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REVIEW

Advances in the application of biosynthesis and metabolic engineering of flavonoids in plants

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Abstract

Flavonoids are secondary metabolites widely distributed in plants. They not only confer a wide spectrum of pigmentation to plant flowers but also protect plants from various biotic and abiotic stresses. Simultaneously, these compounds also offer health benefits to humans. Significant efforts have been made to correlate specific flavonoid production with biosynthetic pathway gene expression. Some structure genes and transcription factors that regulate the biosynthetic pathway have been identified. However, the diverse and complex control of flavonoid accumulation is still not well understood. In this mini-review, we summarized the improvement of flavonoids by genetic engineering from the aspects of flower colour, plant resistance, and benefits on the human diet. A perspective on flavonoid research in plants is provided.

Keywords: flavonoids, flower colour, genetically engineering, human health, resistance.

Introduction

Flavonoids make a large family of plant secondary metabolites. They share a basic C6-C3-C6 skeleton structure that carries two aromatic rings and a heterocyclic ring with one oxygen atom (Forkmann and Martens 2001). Based on the oxidation state and substitution pattern of rings C3, flavonoids can be divided into a number of subgroups: flavanones, dihydroflavonols, flavones, flavonols, flavan-3-ols (catechins and polymeric proanthocyanidins), flavan-3,4-diols (leucoanthocyanidins), anthocyanidins, isoflavonoids with diverse biological activities and so on (Fig. 1). For instance, anthocyanins, one class of flavonoids, offer plant colouration for yellow, red, blue, magenta, *etc.* (Miyagawa *et al.* 2015). Genistein, an isoflavonoid abundant in soybean, shows anticancer activity (Ji *et al.* 2020, Ren *et al.* 2020); quercetin showed a positive effect against hypertension (Maaliki *et al.* 2019). Catechins and condensed tannins (derived from flavonoids) are responsible, at least in part, for plants' resistance to microbes (Desrues *et al.* 2016, Ma *et al.* 2019).

Flavonoid biosynthesis pathways have been extensively studied in a number of plant species, in model species, the main backbone of the pathways is shared (Fig. 2). Most of the structure genes such as *CHS, CHI, F3H, F3'H, DFR,* and *ANS* (encoding chalcone synthase, chalcone isomerase, flavanone 3-hydroxylase; flavonoid 3'-hydroxylase, dihydroflavonol 4-reductase, and anthocyanidin synthase) have been cloned in various plants (Petroni and Tonelli 2011, Goswami *et al.* 2018, Zhang *et al.* 2018b). In addition, a few transcription factors have been characterized, including members from

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Abbreviations: ANS - anthocyanidin synthase; AS - aureusidin synthase; cDNA - complementary DNA; 4'CGT - chalcone 4'-*O*-glucosyltransferase; CHI - chalcone isomerase; CHR - chalcone reductase; CHS - chalcone synthase; DFR - dihydroflavonol 4-reductase; F3H (FHT) - flavanone 3-hydroxylase; F3'H - flavonoid 3'-hydroxylase; F3'5'H - flavonoid 3',5'-hydroxylase; FLS - flavonol synthase; GUS - β-glucuronidase; RNAi - RNA interference; T-DNA - transfer DNA; WBPH - whitebacked planthopper.
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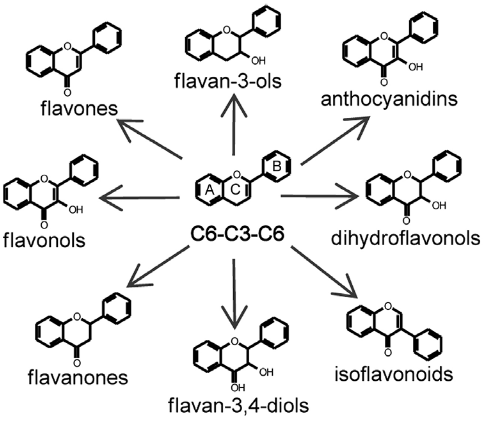

Fig. 1. Basic structural units and major structural classes of flavonoids.

