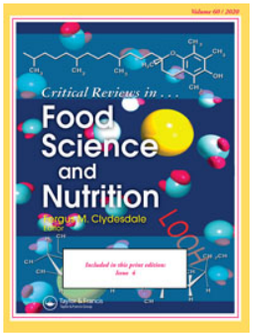

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REVIEW

Biosynthesis of citrus flavonoids and their health effects

Chengying Zhao[a]*, Feng Wang[a]*, Yunhe Lian[a], Hang Xiao[b], and Jinkai Zheng[a]

[a]Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences, Beijing, China; [b]Department of Food Science, University of Massachusetts, Amherst, MA, USA

ABSTRACT

Citrus-derived flavonoids play important roles in the regulation of physiological conditions of citrus plants, including color changes of flower and fruit, flavor development, and anti-stress physiology. Moreover, citrus flavonoids possess multiple health-promoting effects in humans, and they are important ingredients for nutraceuticals and functional foods. The biosynthesis of flavonoids in citrus plants is of special significance because it determines the chemical structures and bioaccumulation of these bioactive compounds in the plants, which consequently influences their physiological functions in both citrus plants and human body. This review systematically summarizes: 1) the biosynthesis pathway of citrus-derived flavonoids, 2) the biosynthesis location and distribution of flavonoids in citrus plants, 3) the factors affecting flavonoid biosynthesis, 4) the biological significance of flavonoid biosynthesis in citrus plants, and 5) the health-promoting properties of citrus-derived flavonoids. The collation of this information provides scientific guidance for the development of healthy citrus foods and other health-promoting products containing citrus flavonoids.

KEYWORDS

Citrus; flavonoids; biosynthesis; health effects

Introduction

Citrus is one of the most popular fruit types worldwide, widely grown in tropical, subtropical, and many other areas. Annually, approximately 121 million tons of citrus are produced (Patsalou et al. 2017). Citrus fruits are consumed in high quantities due to their delicious tastes, attractive colors, and aromas. Moreover, citrus fruits contain various health-promoting components, including dietary fiber, flavonoids, essential oils, and carotenoids (M'hiri, Ioannou, Ghoul, and Boudhrioua 2017; Yang et al. 2017). Flavonoids are among the most important bioactive components of citrus, with a relatively high abundance, especially in their peels (Patil et al. 2009; Pfeiffer and Hegedus 2011; Li and Schluesener 2017). They are built upon a C6 (A ring)-C3 (C ring)-C6 (B ring) flavone skeleton. The hydrogen in the skeleton is usually substituted by various groups, such as hydroxyl, methoxyl, and glycosyl, which results in a large structural diversity of flavonoids. Additionally, the C ring can be subjected to oxidation, and the connection position of the B ring on the C ring can vary (Figure 1). More than 80 flavonoids from citrus plants have been identified and characterized, and they can be categorized into flavanones, flavones, flavonols, and anthocyanins (Tripoli et al. 2007). Citrus flavonoids possess various health-promoting functions, such as anti-inflammatory, anticancer, antioxidant, anti-lipogenic, and metal chelating activities (Pfeiffer and Hegedus 2011; Zvaigzne and Kārkliņa 2013), which significantly contributions to the overall health-promoting benefits of citrus fruits (Yu et al. 2005; Goulas and Manganaris 2012; Barreca et al. 2013; Singanusong et al. 2015; Zhang et al. 2014). Citrus flavonoids have provided valuable resource for commercial applications in pharmaceuticals, food, health products, as well as dyes, and cosmetics.

