017. Chemistry inspired by the colors of fruits, flowers and wine. An. Acad. Bras. Cienc. 90, 681–695.
Ram, M., Prasad, K.V., Kaur, C., Singh, S.K., Arora, A., Kumar, S., 2011. Induction of anthocyanin pigments in callus cultures of *Rosa hybrida* L. in response to sucrose and ammonical nitrogen levels. Plant Cell Tiss. Organ Cult. 104 (2), 171–179.
Ram, M., Prasad, K.V., Singh, S.K., Hada, B.S., Kumar, S., 2013. Influence of salicylic acid and methyl jasmonate elicitation on anthocyanin production in callus cultures of *Rosa hybrida* L. Plant Cell Tiss. Organ Cult. 113 (3), 459–467.
Rehman, R.N.U., You, Y., Zhang, L., Goudia, B.D., Khan, A.R., Li, P., Ma, F., 2017. High temperature induced anthocyanin inhibition and active degradation in Malus profusion. Front. Plant Sci. 8, 1401.
Ruta, L.L., Farcasanu, I.C., 2019. Anthocyanins and anthocyanin-derived products in yeast-fermented beverages. Antioxidants 8, 182), 1–13.
Ryan, J.M., Revilla, E., 2003. Anthocyanin composition of cabernet sauvignon and tempranillo grapes at different stages of ripening. J. Agric. Food Chem. 51, 3372–3378.
Saad, K.R., Parvatam, G., Shetty, N.P., 2018. Medium composition potentially regulates the anthocyanin production from suspension culture of *Daucus carota*. 3 Biotech 8 (3), 134.
Saigo, T., Wang, T., Watanabe, M., Tohge, T., 2020. Diversity of anthocyanin and proanthocyanin biosynthesis in land plants. Curr. Opin. Plant Biol. 55, 93–99.
Saslowsky, D.E., Warek, U., Winkel, B.S., 2005. Nuclear localization of flavonoid enzymes in Arabidopsis. J. Biol. Chem. 280, 23735–23740.
Saw, N.M.M.T., Riedel, H., Cai, Z., Kütük, O., Smetanska, I., 2012. Stimulation of anthocyanin synthesis in grape (*Vitis vinifera*) cell cultures by pulsed electric fields and ethephon. Plant Cell Tiss. Organ Cult. 108 (1), 47–54.
Schiozer, A.L., Barata, L.E.S., 2007. Stability of natural pigments and dyes. Rev. Fitos. 3, 6–23.
Schwarz, M., Wabnitz, T.C., Winterhalter, P., 2003. Pathway leading to the formation of anthocyanin-vinylphenol adducts and related pigments in red wines. J. Agric. Food Chem. 51, 3682–3687.
Seitz, C., Vitten, M., Steinbach, P., Hartl, S., Hirsche, J., Rhthje, W., Treutter, D., Forkmann, G., 2007. Redirection of anthocyanin synthesis in *Osteospermum hybrida* by a two-enzyme manipulation strategy. Phytochemistry 68, 824–833.
Shan, X., Zhang, Y., Peng, W., Wang, Z., Xie, D., 2009. Molecular mechanism for jasmonate-induction of anthocyanin accumulation in Arabidopsis. J. Exp. Bot. 60 (13), 3849–3860.
Shi, M.Z., Xie, D.Y., 2010. Features of anthocyanin biosynthesis in pap1-D and wild-type *Arabidopsis thaliana* plants grown in different light intensity and culture media conditions. Planta 231 (6), 1385–1400.
Shi, M., Xie, D., 2014. Biosynthesis and metabolic engineering of anthocyanins in *Arabidopsis thaliana*. Recent Pat. Biotechnol. 8, 47–60.
Shin, D.H., Choi, M., Kim, K., Bang, G., Cho, M., Choi, S.B., Choi, G., Park, Y.I., 2013. HY5 regulates anthocyanin biosynthesis by inducing the transcriptional activation of the MYB75/PAP1 transcription factor in Arabidopsis. FEBS Lett. 587 (10), 1543–1547.
Shipp, J., Abdel-Aal, E.S.M., 2010. Food applications and physiological effects of anthocyanins as functional food ingredients. Open Food Sci. J. 4, 7–22.
Sigurdson, G.T., Tang, P., Giusti, M.M., 2017. Natural colorants: food colorants from natural sources. Annu. Rev. Food Sci. Technol. 8, 261–280.
Simões, C., Bizarri, C.H.B., da Silva Cordeiro, L., de Castro, T.C., Coutada, L.C.M., da Silva, A.J.R., Albarello, N., Mansur, E., 2009. Anthocyanin production in callus cultures of *Cleome rosea*: Modulation by culture conditions and characterization of pigments by means of HPLC-DAD/ESIMS. Plant Physiol. Biochem. 47 (10), 895–903.