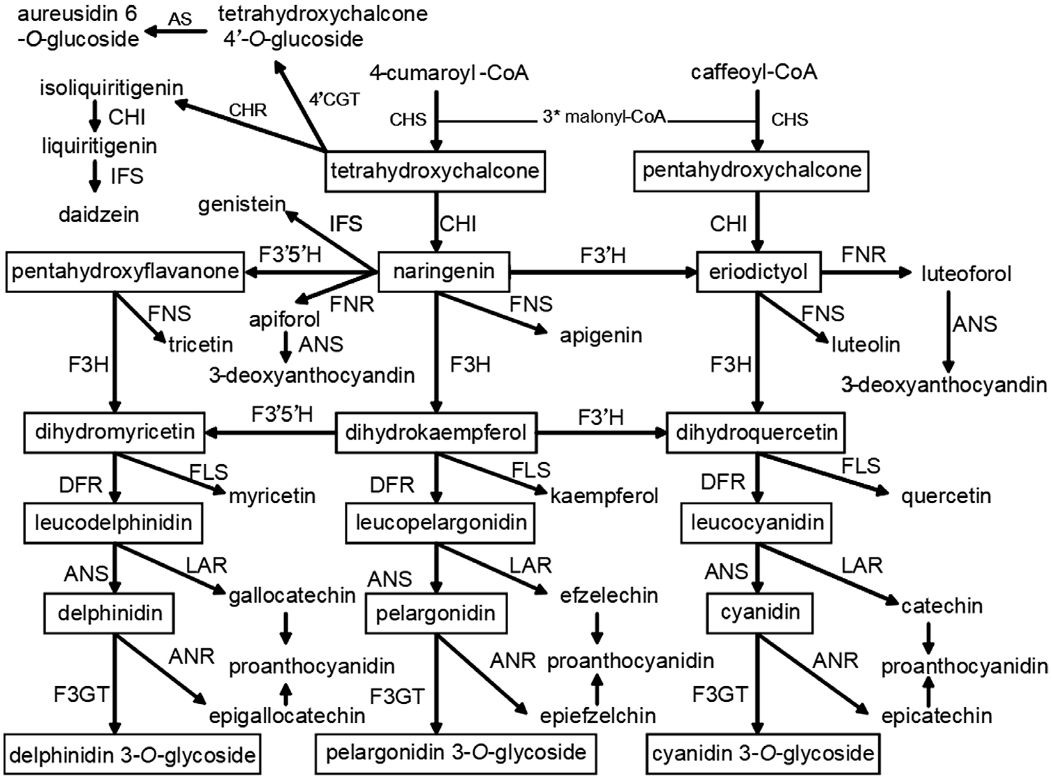

Fig. 2. Main flavonoid biosynthetic pathways in plants.

the v-myb avian myeloblastosis viral oncogene homolog (*MYB*), basic Helix-Loop-Helix (*bHLH*) and WD40 domain-containing protein (*WD40)* families (Wang *et al.* 2018, Zou *et al.* 2018). Structural genes in the pathway are largely regulated at the level of transcription. Some structure genes are regulated by a single transcription factor, for example, production of anthocyanin pigment 1 (*PAP1*) regulating the proanthocyanidin biosynthesis in *Arabidopsis* (Gonzalez *et al.* 2008). Other structure genes are regulated by transcriptional complexes, for example, *transparent testa2* (*TT2*), *transparent testa8* (*TT8*), and *transparent testa glabra1* (*TTG1*) regulated proanthocyanidin biosynthesis (Baudry *et al.* 2004, 2006, Sharma and Dixon 2005).

The rich information on the pathways offers opportunities for genetic engineering for target flavonoids. Various strategies have been adopted; these include over-expression of structural genes or transcription regulator genes, antisense down-regulation of structural genes, and down-regulation of structural genes with RNAi.

Modifying flower colours

Flavonoids are the most common pigments found in flowers (Nishihara and Nakatsuka 2011). The principal flavonoids in flowers are anthocyanidins (pelargonidin, cyanidin, and delphinidin, usually giving red/scarlet, red/magenta,

Table 1. Strategies used to modify flower colours. *** The gene originated from the plant itself unless otherwise specified.

