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Review

Research review

Painting the plant body: pigment biosynthetic pathways regulated by small RNAs

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Summary

Plant pigments are diverse natural molecules involved in numerous biological functions such as development, growth, and metabolism. As plants age, not only new organs will be formed, but also, they will acquire the necessary pigments in response to the environment and endogenous programming in order to achieve reproductive success. Among the endogenous cues, the small RNAs (sRNAs), an endogenous group of ubiquitous regulatory molecules, may regulate the pigments-associated biosynthetic pathways at posttranscriptional level. Although plant pigments and sRNAs have been comprehensively studied in several processes throughout the entire plant cycle in model and nonmodel species, connections among these central players must be revised. Studying these complex networks allow us not only to know the progress that has been made in this area, but also generate research questions to be explored in order to unravel novel mechanisms for improving plant yield; therefore, in this review we have summarized the emerging roles of sRNAs-regulated nodes in mediating plant pigmentation-associated biosynthetic pathways, focused on chlorophylls, flavonoids, carotenoids, and betalains. In addition, we discuss perspectives related to the manipulation of those genes associated with plant pigments for obtaining genetically improved plants.

Introduction

Plant pigments are substances that control different processes during development, growth, and metabolism (Sudhakar *et al.*, 2016). They are involved in diverse functions, including photosynthesis, pollinator attraction and/or repelling herbivores, seed dispersion, and protective mechanisms; in addition, they are economically important for human nutrition and health, and industry such as floriculture (Lee, 2005). Pigments can be grouped into two main groups: lipid-soluble and water-soluble. The first one can be divided into chlorophylls (Chls) and carotenoids, and the second one into flavonoids and betalains (Solovchenko *et al.*, 2019; López-Cruz *et al.*, 2023; Rodríguez-Mena *et al.*, 2023).

Chlorophylls are the most important and abundant lipid-soluble pigments in plants and indispensable for photosynthesis (Qiu *et al.*, 2019; Hu *et al.*, 2021). They are found mainly in vegetative tissues, but also occur in other plant parts such as flowers and fruits (Zhou *et al.*, 2022). Among the different types of Chls, plants contain Chl*a* and Chl*b* (Hu *et al.*, 2021). The biosynthetic pathways of Chl production and the key enzymes have been well characterized (Review in Qiu *et al.*, 2019). Briefly, the precursor D-Aminolevulinic acid is converted into Chl*a* and Chl*b* through a complex enzymatic process that includes DIVINYL REDUCTASE (DVR) to convert 3,8-divinyl-protochlorophyllide into protochlorophyllide (Qiu *et al.*, 2019) and PROTOCHLOROPHYLLIDE OXIDOREDUCTASES (POR) that convert protochlorophyllide into chlorophyllide *a* (Bollivar, 2006). Additionally, chlorophylls must be degraded by a fine-tune-regulated metabolism to allow color modifications in leaves and fruits, from green to yellow or red during plant development and growth (Hu *et al.*, 2021). Carotenoids, the second most abundant lipid-soluble pigments, are found especially in leaves, flowers and fruits, but also in roots and stems (Maoka, 2020). Carotenoid biosynthesis has been widely studied, and it initiates with the precursors isopentenyl pyrophosphate and dimethylallyl pyrophosphate via plastid-localized methylerythritol 4-phosphate

pathway (Review in Sun *et al.*, 2022). Several enzymes are required for carotenoids biosynthesis, including LYCOPENE B-CYCLASE (LCYB), that leads to the formation of β- and α-carotene and LYCOPENE ε-CYCLASE (LCYE), which is involved in α-carotene production from Lycopene (Sun *et al.*, 2022).

In the subgroup of water-soluble pigments, flavonoids are the most common pigments, being anthocyanins the most studied in many plant species (Hughes *et al.*, 2021; Wong *et al.*, 2022). Anthocyanin production is regulated by a set of genes acting between the general phenylpropanoid pathway and the specific flavonoid pathway (Review in Sunil & Shetty, 2022). The PHENYLALANINE AMMONIA LYASE (PAL), 4-COUMARATE--COA LIGASE (4CL), and FLAVONOL-3-GLUCOSYLTRANSFERASE (3GT) act in the general phenylpropanoid pathway to convert phenylalanine into 4-coumaryl-CoA. The flavonoid pathway, in turn, is divided into initial and final stages. The initial stage encompasses biochemical reactions whose precursor is 4-coumaryl-CoA. In this stage, the CHALCONE SYNTHASE (CHS), CHALCONE ISOMERASE (CHI), FLAVANONE 3-HYDROXYLASE (F3H), FLAVONOID 3'-HYDROXYLASE (F3′H), and FLAVONOL SYNTHASE (FLS) are responsible for producing flavonol and other flavonoids. In the final stage, the ANTHOCYANIDIN SYNTHASE (ANS), FLAVONOID 3′,5′-HYDROXYLASE (F3′5′H), UGP GLUCOSE-FLAVONOID 3-O-GLUCOSYLTRANSFERASE (UFGT), and DIHYDROFLAVONOL 4-REDUCTASE (DFR), among other enzymes, are involved to obtain the anthocyanins (Khusnutdinov *et al.*, 2021). Besides these genes, anthocyanins production is also regulated at transcriptional level by the transcription factor complex MYELOBLASTOSIS/BASIC HELIX–LOOP–HELIX/WD REPEAT PROTEIN (MYB-bHLH-WD40), which is highly conserved in plants (Review in Ramsay & Glover, 2005).

Finally, betalains, another group of water-soluble pigments, are present in flowers, fruits and occasionally in vegetative tissues by replacing anthocyanins in plants of the order Caryophyllales (Khan & Giridhar, 2015). In fact, no plants have been found to biosynthesize both pigments, which has been comprehensively studied (Davies, 2015; Timoneda *et al.*, 2019). The biosynthetic pathway derives from the amino acid L-tyrosine and involve several genes including *AROGENATE DEHYDROGENASE 1* (HuADH1), *HuCYP76AD1* (a cytochrome P-450 R), *4,5-DOPA DIOXYGENASE EXTRADIOL* (*HuDODA1*) and *DOPA 5-O-GLUCOSYLTRANSFERASE* (*DOPA5GT1*) to obtain betacyanins and betaxanthins (Review in Timoneda *et al.*, 2019).