Biosynthesis is a multi-step substance-forming process in living organisms, involving the participation of multiple complex enzyme systems, in which secondary metabolites are generated. Many components (e.g., precursor compounds, enzymes, and coenzymes) participate in the process and critically regulate the formation of secondary metabolites. In addition, physiological conditions and environmental factors (e.g., light, temperature, and pH) are important factors influencing the process (Moriguchi et al. 2001). The biosynthesis may occur within a single cellular organelle or in multiple cellular organelles, depending on the locations of the enzymes (Wagner and Hrazdina 1984). Plants have many natural bioactive components with different chemical structures, and the bioactivity of a specific component depends on its chemical structure. With recent scientific developments, the biosynthesis process in different organisms can be manipulated to increase the content of certain bioactive components (Mulder et al. 2015). In addition, novel compounds with special chemical structures and health-promoting properties can be obtained through redesigning the biosynthesis pathway accordingly.

Flavonoid biosynthesis has been subjected to extensive research in different systems. Enzymes that participate in flavonoid biosynthesis have been purified from different cellular

CONTACT Jinkai Zheng zhengjinkai@caas.cn; Hang Xiao hangxiao@foodsci.umass.edu
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*Both authors have contributed equally to this work.

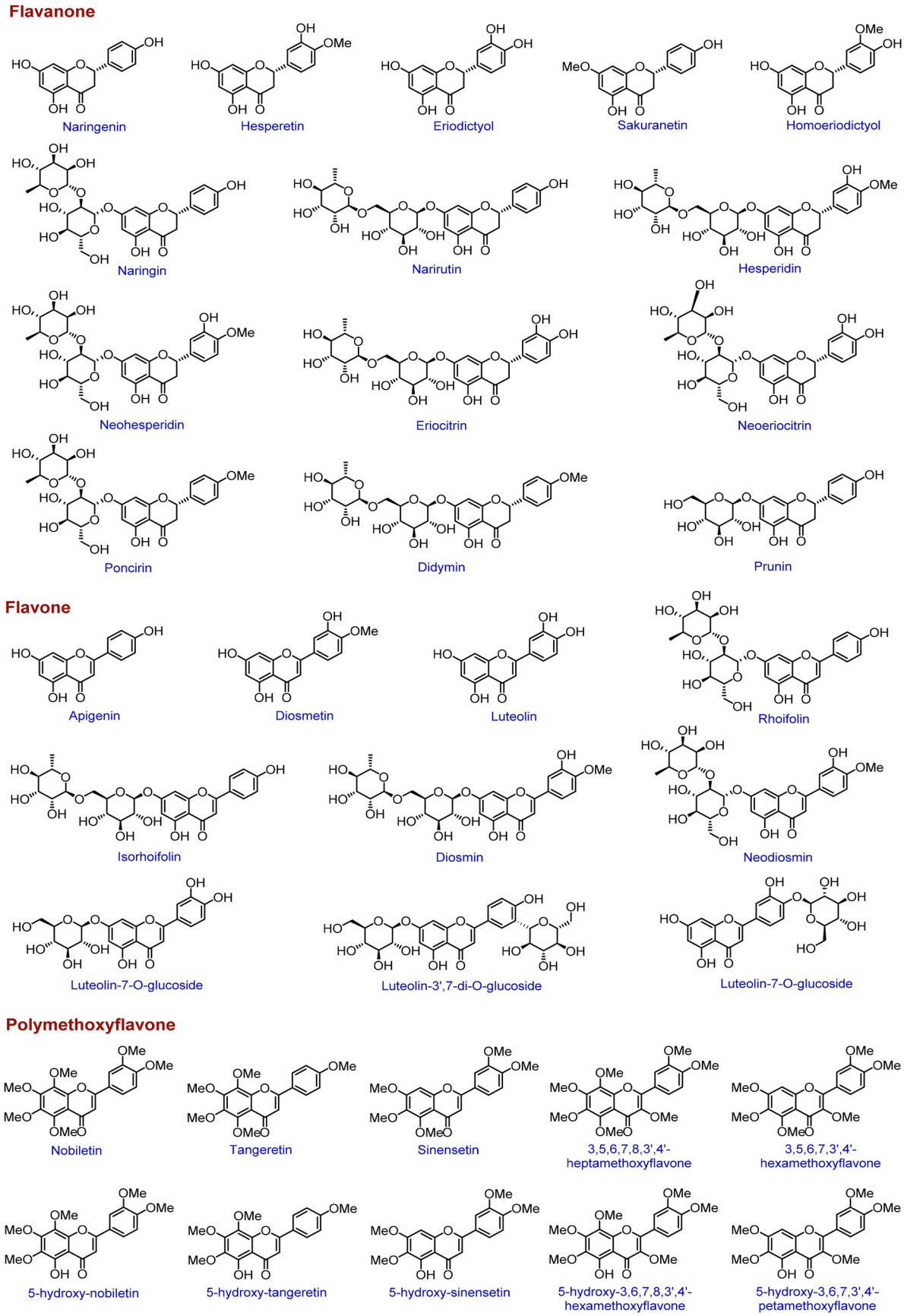

Figure 1. Chemical structures of citrus flavonoids.