Target colour	Host species (original colour)	Strategy***	Major changes of flavonoid (reference)
White	carnation (red)	*F3H*-antisense	pelargonidin (down) (Zuker *et al.* 2002)
	tobacco (pink)	*DFR*-RNAi	dihydro flavonol (up) and flavonoids (up) (Lim *et al.* 2016)
	peony (yellow)	*ANS*-antisense	pelargonidin (up) (Yamamizo *et al.* 2012)
	tobacco (pink)	*Petunia FLS*-sense	flavonol (up) and anthocyanin (down) (Luo *et al.* 2016)
	tobacco (pink)	*Solenostemon scutellarioides MYB3*-sense	condensed tannins (up) and anthocyanin (down) (Zhu *et al.* 2015)
	Tricyrtis sp.(red)	*CHS*-RNAi	anthocyanin (down) (Kamiishi *et al.* 2012)
Red	tobacco (pink)	*Muscari spp. DFR*-sense	dihydromyricetin (up) and anthocyanin (up) (Liu *et al.* 2019)
	Petunia (pale red)	*Clematis patens F3’5’H*-sense	delphinidin (up) (Lee *et al.* 2017)
	Petunia (white)	*MYB1*-sense	anthocyanin (up) (Naing *et al.* 2020)
	Ipomoea Mina lineage (blue)	*F3H*-antisense	anthocyanin (up) (Marais *et al.* 2010)
	Solidago canadensis (yellow)	*Arabidopsis PAP1*-sense	delphinidin (up) (Skaliter *et al.* 2019)
	tobacco (pink)	*Muscari armeniacum MYBA*-sense	anthocyanin (up) (Chen *et al.* 2019)
	Petunia (pink)	*F3’5’H*-antisense *F3’H*-antisense and *DFR*-sense	anthocyanin (up) (Tanaka and Brugliera 2014)
	tobacco (pink)	maize *B-peru*-sense and *Arabidopsis mPAP1*-sense	flavonols (up) and anthocyanin (up) (Kim *et al.* 2018)
Yellow	cyclamen (white)	alfalfa *CHR*-sense	flavonoids (up) (Mizukami *et al.* 2004)
	tobacco (pink)	*Camellia FLS1*-sense	flavones (up) and anthocyanin (down) (Zhou *et al.* 2013)
	Chrysanthemum (red)	*Petunia F3’5’H*-sense	anthocyanin (down) (Seo *et al.* 2007)
	Ipomoea nil (pale yellow)	snapdragon *AmAS1*-sense *Am4’CGT*-sense and *CHI*-antisense	aurone (up) and chalcone glucoside (down) (Hoshino *et al.*2019)
Blue	carnation (white)	*Petunia F3’5’H*-sense and *DFR*-sense	delphinidin (up) and flavone (up) (Fukui *et al.* 2003)
	Chrysanthemum (pink)	rose *F3’5’H*-sense and *F3’H*-RNAi	delphinidin (up) (Brugliera *et al.* 2013)
	rose (red)	*Viola F3’5’H*-sense, *DFR*-RNAi and *Iris DFR*-sense	delphinidin (up) (Katsumoto *et al.* 2007)
	Chrysanthemum (red-purple)	butterfly pea *A3'5'GT*-sense, Canterbury bells *F3'5'H*-sense and *Chrysanthemum F3H*-RNAi	delphinidin (up) and flavone (up) (Noda *et al.* 2017)