Chlorophylls, Flavonoids, Carotenoids, and Betalains production is subject to environmental and endogenous cues. To achieve appropriate levels, endogenous regulators such as transcription factors (TF) and small RNAs (sRNAs) act through a complex network involving transcriptional, posttranscriptional and post-translational regulation (Table 1). sRNAs are single-stranded RNAs that recognize endogenous targets and regulate them through the RNA-induced silencing complex (Bartel, 2004). sRNAs in plants are classified in two main groups, hairpin RNAs (hpRNAs) from a single-stranded precursor, and small interfering RNAs (siRNA) from a double-stranded precursor (Axtell, 2013). In the hpRNAs group, microRNAs (miRNAs) stand out for being involved in numerous biological processes. miRNAs biogenesis and function have been extensively discussed (Barrera-Rojas *et al.*, 2021; Shang *et al.*, 2023). In plants, miRNAs orchestrate development and growth from embryogenesis (Willmann *et al.*, 2011) to senescence (Munk *et al.*, 2017), including the development of meristems (Ferigolo *et al.*, 2023), roots (Barrera-Rojas *et al.*, 2020), leaves (Palatnik *et al.*, 2003), shoots (Barrera-Rojas *et al.*, 2023), flowers and fruits (da Silva *et al.*, 2017). On the other hand, siRNAs comprises sRNA molecules derived from a double-stranded RNA, normally synthesized by a RNA-dependent RNA polymerase such as the *Trans*-acting Small interference RNAs (TAS), acting also in several plant processes (Yoshikawa, 2013).

Both sRNAs and plant pigments have been extensively studied during plant development, growth, and metabolism of model and nonmodel species; therefore, understanding the connections among these players allow us to identify molecular mechanisms for improving plant yield. Therefore, in this review, we have summarized the available information regarding the role of sRNAs in mediating pigment-associated biosynthetic pathways, focusing on Chlorophylls, Flavonoids, Carotenoids, and Betalains. Moreover, we discuss perspectives focused on manipulating these pathways for obtaining genetically improved plants.

The role of sRNAs during chlorophyll biosynthesis

Chlorophylls play a pivotal role in plant development and growth; however, alterations in Chl levels can be critical for the plant (Hu *et al.*, 2021). To achieve a fine-tune balance, Chl biosynthesis and degradation must be precisely regulated (Yin & Bauer, 2013); thus, sRNAs contribute to appropriate endogenous Chl levels. Several miRNAS, have been reported as modulators of Chl content by regulating the expression of Chl biosynthesis-associated genes in several species, including rice, *Arabidopsis*, *Medicago*, Bermudagrass, Broccoli and Blueberry (Table 1).

In rice, alterations in miR171 levels trigger changes in Chl content. miR171 is a highly conserved miRNA that regulates members of the GRAS TFs family (Pei *et al.*, 2023). In leaves, while miR171b overexpression enhances Chl accumulation, the mimicry strategy-based miR171 inhibition leads to reduced Chl content. Up- and downregulation of miR171-targeted *SCARECROW-LIKE6-IIa* (*OsSCL6-IIa*), *OsSCL6-IIb*, and *OsSCL6-IIc*, three negative regulators of Chl biosynthesis, correlates with inhibition or overexpression of miR171b, respectively (Tong *et al.*, 2017). These results resemble the alteration in Chl content in *Arabidopsis* cotyledons, stems and leaves by downregulating of *AtSCL6-II*, *AtSCL6-III* and *AtSCL6-IV* through loss-of-function or miR171c overexpression (Wang *et al.*, 2010; Fig. 1).

Other miR171-regulated *SCL* genes involved in Chl biosynthesis are *AtSCL27* from *Arabidopsis*, and *BolSCL6* and *BolSCL27* from Broccoli. Overexpression of a miR171-resistant *AtSCL27* version decreases Chl content in *Arabidopsis* leaves by inhibiting the *AtPOR* expression (Ma *et al.*, 2014). Similarly, miR171b modifies Chl content in Broccoli through *BolSCL6* and *BolSCL27* (Li *et al.*, 2018). In Bermudagrass, miR171 also affects Chl

Table 1 List of sRNAs and direct or indirect targets that act in the Chlorophyll, Anthocyanin, Carotenoids and Betalains production in the plant body of the different species studied.