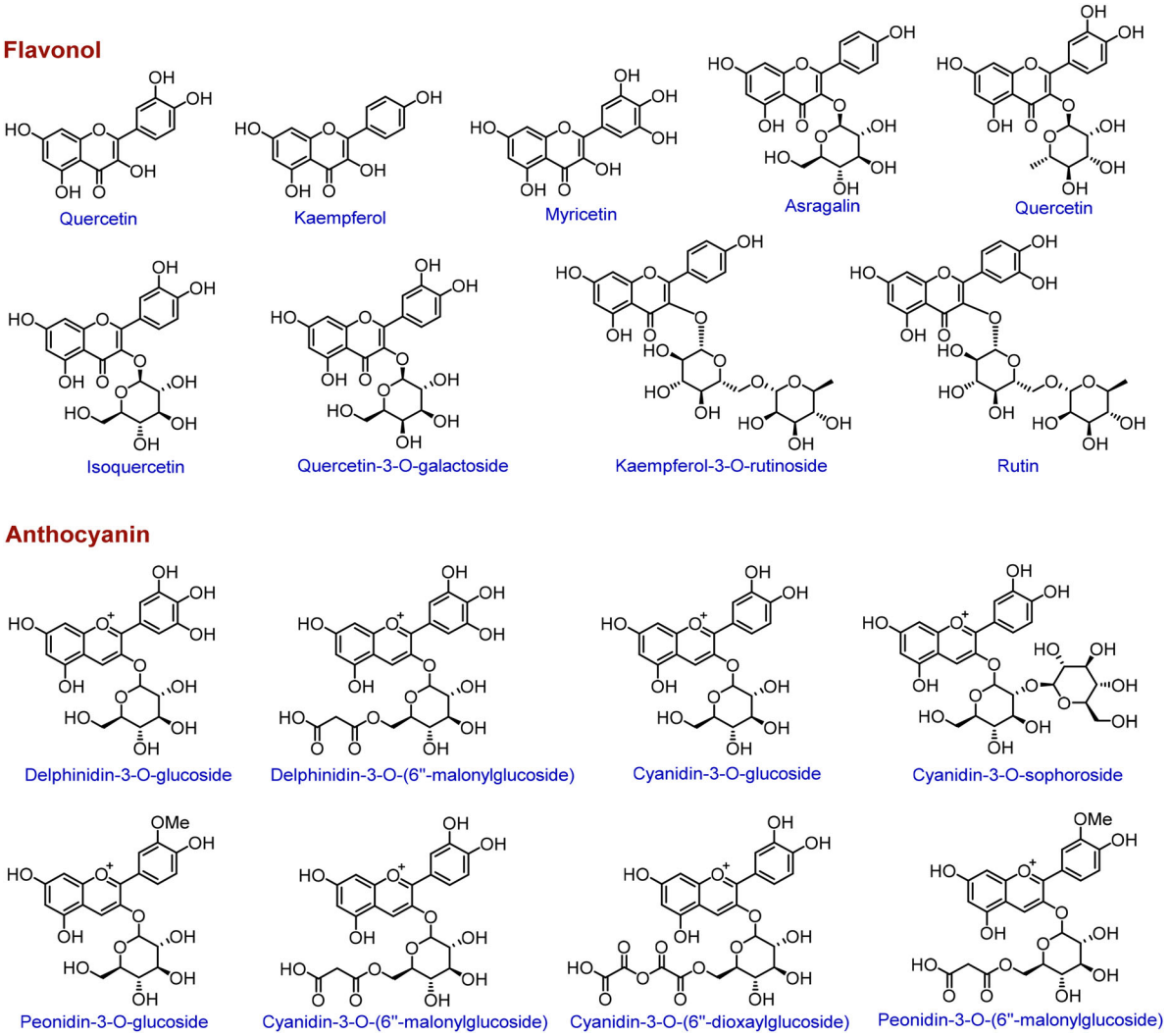

Figure 1. Continued.

organelles in citrus plants, and some bioactive flavonoids have been produced through the co-culture system with microorganisms (Han et al. 2017; Fowler and Koffas 2009). The biosynthesis pathway of citrus-derived flavonoids has been speculated to occur through the general phenylpropanoid pathway. It begins with the transformation of phenylalanine to *p*-coumaroyl-CoA through the sequential actions of three enzymes, i.e. phenylalanine ammonia-lyase (PAL, EC 4.3.1.5), cinnamate 4-hydroxylase (C4H), and 4-coumarate-CoA ligase (4CL). One molecule of *p*-coumaroyl-CoA and three molecules of malonyl-CoA would combine to form chalcone that could be converted to naringenin. Naringenin is the central intermediate of the biosynthesis pathways and further transferred into different classes of flavonoids (i.e. flavanone, flavonols, and anthocyanin) (Pfeiffer and Hegedus 2011). The biosynthesis pathway could be significantly affected by precursors, enzymes, and environmental factors (e.g., UV light, metal mineral elements, and physiological conditions). Citrus flavonoids play important roles in color changes of flower and fruit, flavor development, and regulation of interactions between plants and environments to enhance anti-stress physiology in citrus plants. More importantly, considering the potential health effects of citrus flavonoids in human after their oral consumption, a comprehensive understanding of their biosynthesis would provide scientific guidance for the production of high-value metabolites and the development of healthy citrus foods and health-promoting products containing citrus flavonoids.

Several reviews of flavonoid biosynthesis in citrus plants, focusing mainly on the biosynthesis procedures, have been previously published (Pfeiffer and Hegedus 2011; Owens and McIntosh 2011). However, little attention has been given to the factors affecting flavonoid structure changes during biosynthesis or the implications of these changes to the plants and human health. The aim of this review was to systematically summarize: 1) the biosynthesis pathway of citrus-derived flavonoids, 2) the biosynthesis location and distribution of flavonoids in citrus plants, 3) the factors affecting flavonoid biosynthesis, 4) the biological significance of flavonoid biosynthesis in citrus plants, and 5) the health-promoting properties of citrus-derived flavonoids.

Biosynthesis pathway of citrus-derived flavonoids

With the development of molecular technology and transcriptomics, proteomics, and metabolomics strategies in recent decades, many studies of flavonoid biosynthesis have