or violet/blue colours) (Dai and Hong 2016). The genes *F3’H* and *F3’5’H* (encoding flavonoid 3’-hydroxylase and flavonoid 3’,5’-hydroxylase) determine the availability and relative ratio of the anthocyanidins (Fig. 2). Further modification can occur to anthocyanidins by glycosylation, acylation, and methylation, forming anthocyanins. The latter are eventually transported into vacuoles, making the flowers colourful. It is obvious that enzymes’ substrate specificity is a major factor determining flower colouration. For example, *DFR* in petunia does not utilize dihydrokaempferol (Fig. 2) and, thus, pelargonidin is not accumulated; this is why petunia does not have red colours. It is equally obvious that the presence/absence of particular enzymes (genes) has similar consequences. For example, flowers of rose, lily, and gerbera lack violet-to-blue colours because they do not contain delphinidin-based anthocyanins due to a lack of *F3’5’H*. Flower colours can also be affected by the modification of anthocyanins and other pigments, such as flavones and flavonols (Grotewold 2006). Many genetically engineered flowers are available on the ornamental plant market (examples in Table 1). This is a huge industry. Below are a few strategies used to modify some flower colours.

White flowers are easy to be obtained by antisense or RNAi silencing of structure genes in the flavonoid biosynthesis pathway, such as *CHS* (Kamiishi *et al.* 2012), *F3H* (Zuker *et al.* 2002), *DFR* (Lim *et al.* 2016), and *ANS* (Yamamizo *et al.* 2012). Alternatively, white flowers can be obtained by introducing a dominant gene to synthesize colourless flavonoids and inhibit anthocyanidin biosynthesis (Luo *et al.* 2016, Zhu *et al.* 2015).

Red flowers can be produced by a number of methods; here are a few examples: *1*) introducing a structure gene, which is mutated or missing in the host plant (Lee *et al.* 2017, Liu *et al.* 2019); *2*) introducing an anthocyanidin promoted transcription factor (Chen *et al.* 2019, Skaliter *et al.* 2019, Naing *et al.* 2020); *3*) inhibiting delphinidin accumulation by down-regulating of a structure gene

(Marais *et al.* 2010), and *4*) combing two or more of the above strategies (Tanaka and Brugliera 2014, Kim *et al.* 2018).

To generate yellow flowers, three strategies are commonly used: *1*) introducing the gene for the synthesis of yellow flavonoids, such as the *CHR* gene, into a white cultivar (Mizukami *et al.* 2004); *2*) starting with flowers containing anthocyanidin and yellow pigments (such as carotenoid), down-regulating by antisense or RNAi of structure genes for anthocyanidin biosynthesis, such as *F3H* (Zuker *et al.* 2002), *DFR* (Zhou *et al.* 2013), and *F3'5'H* (Seo *et al.* 2007); *3*) using a combination of these two approaches, chalcone 4'-*O*-glucosyltransferase (*4'CGT*) is essential for aurone biosynthesis and yellow colouration. Coexpression of the *4'CGT* and aureusidin synthase 1 *(AS1)* genes was sufficient for the accumulation of aureusidin 6-O-glucoside in transgenic flowers of *Torenia hybrida*. Furthermore, their coexpression combined with the down-regulation of anthocyanin biosynthesis by RNAi resulted in yellow flowers (Ono *et al.* 2006, Hoshino *et al.* 2019).

Blue flowers could be achieved by over-accumulation of delphinidin, but it seems to be more complicated. Okinaka *et al.* (2003) introduced a *Campanula medium F3'5'H* gene into tobacco, and delphinidin is accumulated in the flowers. However, the flowers changed from pink to purple, but not to blue. Togami *et al.* (2006) transferred a *Clitoria ternatea F3'5'H* gene into *Verbena*, and high amounts of delphinidin were found in the flowers. But the flowers changed from pink to violet, not to blue. Success was reported in rose by introducing a viola *F3'5'H* and iris *DFR*, in combination with RNAi for the endogenous *DFR*. In this case, in addition to delphinidin, a suitable amount of flavonols ("co-pigments") and a higher vacuolar pH are necessary to achieve blue colour (Katsumoto *et al.* 2007). Since then, blue dianthus and chrysanthemum have also been successfully obtained (Fukui *et al.* 2003, Noda *et al.* 2017, Brugliera *et al.* 2013).