Pigments	sRNA	Targets	Specie	Tissue	References
Chlorophylls	miR171	*OsSCL6-IIa/b/c*	Rice	Leaves	Tong *et al*. (2017)
		AtSCL6-II/III/IV	Arabidopsis	Cotyledons, Stems, Leaves	Wang *et al*. (2010)
		AtSCL27	Arabidopsis	Leaves	Ma *et al*. (2014)
		BolSCL6/27	Broccoli	Leaves	Li *et al*. (2018)
		CdLHC1	Bermudagrass	Leaves	Fan *et al*. (2023)
		MtLHC1	Medicago	Leaves	Fan *et al*. (2023)
	miR398	*AtCSD2*	Arabidopsis	Leaves	Lu *et al*. (2013)
	miR156	*VcSPL12*, *VcDVR*, *VcPORA*, *VcLPA3*, *VcCIA2*	Blueberry	Fruits	Sun *et al*. (2022), Li *et al*. (2024)
		VcSPL12	Arabidopsis	Leaves	X. Li *et al*. (2020)
Anthocyanin	miR828/ta-siRNAs	*MYB12*	Lilies	Flowers	Yamagishi & Sakai (2020), Suzuki *et al*. (2016)
		AtTAS4, *AtPAP1/2*, *AtMYB113*	Arabidopsis	Vegetative tissues	Luo *et al*. (2012)
		StTAS4, *StR2R3MYB*, *StMYB36284*	Potato	Tuber skin, Tuber flesh	Bonar *et al*. (2018)
		VvMYB114, *VvFLS*, *VvF3MO*	Grapes	Fruits	Tirumalai *et al*. (2019)
		MdTAS4, *MdbHLH3*, *MdbMYB1*, *MdDFR*, *MdANS*, *MdUFGT*	Apple	Fruits	Zhang *et al*. (2020)
		AtMYB113, *AtMYB75*, *AtMYB90*, *AtDFR*, *AtCHS*, *AtF3H*	Arabidopsis	Seedlings	Zhang *et al*. (2020)
		BrPAP1, *BrMYB82*, *BrTAS4*	Turnip	Hypocotyl	Zhou *et al*. (2020)
	miR156	*AtSPL9*, *AtANS*, *AtF3′H*, *AtDFR*, *AtMYB*, *AtbHLH*, *AtWD40*, *AtPAP1*	Arabidopsis	Internodes	Gou *et al*. (2011) Cui *et al*. (2014)
		PpMYB10, *PpMYB*, *PpbHLH*, *PpWD40*, *PpPpCHS*, *PpCHI*, *PpF3H*, *PpANS*, *PpUFGT*	Pear	Fruits	Qian *et al*. (2017)
		PsSPL2, *PsF3′H*, *PsDFR*	Peony	Petals	Luo *et al*. (2022)
		NtF3′H, *NtDFR*	Tobacco	Leaves	Luo *et al*. (2022)
		AtSPL1, *AtDFR*	Arabidopsis	Lateral branches	Zhao *et al*. (2017)
		VcSPL12, *VcPALs*, *Vc4CLs*, *VcCHSs*, *VcF3Hs*, *VcUFGTs*, *VcMYBA*, *VcF3′5′H*, *VcANS*	Blueberry	Fruits	Li *et al*. (2024)
		Unravel	Poplar	Stems, leaves, and petioles	Wang *et al*. (2020)
		PAL, *DFR*, *ANS*, *F3′H*, *F3H*, *bHLH1*	Phalaenopsis	Sepals	Zhao *et al*. (2019)
	miR858	*AaMYBC1*, *AabHLH42*, *AaCHS*, *AaF3H*, *AaUFGT*	Kiwifruit	Fruits	Li *et al*. (2019) Y. Li *et al*. (2020)
		MdMYB9, *MdMYBPA1*, *MdANS*, *MdUFGT*	Apple	Fruits	Wang *et al*. (2020)
		SlMYB7-like, *SlMYB48-like*, *SlPAL*, *SlCHS*, *SlDFR*, *SlANS*, *Sl3GT*	Tomato	Seedlings, leaves, stems	Jia *et al*. (2015)
		PePAL, *PeDFR*, *PeANS*, *PeF3′H*, *PeF3HI*, *PeF3H*, *PebHLH1*	Phalaenopsis	Petals	Zhao *et al*. (2019)
	miR399	*McMYB10*, *McPAL*, *McCHS*, *McANS*	Apple	Leaves	Peng *et al*. (2020)
	miR778	*AtPAP1*, *AtSUVH6*	Arabidopsis	Roots, flowers	Wang *et al*. (2015)
Carotenoids	miR1857	*CcLYCb*	Orange	Fruits	Xu *et al*. (2010)
	miR159	*DcLCYE*	Carrot	Taproot	Bhan *et al*. (2019) Wang *et al*. (2023)
Betalains	miR156	*HuSPL12*, *HuWRKY40*, *HuCYP76AD1*, *HuMYB132*, *HuWRKY42*	Pitaya	Fruit	Zeng *et al*. (2023)

production. By heterologous overexpression, miR171 induced morphological changes in *Medicago* including greener leaves suggesting that miR171, through the *LIGHT HARVESTING COMPLEX 1* (*LHC1*), regulates Chl production (Fan *et al*., 2023). Collectively, these data suggest a conserved role of miR171 in Chl accumulation. However, further analyses involving loss- and/or gain-of-function mutants of miR171 targets are necessary to unravel more precisely this molecular mechanism (Fig. 1). Besides miR171, another miRNA that positively regulates Chl production is miR398, a conserved miRNA that controls stress responses and plant growth. This miRNA has both conserved and species-specific target genes (Li *et al*., 2022), including the *COPPER/ZINC SUPEROXIDE DISMUTASE 2* (*CSD2*) involved in detoxifying superoxide radicals in response to oxidative stress

New Phytologist

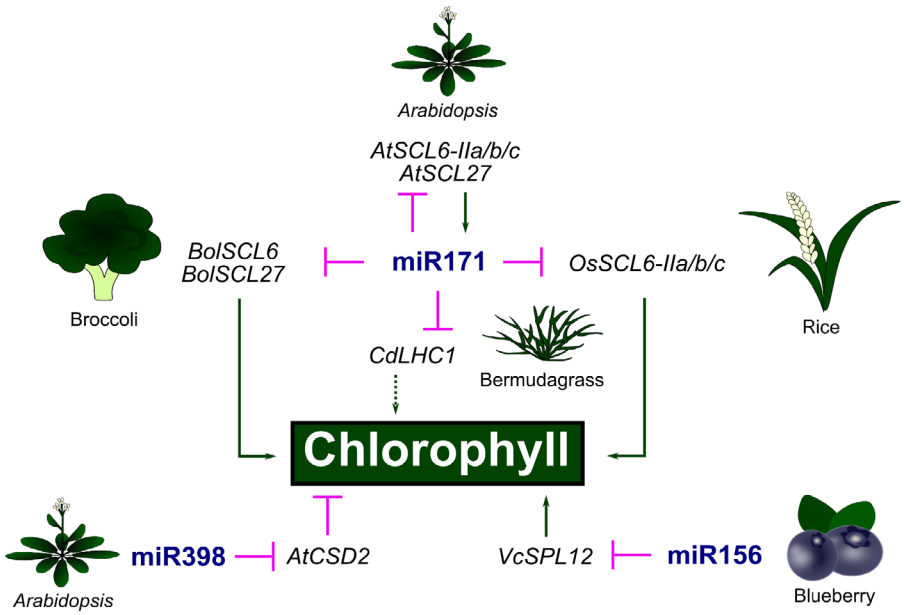

Fig. 1 Harpin-derived sRNAs during Chlorophyll (Chl) biosynthesis. Schematic representation of the sRNAs-regulated Chl production in *Arabidopsis*, Bermudagrass (*Cynodon dactylon*), Rice (*Oryza sativa*), Broccoli (*Brassica oleracea*) and Blueberry (*Vaccinium corymbosum*). miRNAs are highlighted in bold blue. Green arrows indicate positive regulation, magenta blunt-ended bars indicate negative regulation, and dashed lines or dashed arrows indicate hypothetical interactions.