Simões, C., Albarello, N., Castro, T.C., Mansur, E., 2012. Production of anthocyanins by plant cell and tissue culture strategies. In: Biotechnological Production of Plant Secondary Metabolites. Bentham Science Publishers, pp. 67–86.
Simoes-Gurgel, C., da Silva Cordeiro, L., de Castro, T.C., Callado, C.H., Albarello, N., Mansur, E., 2011. Establishment of anthocyanin-producing cell suspension cultures of *Cleome rosea* Vahl ex DC. (Capparaceae). Plant Cell Tiss. Organ Cult. 106, 537–545.
Sinilal, B., Ovadia, R., Nissim-Levi, A., Perl, A., Carmeli-Weissberg, M., Oren-Shamir, M., 2011. Increased accumulation and decreased catabolism of anthocyanins in red grape cell suspension culture following magnesium treatment. Planta 234 (1), 61–71.
Smeriglio, A., Bareca, D., Belloccoco, E., Trombetta, D., 2016. Chemistry, pharmacology and health benefits of anthocyanins. Phytother. Res. 30 (8), 1256–1286.
Solopova, A., Tilburg, A.Y., Van Foito, A., Allwood, J.W., Stewart, D., Kulakauskas, S., Kuipers, O.P., 2019. Engineering *Lactococcus lactis* for the production of unusual anthocyanins using tea as substrate. Metab. Eng. 54, 160–169.
Soubeyrand, E., Basteau, C., Hilbert, G., van Leeuwen, C., Delrot, S., Gomès, E., 2014. Nitrogen supply affects anthocyanin biosynthetic and regulatory genes in grapevine cv. Cabernet-Sauvignon berries. Phytochemistry 103, 38–49.
Stintzing, F.C., Carle, R., 2004. Functional properties of anthocyanins and betalains in plants, food, and in human nutrition. Trends Food Sci. Technol. 15 (1), 19–38.
Stommel, J.R., Lightbourn, G.J., Winkel, B.S., Griesbach, R.J., 2009. Transcription factor families regulate the Anthocyanin biosynthetic pathway in *Capsicum annuum*. J. Am. Soc. Hortic. Sci. 134, 244–251.
Suan-See, K., Bhatt, A., Keng, C.L., 2011. Effect of sucrose and methyl jasmonate on biomass and anthocyanin production in cell suspension culture of *Melastoma malabathricum* (Melastomaceae). Rev. Biol. Trop. 59 (2), 597–606.
Sudha, G., Ravishankar, G.A., 2003. Elicitation of anthocyanin production in callus cultures of *Daucus carota* and the involvement of methyl jasmonate and salicylic acid. Acta Physiol. Planta. 25 (3), 249–256.
Sun, Y., Li, H., Huanf, J.-R., 2012. Arabidopsis TT19 functions as a carrier to transport anthocyanin from the cytosol to tonoplasts. Mol. Plant 5, 387–400.
Sun, J., Wang, Y., Chen, X., Gong, X., Wang, N., Ma, L., Qiu, Y., Wang, Y., Feng, S., 2017. Effects of methyl jasmonate and abscisic acid on anthocyanin biosynthesis in callus cultures of red-fleshed apple (*Malus sieversii* f. *niedzwetzkyana*). Plant Cell Tiss. Org. Cult. 130 (2), 227–237.
Sun, C., Deng, L., Du, M., Zhao, J., Chen, Q., Huang, T., Jiang, H., Li, C.B., Li, C., 2020. A transcriptional network promotes anthocyanin biosynthesis in tomato flesh. Mol. Plant 13 (1), 42–58.
Suzuki, H., Nakayama, T., Yonekura-sakakibara, K., Fukui, Y., Nakamura, N., Yamaguchi, M.A., Tanaka, Y., Kusumi, T., Nishino, T., 2002. cDNA cloning, heterologous expressions, and functional characterization of malonyl-coenzyme A: anthocyanidin 3-O-glucoside-6″-O-malonyltransferase from Dahlia flowers. Plant Physiol. 130 (4), 2142–2151.
Tanaka, Y., Ohmiya, A., 2008. Seeing is believing: Engineering anthocyanin and carotenoid biosynthetic pathways. Curr. Opin. Biotechnol. 19, 190–197.
Tanaka, Y., Sasaki, N., Ohmiya, A., 2008. Biosynthesis of plant pigments: Anthocyanins, betalains and carotenoids. Plant J. 54, 733–749.
Tarrahi, R., Rezanejad, F., 2013. Callogenesis and production of anthocyanin and chlorophyll in callus cultures of vegetative and floral explants in *Rosa gallica* and *Rosa hybrida* (Rosaceae). Turk. J. Bot. 37 (6), 145–1154.