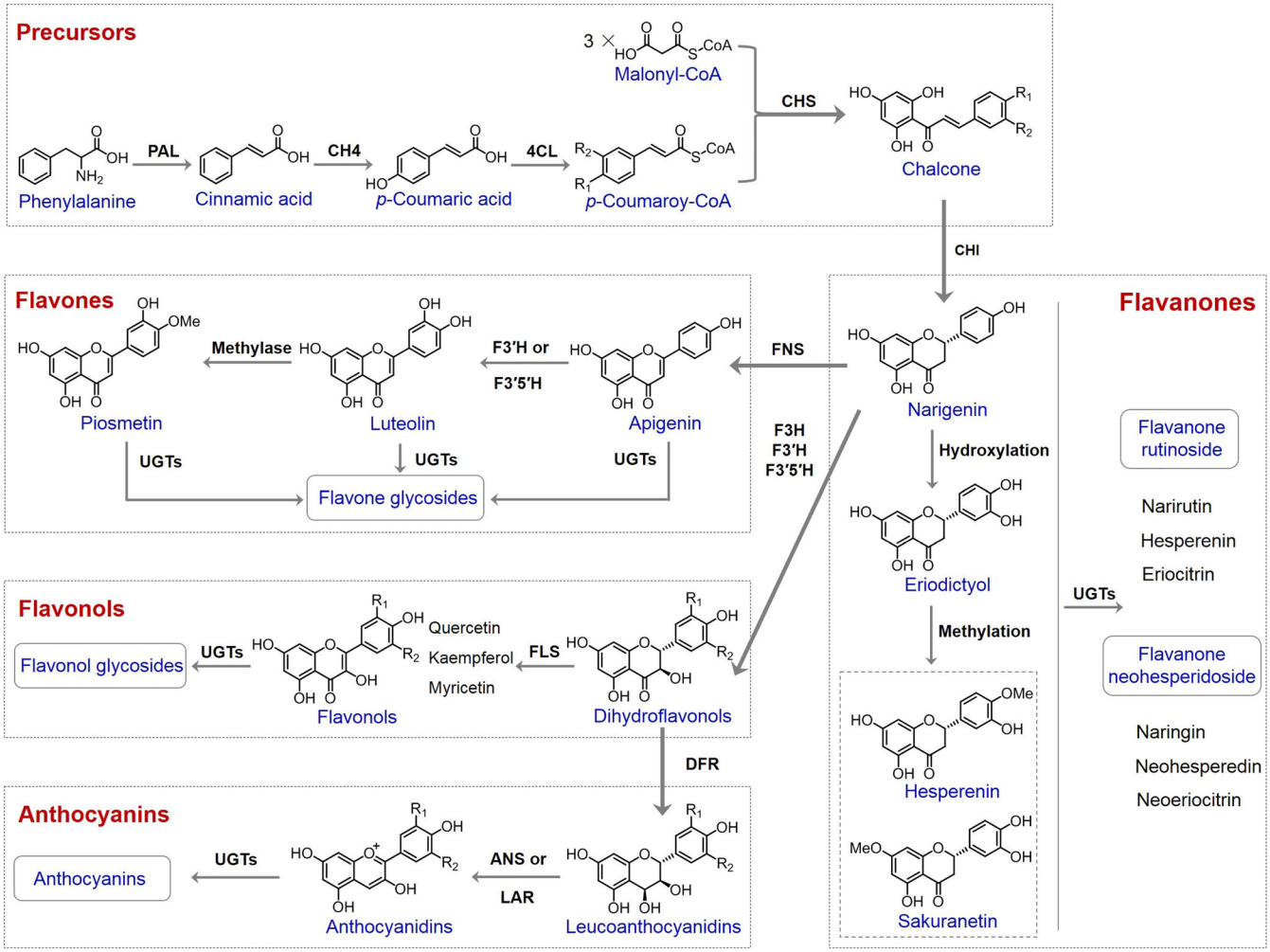

Figure 2. Major biosynthesis pathway of flavonoids in citrus plants.