Enhancing plant resistance

Many flavonoids are considered to play important roles in plants' tolerance to biotic and abiotic stresses. (Brunetti *et al.* 2013, Zhang *et al.* 2017, Jakl *et al.* 2021a,b). For instance, transgenic *Arabidopsis* with high total flavonoids exhibited enhanced tolerance to drought stress compared to wild-type plants (Rao *et al.* 2020). The increased synthesis of flavonoids in plants can also enhance the resistance of plants to salt stress (Song *et al.* 2016). Flavan-3-ols in apple were considered useful in resistance to bacterial and fungal diseases, such as fire blight and apple scab (Leser and Treutter 2005, Pontais *et al.* 2008). Red mangoes with higher anthocyanins have stronger resistance to anthracnose (Sivankalyani *et al.* 2016). Genetic engineering of the flavonoid biosynthesis pathway opens new opportunities to enhance plant defence. After the overexpression of *AtDFR* from *Arabidopsis thaliana* in *Brassica napus*, it was found that the anthocyanin content was significantly increased, and the resistance of transgenic plants to salt stress was significantly enhanced (Kim *et al.* 2017). In *Oryza sativa*, a WBPH-resistant cultivar was developed by overexpressing the *OsF3H*, and the content of quercetin and delphinidin was increased by 2.68- and 2.77-times, respectively, compared with wild-type rice (Jan *et al.* 2020). Plant resistance to pathogens can be enhanced by multiple genes. When cDNAs for *CHS*, *CHI*, and *DFR* were transferred into flax through a multigene construct, simultaneous expression of these genes resulted in significant increases in flavanones (1.53-fold) and anthocyanins (3.67-fold), and plants showed improved resistance to *Fusarium*, the main pathogen for flax (Lorenc-Kukula *et al.* 2007).

Manipulation of transcription factors involved in the flavonoid biosynthesis pathway is another way to achieve multiple gene expression. Transgenic *Begonia* overexpressing the maize *Lc* (bHLH) transcription factor synthesized large amounts of anthocyanins (12-fold) and flavan-3-ols (14-fold) (Li *et al.* 2007). These plants were resistant against fire blight (caused by the bacterium *Erwinia amylovora*) and against scab (caused by the fungus *Venturia inaequalis*) (Flachowsky *et al.* 2010). The *Arabidopsis* MYB transcription factor *MYB111* regulates salt stress by regulating flavonoid biosynthesis. After overexpression of *MYB111*, the content of flavonoids increased by about 1.85-times, and the salt tolerance of transgenic plants was also greatly improved (Li *et al.* 2019). It was found that the flavonoids in the transgenic tobacco of *AtMYB12* were increased, especially the rutin content was more than 10-times higher than that of the wild-type tobacco, and the resistance of the transgenic tobacco to aphids and *Bemisia tabaci* was improved (Li *et al.* 2012).

In another line of research, the accumulation of flavonoid glycosides in transgenic plants can improve their resistance to pathogen infection. Ectopic expression of 5-*O*-glucosyltransferase cDNA in the potato tuber improved the plant's defence against *Erwinia carotovora* (Lorenc-Kukula *et al.* 2005). Flax overproducing *Solanum sogarandinum* 7-O-glycosyltransferase showed enhanced resistance to *Fusarium* infection (Lorenc-Kukula *et al.* 2009). The observed benefit is attributable to the fact that flavonoid glycosides stabilize flavonoids.

Nutritional enhancement for human diet

Some flavonoids have great nutritional value. Consumption of some flavonoids, such as genistein or quercetin, through vegetables and fruits, can reduce the risk of the stomach-, rectum-, cervical-, breast-, and lung cancer (Luo *et al.* 2016, Oin *et al.* 2016, Fu *et al.* 2017, Zhang *et al.* 2017, Xing *et al.* 2018, Wang *et al.* 2020). In cultures of pancreatic â-cells, anthocyanins and anthocyanidins were found to induce insulin secretion; indicating the potential application of these flavonoids in treating diabetes (Matkowski *et al.* 2021). Flavonoids are also the quantitatively most frequent and most effective anti-oxidative compounds in apples, onions, tea, red wines, and other berries (Christensen *et al.* 2012, Rossi *et al.* 2012, Zhang *et al.* 2018, Han *et* *al*. 2019). They are natural antioxidants, which not only prevent food oxidation but also enhance the health value of foods. A further important function of flavonoids for human health was apoptosis induction, antimutagenesis, enzyme inhibition, the influence of the blood clotting, anti-inflammatory functions, beneficially influence on rheumatoid arthritis, and protective effect in cardiovascular diseases (Cassidy *et al*. 2013, Hatier *et al*. 2013, Yousuf *et al*. 2016, Meng *et al*. 2018, You *et al*. 2019).