Tian, J., Li, K.T., Zhang, S.Y., Zhang, J., Song, T.T., Zhu, Y.J., Yao, Y.C., 2017. The structure and methylation level of the McMYB10 promoter determine the leaf color of *Malus crabapple*. HortScience 52 (4), 520–526.
Toda, K., Kuroiwa, H., Senthil, K., Shimada, N., Aoki, T., Ayabe, S., Shimada, S., Sakuta, M., Miyazaki, Y., Takahashi, R., 2012. The soybean F3'H protein is localized to the tonoplast in the seed coat hilum. Planta 236, 79–89.
Tonelli, M., Pellegrini, E., D'Angiolillo, F., Petersen, M., Nali, C., Pistelli, L., Lorenzini, G., 2015. Ozone-elicited secondary metabolites in shoot cultures of *Melissa officinalis* L. Plant Cell Tiss. Organ Cult. 120 (2), 617–629.
Turnbull, J.J., Nakajima, J., Welford, R.W., Yamazaki, M., Saito, K., Schofield, C.J., 2004. Mechanistic studies on three 2-oxoglutarate-dependent oxygenases of flavonoid biosynthesis: anthocyanidin synthase, flavonol synthase, and flavanone 3β-hydroxylase. J. Biol. Chem. 279 (2), 1206–1216.
Vasconsuelo, A., Boland, R., 2007. Molecular aspects of the early stages of elicitation of secondary metabolites in plants. Plant Sci. 172 (5), 861–875.
Vilela, A., Cosme, F., 2016. Drink red: Phenolic composition of red fruit juices and their sensorial acceptance. Beverages 2, 29.
Vitrac, X., Larronde, F., Krisa, S., Decendit, A., Deffieux, G., Mérillon, J.M., 2000. Sugar sensing and Ca2+–calmodulin requirement in *Vitis vinifera* cells producing anthocyanins. Phytochemistry 53 (6), 659–665.
Wada, T., Kunihiro, A., Tominaga-wada, R., 2014. *Arabidopsis* CAPRICE (MYB) and GLABRA3 (bHLH) control tomato (*Solanum lycopersicum*) anthocyanin biosynthesis. PLoS One 9, e109093.
Walker, R.S.K., Pretorius, I.S., 2018. Application of yeat synthetic biology geared towards the production of biopharmaceuticals. Genes 9, 340.
Wang, N., Zhang, Z., Jiang, S., Xu, H., Wang, Y., Feng, S., Chen, X., 2016. Synergistic effects of light and temperature on anthocyanin biosynthesis in callus cultures of red-fleshed apple (*Malus sieversii* f. *niedzwetzkyana*). Plant Cell Tiss. Org. Cult. 127 (1), 217–227.
Wang, Y.C., Wang, N., Xu, H.F., Jiang, S.H., Fang, H.C., Su, M.Y., Zhang, Z.Y., Zhang, T.L., Chen, X.S., 2018. Auxin regulates anthocyanin biosynthesis through the Aux/IAA–ARF signaling pathway in apple. Hortic. Res. 5 (1), 1–11.
Wang, Y., Liu, W., Jiang, H., Mao, Z., Wang, N., Jiang, S., Xu, H., Yang, G., Zhang, Z., Chen, X., 2019. The R2R3-MYB transcription factor MdMYB24-like is involved in methyl jasmonate-induced anthocyanin biosynthesis in apple. Plant Physiol. Bioch. 139, 273–282.
Wen, P.-F., Chen, J.-Y., Kong, W.-F., Pan, Q.-H., Wan, S.-B., Huang, W.-D., 2005. Salicylic acid induced the expression of phenylalanine ammonia-lyase gene in grape berry. Plant Sci. 169, 928–934.
Wink, M., 2008. Plant secondary metabolism: diversity, function and its evolution. Nat. Prod. Commun 3 (8), 1205–1216.
Winkel-Shirley, B., 2001. Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. 126, 485–493.
Xie, D., Jackson, L.A., Cooper, J.D., Ferreira, D., Paiva, N.L., 2004. Molecular and biochemical analysis of two cDNA clones encoding dihydroflavonol-4-reductase from *Medicago truncatula*. Plant Physiol. 134 (3), 979–994.
Xie, D., Tan, H., Ma, Z., Huang, J., 2016. DELLA proteins promote anthocyanin biosynthesis via sequestrating MYBL2 and JAZ suppressors of MYB/bHLH/WD40

complex in *Arabidopsis thaliana*. Mol. Plant 9, 711–721.
Xu, Y., Charles, M.T., Luo, Z., Mimee, B., Veronneau, P.Y., Rolland, D., Roussel, D., 2017a. Preharvest Ultraviolet C irradiation increased the level of polyphenol accumulation and flavonoid pathway gene expression in strawberry fruit. J. Agr. Food Chem. 65 (46), 9970–9979.