(Sunkar *et al.*, 2006). *Arabidopsis* plants overexpressing a miR398-resistant version of the *AtCSD2* display reduced Chl content (Lu *et al.*, 2013), indicating that the miR398/*AtCSD* module is involved in Chl production; nevertheless, it needs to be further investigated (Fig. 1).

While miR171 and miR398 promote Chl accumulation, miR156 acts in the opposite way. This highly conserved miRNA and its targets, members of the *SQUAMOSA PROMOTER-BINDING-LIKE* (*SPL*) genes, have been extensively studied (Morea *et al.*, 2016; Yuan *et al.*, 2023). In blueberry, miR156 overexpression reduces Chl content by downregulating *VcDVR* and *VcPORAs*, two important Chl biosynthesis-associated genes, and *LOW PSII ACCUMULATION3* (*VcLPA3*) and *CHLOROPLAST IMPORT APPARATUS 2* (*VcCIA2*), two important genes associated with Chl-protein complex formation and chloroplast biogenesis, respectively (Li *et al.*, 2024). Among the miR156-targeted *VcSPLs*, *VcSPL12* enhanced Chl accumulation by altering the expression of several Chl-associated genes; in fact, heterologous expression of the *VcSPL12* in *Arabidopsis* also enhanced Chl accumulation (X. Li *et al.*, 2020). In addition, *VcSPL12* promotes Chl accumulation in blueberries by increasing the expression of *VcDVR* by binding to its promoter (Li *et al.*, 2024; Fig. 1). Thus, Chl production-associated regulators such as *SPL12* and/or homologous genes are potential candidates for genome editing at the miRNA response element through CRISPR-mediated single base edition to obtain gain-of-function mutants and consequently increased Chl levels.

sRNA-dependent regulation of anthocyanin production

While Chl is responsible for the green pattern on plant body, anthocyanin is the main pigment responsible for reddish, bluish, and purple hues (Qian *et al.*, 2017). Some studies have shown antagonism between these pathways that deserve a comprehensive review (X. Li *et al.*, 2020; Li *et al.*, 2024). The molecular mechanisms of anthocyanin production have been extensively studied in different species. Such pathways are conserved among plant groups and demonstrate the protagonism of sRNAs. Among them, miR828 has been extensively studied in *Arabidopsis*, potato, grapes, lilies, Turnip and apples, and miR156 in *Arabidopsis*, the Chinese peony, blueberry, the tree peony, and pear (Table 1).

The miR828, a highly conserved miRNA, negatively regulates anthocyanin-derived pigment patterns by repressing the expression of *R2R3-MYB* genes in flowers, vegetative tissues, tubers and fruits through a mechanism involving the *trans*-acting small interfering RNA4 (*TAS4*)-derived sRNAs (ta-siRNAs; Hsieh *et al.*, 2009). In flowers of the Asiatic-hybrid lilies, the miR828-mediated regulation of *MYB12* inhibits the anthocyanin biosynthesis, which leads to a bicolor pattern. The *MYB12* is a member of the R2R3-MYB TF family with a central role in anthocyanin-dependent pigmentation. Interestingly, the miR828-guided cleavage of *MYB12*-derived transcripts triggers the production of secondary siRNAs that can potentially regulate downstream targets, even anthocyanin-associated genes, as previously found in other species (Review in Sanan-Mishra *et al.*, 2021) which requires further analysis. Both, miR828 and *MYB12* are expressed at lower levels in tepals; however, differential transcript accumulation in the tepals is the responsible for the anthocyanin-associated bicolor patterns in lilies (Yamagishi & Sakai, 2020; Suzuki *et al.*, 2016; Fig. 2).

Besides flowers, miR828 regulates anthocyanin production in vegetative tissues. In *Arabidopsis*, the miR828-guided cleavage of *AtTAS4* triggers the production of ta-siRNAs. These ta-siRNAs target *PRODUCTION OF ANTHOCYANIN PIGMENT* 1 (*PAP1*), *PAP2*, and *MYB113*, and, consequently, suppress anthocyanin biosynthesis (Luo *et al.*, 2012; Fig. 2). In addition, miR828 is also associated with the formation of purple tuber skin and flesh color in potato, through a similar mechanism of *TAS4*-