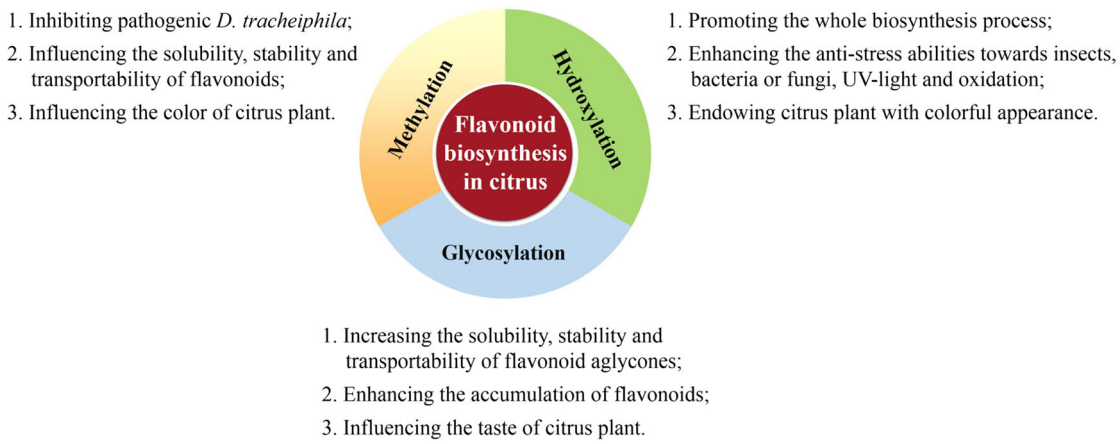

Figure 3. Biological significances of flavonoid biosynthesis in citrus plants.

been conducted. Although there are various kinds of citrus flavonoids with diverse chemical structures, the synthesis route of these flavonoids has been elucidated in three major stages (Figure 2): synthesis of the precursor (*p*-coumaroyl-CoA), synthesis of flavonoids (e.g., flavanones, flavonols, flavones, and anthocyanins), and glycosylation to form various glycosides, which are very important flavonoids in citrus.

Synthesis of precursors

The phenylpropanoid pathway is the upstream stage of the whole process of citrus-derived flavonoid synthesis. In this pathway, the precursor, *p*-coumaroyl-CoA, is synthesized in three steps. First, phenylalanine, a primary metabolite in plants, is transformed to cinnamic acid (cinnamate) through

the elimination of amine by PAL. Then, hydroxylation occurs at C4 under the catalysis of C4H, forming *p*-coumaric acid (*p*-coumarate), which is further acylated with acetyl-CoA to *p*-coumaroy-CoA by 4CL (Figure 2). All intermediates and enzymes involved in the upstream of the whole pathway are of significance, as they play important roles in the regulation of subsequent reactions in the downstream steps of flavonoid biosynthesis (Fowler and Koffas 2009; Khan, Zill-E-Huma, and Dangles 2014; McIntosh and Owens 2016).

Synthesis of flavanones

The flavonoid pathway is the main process by which various flavonoids are produced in citrus plants. In addition to *p*-coumaroyl-CoA, the other precursor for flavonoid biosynthesis is malonyl-CoA, which originates from carbohydrate metabolism (the shikimate and arogenate pathways). Consequently, naringenin chalcone (2′,4,4′,6′-tetrahydroxychalcone) is formed by the condensation of one *p*-coumaroyl-CoA and three malonyl-CoA under the catalysis of chalcone synthase (CHS, EC 2.3.1.74). The chalcone then cyclizes to the general chiral flavanone structure (4′,5,7-trihydroxyflavanone, naringenin) through the addition of 5-hydroxyl on A ring and α-alkene with the catalysis of chalcone isomerase (CHI, EC 5.5.1.6). A ring originates from three molecules of malonyl-CoA and C ring originates from one molecule of *p*-coumaroyl-CoA, which has been verified through isotope labeling experiments. The general chiral flavanone (naringenin) is a central intermediate, which can be further transferred into different classes of flavonoids (flavanones, flavones, flavonols, and anthocyanins) by a well-characterized enzymatically derived process in citrus plants (Pfeiffer and Hegedus 2011). Naringenin can be converted to different flavanones (e.g., hesperetin, eriodictyol, and sakuranetin) through hydroxylation and methylation of A or B ring, which could be further glucosylated and rhamnosylated to various glycosides (Figure 2).