Xu, Z., Feng, K., Que, F., Wang, F., Xiong, A., 2017b. A MYB transcription factor, DcMYB6, is involved in regulating anthocyanin biosynthesis in purple carrot taproots. Sci. Rep. 7, 1–9.
Xu, Z.S., Yang, Q.Q., Feng, K., Xiong, A.S., 2019. Changing carrot color: insertions in DcMYB7 alter the regulation of anthocyanin biosynthesis and modification. Plant Physiol. 181 (1), 195–207.
Yamane, T., Jeong, S.T., Goto-Yamamoto, N., Koshita, Y., Kobayashi, S., 2006. Effects of temperature on anthocyanin biosynthesis in grape berry skins. Am. J. Enol. Viticult. 57 (1), 54–59.
Yan, Y., Chemler, J., Huang, L., Martens, S., Koffas, M.A.G., 2005. Metabolic engineering of anthocyanin biosynthesis in *Escherichia coli*. Appl. Environ. Microbiol. 71, 3617–3623.
Yan, Y., Li, Z., Koffas, M.A.G., 2008. High-yield anthocyanin biosynthesis in engineered *Escherichia coli*. Biotechnol. Bioeng. 100, 126–140.
Yuan, L., Grotewold, E., 2015. Metabolic engineering to enhance the value of plants as green factories. Metab. Eng. 27, 83–91.
Zha, J., Koffas, M.A.G., 2017. Production of anthocyanins in metabolically engineered microorganisms: Current status and perspectives. Synth. Syst. Biotechnol. 2, 259–266.
Zha, J., Zang, Y., Mattozzi, M., Plassmeier, J., Gupta, M., Wu, X., Clarkson, S., Koffas, M.A.G., 2018. Metabolic engineering of *Corynebacterium glutamicum* for anthocyanin production. Microb. Cell. Fact. 17, 1–13.
Zha, J., Wu, X., Koffas, M.A.G., 2020. Making brilliant colors by microorganisms. Curr. Opin. Biotechnol. 61, 135–141.
Zhang, W., Curtin, C., Kikuchi, M., Franco, C., 2002. Integration of jasmonic acid and light irradiation for enhancement of anthocyanin biosynthesis in *Vitis vinifera* suspension cultures. Plant Sci. 162 (3), 459–468.
Zhang, Y., Butelli, E., Martin, C., 2014. Engineering anthocyanin biosynthesis in plants. Curr. Opin. Chem. Biol. 19, 81–90.
Zhang, Y., Jiang, L., Li, Y., Chen, Q., Ye, Y., Zhang, Y., Luo, Y., Sun, B., Wang, X., Tang, H., 2018. Effect of red and blue light on anthocyanin accumulation and differential gene expression in strawberry (*Fragaria*× *ananassa*). Molecules 23 (4), 820.
Zhang, X., Xu, Z., Yu, X., Zhao, L., Zhao, M., 2019. Identification of two novel R2R3-MYB transcription factors, PsMYB114L and PsMYB12L, related to anthocyanin biosynthesis in *Paeonia suffruticosa*. Int. J. Mol. Sci. 20, 1–18.
Zhao, J., Dixon, R.A., 2010. The 'ins' and 'outs' of flavonoid transport. Trends Plant Sci. 15, 72–80.
Zhao, J., Davis, L.C., Verpoorte, R., 2005. Elicitor signal transduction leading to production of plant secondary metabolites. Biotechnol. Adv. 23 (4), 283–333.
Zhao, C.L., Chen, Z.J., Bai, X.S., Ding, C., Long, T.J., Wei, F.G., Miao, K.R., 2014. Structure-activity relationships of anthocyanidin glycosylation. Mol. Diversity 18, 687–700.
Zhou, L.L., Zeng, H.N., Shi, M.Z., Xie, D.Y., 2008. Development of tobacco callus cultures over expressing Arabidopsis PAP1/MYB75 transcription factor and characterization of anthocyanin biosynthesis. Planta 229, 37–51.
Zhu, L., Huang, Y., Zhang, Y., Xu, C., Lu, J., Wang, Y., 2017. The growing season impacts the accumulation and composition of flavonoids in grape skins in two-crop-a- year viticulture. J. Food Sci. Technol. 54, 2861–2870.
Zong, Y., Zhu, X., Liu, Z., Xi, X., Li, G., Cao, D., Wei, L., Li, J., Liu, B., 2019. Functional MYB transcription factor encoding gene AN2 is associated with anthocyanin biosynthesis in *Lycium ruthenicum* Murray. BMC Plant Biol. 19, 1–9.