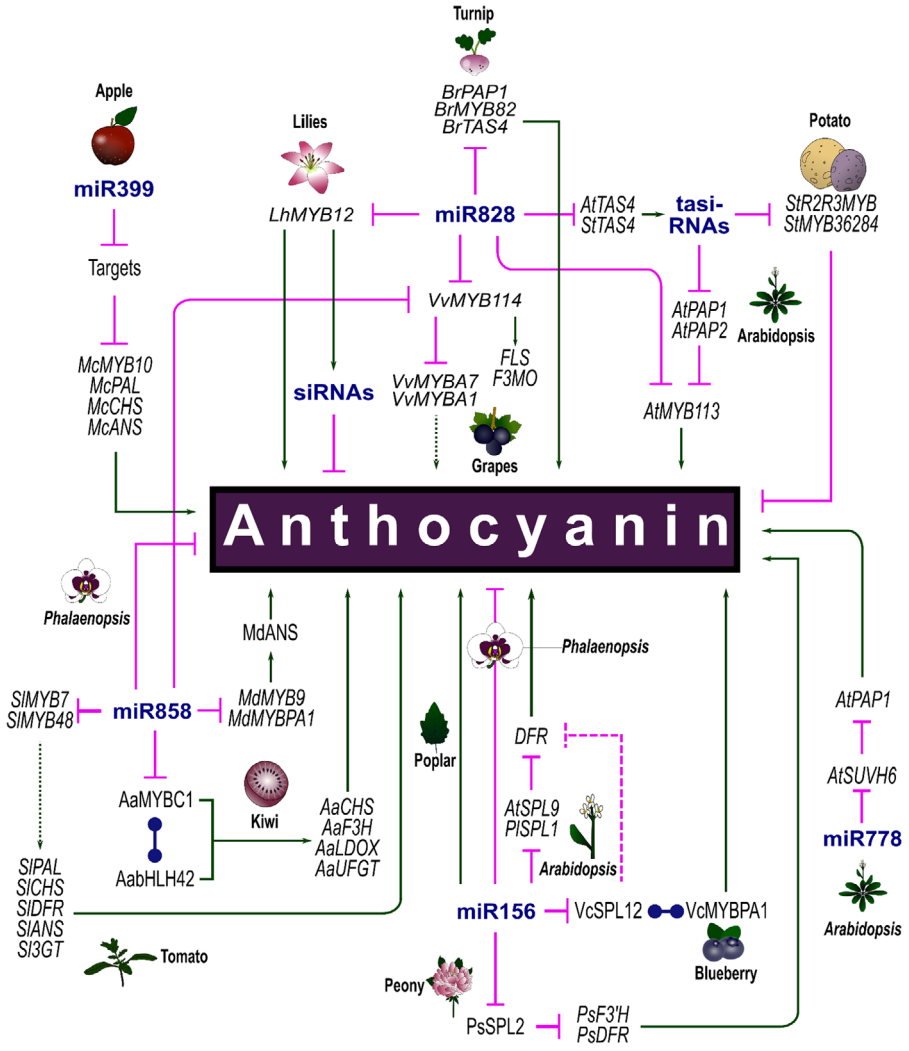

Fig. 2 Small RNA-controlled anthocyanin biosynthesis. Schematic representation of the sRNAs-regulated anthocyanin production in Apple (*Malus domestica*), Lilies (*Lilium* sp.), Turnip (*Brassica rapa*), Potato (*Solanum tuberosum*), *Arabidopsis*, Grapes (*Vitis vinifera*), *Phalaenopsis*, Tomato (*Solanum lycopersicum*), Kiwi (*Actinidia arguta*), Poplar (*Populus* sp.), Peony (*Paeonia* × *suffruticosa*), and Blueberry (*Vaccinium corymbosum*). Small RNAs are highlighted in bold blue. Green arrows indicate positive regulation, magenta blunt-ended bars indicate negative regulation, dashed lines or dashed arrows indicate hypothetical regulation, and blue lines with circular termination indicate protein–protein interactions.

derived siRNAs production; while high levels of miR828 and *StTAS4* transcripts were detected on pigmented sectors, low levels were detected in nonpigmented sectors. The miR828-dependent ta-siRNA4s target *StR2R3MYB* and *StMYB36284*, two MYB TFs and important inhibitors of anthocyanin biosynthesis, forming a regulatory pathway to control the purple tuber skin and flesh color in potatoes (Bonar *et al.*, 2018; Fig. 2).

In grapes, miR828 positively regulates anthocyanin accumulation by targeting *VvMYB114*. The miR828-directed *VvMYB114* cleavage triggers the production of RNA-dependent RNA polymerase 6 (RDR6)-dependent siRNAs that can potentially regulate the expression of downstream targets. miR828 is predominantly expressed in anthocyanin-rich grapes while *VvMYB114* is weakly expressed. Interestingly, transgenic grapes overexpressing *VvMYB114* display higher accumulation of flavonols through the upregulation of *FLS* and *FLAVONOID 3′-MONOOXYGENASE* (*F3MO*) expression, a downstream regulator of *FLS* (Tirumalai *et al.*, 2019; Fig. 2) indicating that *VvMYB114* negatively regulates anthocyanin production and also induces flavonols biosynthesis.

In apples and turnips, miR828 negatively regulates anthocyanin production. By transient overexpression of miR828 in fruit peel the *MdTAS4*, *MdbHLH3*, *MdbMYB1*, *MdDFR*, *MdANS*, and *MdUFGT* expression, and consequently, anthocyanin content were reduced; on the other hand, transient overexpression of MdbHLH3 promoted *MdTAS4*, *MdbMYB1*, *MdDFR*, *MdANS*, and *MdUFGT* expression, triggering anthocyanin accumulation. Interestingly, MdMYB1 promotes miR828 expression by binding to its promoter forming a feedback regulatory loop to control anthocyanin content in apple peel. Besides that, heterologous overexpression of MdmiR828 in *Arabidopsis* inhibited anthocyanin accumulation through the downregulation of the anthocyanin biosynthesis-related genes *AtMYB113 AtMYB75* and *AtMYB90*, and the structural genes *AtDFR*, *AtCHS*, and *AtF3H* (Zhang *et al.*, 2020; Fig. 2). Moreover, in Turnip, miR828 negatively regulates light-induced anthocyanin production in the hypocotyl by targeting *BrPAP1*, *BrMYB82*, and *BrTAS4* genes. Although it was demonstrated that *BrPAP1* participates in anthocyanin production in Turnip, *BrMYB82*, and *BrTAS4* functions need to be confirmed (Zhou *et al.*, 2020; Fig. 2). Those data reveal that the

miR828-regulated anthocyanin production is a complex genetic regulatory pathway involving several players that constitute a source for reverse genetics studies to improve the anthocyanin content in economically important species.