Some important crop plants are not equipped with the machinery for biosynthesis pathways of certain flavonoids (Willits *et al*. 2005) and, thus, expression of the required structure genes from other species would be able to overcome the rate-limiting enzymatic steps. In this way, the flux through an already existing pathway can be increased, leading to high amounts of desired flavonoids or even new flavonoids. Most tomato cultivars do not synthesize anthocyanins in fruits, but expressing petunia *CHI* in tomato can lead to an increase of up to 78-fold of fruit peel flavonols, mainly due to rutin accumulation (Muir *et al*. 2001). In potato, several attempts have been made to increase the flavonoid production in the tubers by introducing structural genes. Over-expression of a petunia *CHS* cDNA resulted in an increase in petunidin and pelargonidin-type anthocyanins in potato tubers (Lukaszewicz *et al*. 2004). Transgenic potato plants over-expressing a petunia *DFR* gene show a 3-fold increase in the content of petunidin and a 4-fold increase in pelargonidin derivatives (Lukaszewicz *et al*. 2004).

Another approach to enhance target flavonoid synthesis is to use transcription factors. Overexpression of the MYB-type C1 family (*C1*) and the basic helix-loop-helix, MYC-type R family (*Lc*) maize transcription factor genes in the flavonoid pathway, in tomato significantly increased flavonoid levels (Bovy *et al*. 2002). Aiming at enriching anthocyanins in tomato fruit, two transcription factor genes *Delila* (*Del*) and *Rosea1* (*Ros1*) from snapdragon were introduced into tomato under the control of the fruit-specific *E8* promoter. Transgenic tomato plants accumulated anthocyanins in fruits and antioxidant capacity was enhanced 3-fold. When fed with these tomato fruits, cancer-susceptible mice showed a significant extension in life span (Butelli *et al*. 2008). With soybean, ectopic expression of the maize *C1* and *R* chimeric transcription factors caused a 2-fold increase in isoflavonoid content in seeds, while expression of *C1* and *R* chimeric transcription factors in conjunction with suppressing *F3H* to block the anthocyanin and flavonol pathways, resulted in 4-fold higher content of isoflavone in soybean seeds (Yu *et al*. 2003).

To date, the genetic engineering of plants used for human nutrition has been concentrated on metabolic engineering and in several studies, the antioxidant activity has been tested. But the health-promoted function of transgenic plants is only demonstrated by theory. Feeding the model animals (such as a rat, and mouse), which have the symptom of hypotension, cancer, diabetes, *etc*., with the transgenic product should be a feasible method to test the health-promoted activity of the transgenic product.

Accumulating bioactive flavonoids in medicinal plants

Medicinal plants have great value for human health. This is especially true for many developing countries; more than 80 % of the populations in those countries rely on plant-based medication for healthcare needs. The active ingredients in many medicinal plants, such as jaceosidin, hispidulin, apigenin, xanthohumol, wogonin, and ellagic acid, are all derivatives of flavonoids (Duan *et al*. 2002, Gerhauser *et al*. 2002, Lee *et al*. 2003, Kang *et al*. 2006). But medicinal plants need a long time to accumulate high flavonoid content. Many only grow in limited regions or under certain conditions, however, plant tissue culture techniques have been used to break these limitations (Fonseca *et al*. 2006, Qiu *et al*. 2010). Hairy root culture through *Agrobacterium rhizogenes*-mediated genetic modification has been another powerful tool (Guillon *et al*. 2006). Hairy roots from many plants, such as *Codonopsis* (Yang *et al*. 2020), periwinkle (Chung *et al*. 2009), American ginseng (Kochan *et al*. 2020), watercress (Chung *et al*. 2009), and *Scutellaria* (Chang *et al*. 2021) were successfully obtained using an empty root-inducing (Ri) plasmid. A large number of hairy root lines can be made available for stable and extensive production of flavonoids.