Another well-studied miRNA and regulator of pigment biosynthesis of vegetative tissues, flowers and fruits in *Arabidopsis*, pear, tree peony, blueberry, poplar and, also, *Phalaenopsis* is miR156. *Arabidopsis* plants overexpressing miR156 exhibits hyperaccumulation of purple pigments in the internodes, whereas the mimicry strategy-based miR156 inhibition causes loss of these pigments. These observations indicate that miR156 positively regulates anthocyanin production. Among the *Arabidopsis* miR156-targeted *SPLs*, *AtSPL9* and *AtSPL15* have key roles in anthocyanin accumulation because the *spl9;spl15* double-mutant does not accumulate these pigments, suggesting redundant regulation of anthocyanin biosynthesis. Indeed, plants expressing a miR156-resistant version of *AtSPL9* show reduced anthocyanin accumulation by downregulation of *AtANS*, *AtF3′H*, and *AtDFR* genes, through destabilization of the MYB/bHLH/WD40 transcriptional activation complex (Gou *et al.*, 2011; Fig. 2). This indicates that upregulation of *AtSPL9* is responsible for the phenotype of mimicry plants, and suggests that miR156-targeted *SPLs* negatively regulate anthocyanin biosynthesis genes.

The miR156-mediated anthocyanin accumulation is higher under stress and bagging conditions. A comprehensive review about noncoding RNAs during stress-induced anthocyanin biosynthesis has been reported (Zhou *et al.*, 2023). In *Arabidopsis*, while miR156 expression was higher under salt and drought stress compared to nonstressed conditions, the *AtSPL9* responded in an opposite manner; thus, stress-mediated anthocyanin production correlates with miR156 and the *AtSPL9* expressions. In addition, the *DFR* and the *PAP1* were also responsive to stress, indicating that the miR156/*SPL9*/*DFR* is a stress-dependent circuit (Cui *et al.*, 2014; Fig. 2). Because this molecular circuit may be conserved in regulating stress tolerance in plants, studying this mechanism in two separated groups of plants, such as monocots and eudicots, could confirm this hypothesis. Regarding bag treatments, in the Chinese sand pear, miR156 increases in response to bagging treatments. The miR156-mediated *SPL* genes regulation leads to the *PpMYB10* upregulation and, consequently, the formation of the MYB/bHLH/WD40 complex that activate the expression of *PpCHS*, *PpCHI*, *PpF3H*, *PpANS*, and *PpUFGT* genes (Qian *et al.*, 2017; Fig. 2); these data suggest that miR156 and *SPL* genes should be targets for further research during bagging.

In plants of *Paeonia*, blueberry and poplar, miR156 also positively regulates anthocyanin accumulation. In the tree peony, miR156 promotes the anthocyanin production in petals by negatively regulating the *PsSPL2*. Total anthocyanins were negatively correlated with *PsSPL2*. While plants with silenced *PsSPL2* display increased *PsF3′H* and *PsDFR* expression in petals, heterologous overexpression of *PsSPL2* in tobacco resulted in downregulation of *NtF3′H* and *NtDFR* transcripts and consequently decrease in anthocyanin content (Luo *et al.*, 2022; Fig. 2), indicating that *PsSPL2* may regulate *PsF3′H* or *PsDFR* expression. In addition, heterologous overexpression of the Chinese peony *MIR156E* precursor in *Arabidopsis* triggers anthocyanin accumulation in lateral branches by repressing *AtSPL1*, a homolog of the *PlSPL1*, and consequently leading to *AtDFR* expression (Zhao *et al.*, 2017; Fig. 2). This molecular mechanism was also observed in blueberry, tomato and Arabidopsis. Heterologous overexpression of *VcMIR156a* in tomato enhances anthocyanin biosynthesis, and overexpression of the miR156-targeted *VcSPL12* in *Arabidopsis* downregulates anthocyanin biosynthetic and regulatory genes, including *DFR*, *PAP1*, *F3H*, *ANS* by a physical interaction between VcSPL12 and VcMYBPA1, a MYBPA1-type TF that regulates proanthocyanidin synthesis (X. Li *et al.*, 2020; Fig. 2). In addition, blueberry plants overexpressing miR156 display higher content of anthocyanin and correlated upregulation of anthocyanin production-related genes such as *VcPALs*, *Vc4CLs*, *VcCHSs*, *VcF3Hs* and *VcUFGTs* and *VcMYBA*. Thus, miR156 enhances anthocyanin by promoting not only biosynthetic genes, but also anthocyanin regulatory genes. In fact, anthocyanin content decreased in plants overexpressing VcSPL12 due to the VcSPL12-dependent repression of *CHS F3H*, *F3′5′H*, *ANS*, *UFGT*. These findings indicated that VcSPL12 regulates color change in blueberries by affecting the expression of anthocyanin as well as Chl-associated genes as previously shown (Li *et al.*, 2024; Fig. 2). As in peony and blueberry, in poplar miR156 promotes anthocyanin production as observed in plants overexpressing miR156 that increase the accumulation of anthocyanins via multiple factors (Wang *et al.*, 2020), however, whether this complex regulatory network of anthocyanin biosynthesis is conserved in poplar deserves further research.

While in *Arabidopsis*, pear, tree peony, blueberry, and poplar, miR156 enhances anthocyanin accumulation, in *Phalaenopsis* this miRNA seems to act in an opposite way. miR156 has different expression patterns from spot and nonspot sepal tissues of *Phalaenopsis* cv. 'Panda', being higher in nonspotted sepals where the anthocyanin amount is lower compared to the spotted ones. Moreover, the expression level of anthocyanin-associated genes, including *PAL*, *DFR*, *ANS*, *F3′H*, *F3H*, *bHLH1*, was lower (Zhao *et al.*, 2019; Fig. 2), suggesting that downstream targets of miR156 might fulfill opposite roles in different clades. Because this work does not present functional experiments, it is hard to assess whether the miR156-dependent circuit acts differently in this orchid; thus, more detailed experiments are necessary to evaluate the role of miR156 and downstream genes in the anthocyanin pathway of *Phalaenopsis* and other Orchidaceae members.