Hairy root cultures expressing structure genes for target flavonoids would be preferred, and this approach has been used for several medicinal plants. Using an *Agrobacterium rhizogenes*-mediated transformation system, the chi overexpression casette was incorporated into the genome of *Saussurea involucrata*, and transgenic hairy root lines grew faster and produced higher content of apigenin (12-fold) and total flavonoids (4-fold) than wild-type hairy roots (Li *et al*. 2006). The introduction of soybean 2-hydroxyisoflavanone dehydratase (*HID*) into the hairy root culture of *Lotus japonicus* can cause an accumulation of daidzein and genistein (Shimamura *et al*. 2007). Previous studies in *Scutellaria baicalensis* showed a similar result, almost all of the *SbCHI*-overexpressed hairy root lines accumulated more flavones than the control hairy root line, and baicalin content reached 42.2-60.2 $\mu g\ mg^{-1}$, which was 2.4 - 3.4 times higher than that in the control hairy root line (Park *et al*. 2011).

Manipulating the expression of transcription factors in hairy root cultures can be promising. After overexpression of the flavonol-specific transcription factor *AtMYB12* in buckwheat, the production of rutin (0.53-0.88 $\mu g\ mg^{-1}$) was found to be 2.2 - 3.7 times higher than that of control plants (Park *et al*. 2012). Another MYB transcription factor had similar results, after overexpression of *FtMYB116* into Tartary buckwheat hairy roots, rutin (2.75 - 9.4-fold) and quercetin (2.4 - 10.4-fold) were significantly increased (Zhang *et al*. 2019). When *FtbZIP5* was overexpressed in the hairy roots of Tartary buckwheat, the total flavonoid content reached 0.96 $mg\ g^{-1}$, which was approximately twice that in the wild strain (Weng *et al*. 2021).

In the empty root-inducing (Ri) plasmid's T-DNA region, there are genes for auxin and cytokinin synthesis; hence, in addition to the massive growth, the altered (elevated) hormone content in hairy roots can trigger the relocation of secondary metabolism (Kovacs *et al*. 2004, Fu *et al*. 2006). However, generated hairy root cultures have limited applications. Since genes from the T-DNA are not directly involved in flavonoid biosynthesis, the outcome is hard to predict. For example, baicalin is the most abundant flavonoid in *Scutellaria*, whereas in its transgenic hairy roots the glycoside of wogonin dominates (Kovacs *et al*. 2004). Generally, many high-value flavonoids exist in trace amounts and may be structurally unique according to the plant species, and hairy root cultures normally do not improve the accumulation of such compounds.

In future, the genetic modification of flavonoids in medicinal plants will concern the identification of key genes involved in bioactive flavonoid biosynthesis. It is still a major challenge in the genetic engineering of medicinal plants. Genomic sequencing, flavonoid profiling, and bioactive composition screening of the important medicinal species would facilitate high-quality medicinal plants through flavonoid genetic engineering.

Concluding remarks

Genetic engineering for a number of flavonoids has been successful, but there are some critical restraints. One major constraint is the lack of knowledge of flavonoid pathways in many plant species. Both structural genes and regulatory genes have target specificity. Additionally, certain genes involved in the pathways may be silenced due to natural mutation. An improved understanding of the molecular mechanism underlying the biosynthesis pathways of flavonoids is necessary before major progress can be made in the production of target compounds.

In addition, although genetic engineering has brought us a lot of conveniences, it also has biosafety issues that cannot be ignored. Because the metabolic pathways in plants and other organisms are intertwined with one another to form a complex system, it is expected that perturbation of a single step in the network usually has extensive effects on the whole metabolic flux. There may be problems leading to toxicity and food allergies. In other scenarios, can promiscuous transgenic plants spread genes to other plants? So far, too little attention has been paid to these questions. Research to support the risk assessment of novel transgenic plants should be prioritized.

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