In addition to miR828 and miR156, the miRNAs miR858, miR399 and miR778 were also reported as players in anthocyanin regulation. While miR858 regulates anthocyanin content in kiwifruit, apple, tomato and *Phalaenopsis*, miR399 and miR778 regulate it in apple and *Arabidopsis*, respectively. A preliminary study of fruit coloring-involved sRNAs identified miR858, a miRNA that regulates members of the MYB TFs family, as a strong candidate involved in anthocyanin biosynthesis in kiwifruit (Li *et al.*, 2019; Fig. 2). Further analysis confirmed that miR858 negatively regulates anthocyanin biosynthesis by repressing *AaMYBC1*. Transient overexpression of miR858 in kiwifruit fruits decrease the transcript levels of *AaMYBC1*, *AabHLH42*, *AaCHS*, *AaF3H*, and *AaUFGT* and, consequently, reduced anthocyanin content; this effect phenocopies the *AaMYBC1* silencing.

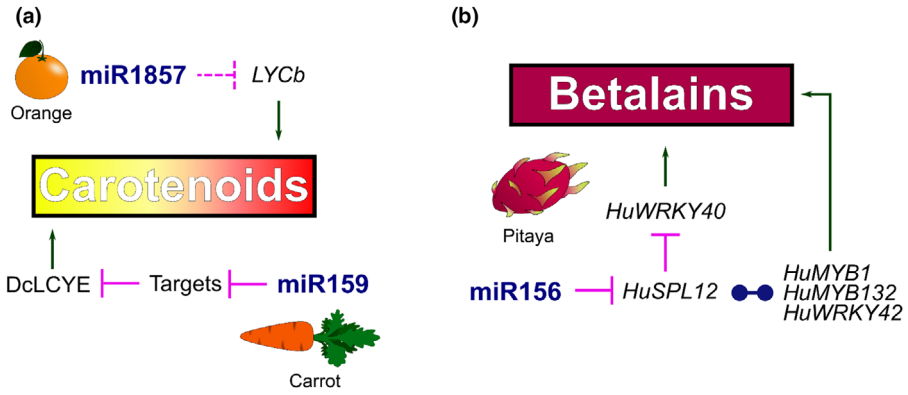

Fig. 3 Small RNAs modulate carotenoid and betalain biosynthesis. (a) Schematic representation of the misRNAs-regulated carotenoid production in Orange (*Citrus sinensis*) and Carrot (*Daucus carota*). (b) Schematic representation of the small RNAs-regulated betalains production in Pitaya (*Hylocereus* sp.). miRNAs are highlighted in bold blue. Green arrows indicate positive regulation, magenta blunt-ended bars indicate negative regulation, dashed lines or dashed arrows indicate hypothetical regulation, and blue lines with circular termination indicate protein–protein interactions.

Moreover, AaMYBC1 interacts with AabHLH42 to form a transcriptional complex and activate anthocyanin biosynthesis genes (Y. Li *et al.*, 2020; Fig. 2).

In apples, miR858 is expressed in the fruit flesh and its expression increases during development. In red-fleshed apples, the miR858 expression is significantly lower compared to white-fleshed ones, indicating a negative correlation between miR858 and anthocyanin content. Alteration in miR858 levels confirms this observation, high levels of miR858 decreased the *MdANS* and *MdUFGT* transcript levels leading to inhibition of anthocyanin accumulation. On the other hand, the mimicry strategy-based miR858 inhibition leads to an increase in *MdANS* and *MdUFGT* transcript levels, promoting anthocyanin biosynthesis. In addition, *MdMYB9* and *MdMYBPA1*, targets of miR858, participate in anthocyanin accumulation by binding to *MdANS* promoter and triggering anthocyanin biosynthesis (Li *et al.*, 2023; Fig. 2).

In tomato and *Phalaenopsis*, miR858 is also a negative regulator of anthocyanin biosynthesis in young seedlings, leaves, stems and leaf buds and petals. Tomato miR858 mediates the cleavage of *SlMYB7-like* and *SlMYB48-like*, two R2R3 MYB TFs. Mimicry strategy-based inhibition of miR858 increased expression of these genes, and also several anthocyanin biosynthesis genes, including *SlPAL*, *SlCHS*, *SlDFR*, *SlANS* and *Sl3GT* (Jia *et al.*, 2015). In *Phalaenopsis* cv. 'Panda', expression of miR858 is higher in nonspotted sepals where the anthocyanin amount is lower compared to the spotted ones, and the gene expression level of anthocyanin structure and regulation genes, including *PePAL*, *PeDFR*, *PeANS*, *PeF3′H*, *PeF3HI*, *PeF3H*, *PebHLH1* among others was lower as well (Zhao *et al.*, 2019), indicating that the mechanism of negative regulation on anthocyanin content by miR858 is highly conserved (Fig. 2).

In apples, miR399 promotes anthocyanin content. miR399 is involved in the phosphorus homeostasis. In leaves, anthocyanin and inorganic phosphorus display opposite patterns, indicating that low-phosphate concentrations induce miR399 expression to positively regulate anthocyanin biosynthesis. In fact, miR399 overexpressing plants display higher anthocyanin content in leaves and stems by upregulating anthocyanin-related biosynthesis genes *McMYB10*, *McPAL*, *McCHS*, and *McANS*; however, silenced miR399 did not induce these anthocyanin-related genes under phosphorus deficiency, indicating that inorganic phosphorus indirectly modulates anthocyanin content through miR399 expression (Peng *et al.*, 2020; Fig. 2).

In *Arabidopsis*, miR778 enhances anthocyanin production by increasing the expression of *AtPAP1* probably through its target *KRYPTONITE* (*AtSUVH6*), a SET domain-containing protein involved in regulation of histone methylation. Phosphate deficiency enhances miR778 expression and, under this condition, miR778 overexpression increases *AtPAP1* and decreases *AtSUVH6* expression compared to WT. In addition, *AtPAP1* and *AtSUVH6* transcript levels were lower in MIM778 plants compared to WT (Wang *et al.*, 2015; Fig. 2).

The sRNA-controlled biosynthesis of carotenoids and betalains

Few studies have shown, at posttranscriptional level, the role of the sRNAs, especially miRNAs in carotenoids production (Table 1). Those studies were carried out in oranges and carrots. In the sweet orange, differentially expressed miRNAs and putative targets were identified between the red-flesh mutant and WT; among them, miR1857 and a putative target *LYCOPENE B-CYCLASE* (LYCb) were identified as possible regulators of carotenoids biosynthesis (Xu *et al.*, 2010; Fig. 3a); however, further experiments need to be done to confirm whether miR1857 is a regulator of carotenoids production. In addition, a preliminary study in carrots found that miR159 seems to be involved in the regulation of carotenoids biosynthesis and accumulation (Bhan *et al.*, 2019). miR159 displayed higher expression in the orange red variant compared to the purple black one; orange red carrots display dark orange color by the accumulation of carotenoids. miR159 seems to regulate *LYCOPENE EPSILON-CYCLASE* (DcLCYE), a key enzyme in the carotenoids biosynthetic pathway, likely through its targets (Wang *et al.*, 2023; Fig. 3a); thus, miR159 emerges as a potential

regulator of carotenoids accumulation, although additional research needs to be done to confirm this hypothesis.

Betalains provide plants with purple color shades by totally replacing anthocyanins (Khan & Giridhar, 2015). Betalain biosynthesis is simpler compared to other pigments; however, the study of their regulation, especially at transcriptional level, is incipient. So far, the role of sRNAs in betalain biosynthesis has been poorly explored. Pitaya has been the focus of research because it is the only large-scale commercially grown fruit containing abundant betalains. Therefore, an initial work has identified several candidate miRNAs and their target genes related to pitaya fruit coloration and betalain accumulation, including miR157, miR160, miR6020, miR828 and miR858 (Chen *et al.*, 2020); nevertheless, for a better understanding of betalain biosynthesis in Pitaya, further analyses involving up- and downregulation of these miRNAs and its targets are necessary. A recent study has found that miR156 seems to positively regulate betalain biosynthesis in Pitaya (Zeng *et al.*, 2023; Fig. 3b). The higher expression of miR156 the higher the content of betalains whereas, intriguingly, the higher *HuSPL12* expression, one of miR156 targets in Pitaya, the lower the content of betalains, indicating that *HuSPL12* negatively regulates betalain biosynthesis during fruit development. Further analysis confirmed that *HuSPL12* is involved in betalain biosynthesis by repressing the promoter activity of *HuWRKY40*, a TF involved in betalain biosynthesis. This repression influences the expression of *HuCYP76AD1*, a key structural gene. In addition, *HuSPL12* interacts at protein level with *HuMYB1*, *HuMYB132*, and *HuWRKY42*, three positive regulators involved in pitaya betalain biosynthesis. These interactions seem to block the gene expression of betalain biosynthesis-related structural genes including *HuADH1*, *HuCYP76AD1*, *HuDODA1* and *DOPA5GT1* (Zeng *et al.*, 2023); however, these expression patterns deserve further analyses.

Future directions

The study of the regulation of pigment production by sRNAs has increased during the last decades in both model and nonmodel species. This knowledge allows us not only to know the interactions of these players but also being the basis for future research aimed to manipulate these pathways; thus, investigating how pigment biogenesis is modulated by sRNAs may help to open new venues to manipulate economically important plants in a world of growing population, climate change and limited land use.

Few sRNAs, mostly miRNAs, have been explored during the processes associated with pigment production; thus, further studies are still necessary to unravel novel sRNAs that might contribute to pigment biogenesis. Moreover, new technologies should be employed to manipulate pigment-associated biosynthetic pathways via sRNAs, which may help improve yield productivity. In this sense, miRNA/sRNA targets are potential candidates for CRISPR-mediated single base editing to fine-tuning control the levels of the desired pigment. In addition, knocking out the negative regulators of pigment production to generate transgenic-free lines is an approach that should be explored. More recently, exciting progress has been made on the roles of extracellular miRNAs in plant to plant communication (Betti *et al.*, 2021; Strzyz, 2021). The controlled delivery of extracellular sRNAs to plants may become in the near future a new transgenic-free technology to modulate pigment production in a tissue- and developmental-specific manner.

While sRNA-mediated regulation of anthocyanins dominate this research field, Chls, carotenoids and betalains are still in the beginning; thus, further research, including spatio-temporal regulation, devoted to those pigments that were not fully explored will allow a better understanding of the regulatory mechanisms of plant pigments. The results of these studies may help us to envision novel technologies in areas such as pharmacy and human nutrition, main areas of great commercial interest where obtaining plants with higher pigment content or the development of alternative systems for pigments production, such as cell factories, could bring benefits to human health.

Another field of interest involving pigments is floriculture. In some plant genera, the traditional development of new commercial cultivars, through intra- and interspecies crossings, can be time consuming, labor intensive and highly expensive due to the unpredictable interaction between genomes. Thus, getting new varieties of ornamental plants, by molecular approaches, is an important issue that needs to be explored; therefore, the knowledge of the regulation of pigments biosynthesis by sRNAs can guide these efforts in order to editing pigmentation-associated genes for obtaining new commercial cultivars and, consequently, save time and efforts.

Conclusion

Each of the plant pigment groups is synthesized endogenously to fully support the plant's needs. These biosynthetic pathways involve environmental signals and multiple endogenous factors at different levels through complex networks, including TFs, and sRNAs, specially miRNAs. Although advances in research suggest a complexity of these processes, we are still at the beginning of fully elucidating the regulation of plant pigments by sRNAs. Understanding the complexity of these molecular mechanisms will provide novel insights to modify the biological systems in order to improve nutritional value of food crops, obtain new commercial cultivars of ornamental plants, and develop new ways to obtain high levels of pigments for industry, especially in a world of increasing nutritional and health demands.

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Competing interests

None declared.

Author contributions

CHB-R conceptualized and wrote the manuscript. CHB-R, FTSN, and CvdB reviewed the manuscript. All authors have read and agreed to the published version of the manuscript.

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