Synthesis of other flavonoids

As mentioned above, naringenin is formed after the common biosynthesis pathway, and is a precursor of other flavonoids (e.g., flavones, flavonols, and anthocyanins). Flavone biosynthesis starts with the hydrogenation of naringenin at C2-C3 to form apigenin by flavone synthase (FNS). Then, luteolin and diosmetin are produced with the help of F3′H/F3′5′H and methylases, respectively. Apigenin (in *Citrus paradisi*), diosmin (in *C. sinensis* and *C. limonia*), and luteolin (in *C. limonia* and *C. aurantium*) are the three main flavone aglycones in citrus, and can be converted to various glucosides by a series of glycosyltransferases (Lucheta et al. 2007). Citrus fruits are a rich source of O-methylated flavonoids and polymethoxylated flavones (PMFs) almost exclusively exist in citrus, especially in sweet orange (*C. sinensis* L. Osbeck) and mandarin (*C. reticulata* Blanco) (Green et al. 2007; Li, Lo, and Ho 2006). A previous study showed that two O-methyltransferase (OMT) genes of flavonoids had higher expression levels in the peel and pulp of *C. sinensis* during fruit development, respectively, which led to higher levels of O-methylated flavonoids (Gana et al. 2013; Guidetti-Gonzalez et al. 2007; Liu et al. 2016b). Five other OMT genes (*CdFOMT 1*, *3*, *4*, *5*, and *6*) isolated from *C. depressa* promoted the accumulation of nobiletin in the peels (Itoh, Iwata, and Toda 2016). Therefore, PMFs might be synthesized from flavone aglycones by OMTs (Roje 2006). Another potential biosynthesis pathway is the methoxylation of flavonoids with the catalysis of methoxylase (Itoh, Iwata, and Toda 2016). However, no study has completely verified PMF biosynthesis until now.

Flavonols are pale-yellow, poorly soluble substances that consist mainly of kaempferol, quercetin, and myricetin (Lucheta et al. 2007), which can form co-pigments with anthocyanin. Dihydrokaempferol, the common direct precursor of flavonols, is formed through the hydroxylation of naringenin at C3 under the catalysis of flavanone 3-hydroxylase (F3H). It can be further converted to dihydroquercetin and dihydromyricetin by flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′,5′-hydroxylase (F3′5′H), respectively (Lucheta et al. 2007; Yáñez et al. 2012). Flavonols (e.g., kaempferol, quercetin, and myricetin) are then synthesized from the dihydroflavonols under the catalysis of flavonol synthase (FLS).

Anthocyanidins are common plant pigments that are present mainly in blood oranges. For anthocyanin biosynthesis, dihydroflavonol 4-reductase (DFR, EC 1.1.1.219) catalyzes the reduction of dihydroflavonols to flavan-3,4-diols (leucoanthocyanidins), which are further reduced to anthocyanidins by anthocyanidin synthase (ANS, EC 1.14.11.19) or catechin by leucoanthocyanidin reductase (LAR, EC 1.17.1.3) (Yáñez et al. 2012). The 'Moro' and 'Sanguinello' blood oranges are rich in a variety of anthocyanins, with the main pigments of cyanidin 3-glucoside and cyanidin 3-(6″-malonyl-glucoside) (Fabroni et al. 2016) being the most abundant, accounting for more than 60% of anthocyanins (Lee 2002; Dugo et al. 2003; Hillebrand, Schwarz, and Winterhalter 2004; Kelebek, Canbas and Selli 2008). In addition, anthocyanidins can be synthesized from delphinidin and paeonidin through glycosylation and acylation, respectively (Dugo et al. 2003).

Glycosylation procedures

In natural citrus plants, flavonoids exist predominantly in glycosylated forms, especially flavanones-O-glycoside, flavone-O-glycoside, and flavone-C-glycoside, which are generally water soluble and accumulate in the vacuoles of plant cells (Pfeiffer and Hegedus 2011). These glycoside flavonoids are obtained from the binding of the sugar group with a receptor by the uridine diphosphate (UDP)-dependent glycosyltransferases (UGTs), including flavonoid 3-O-glycosyltransferases, 5-O-glycosyltransferases, 7-O-glycosyltransferases, 3′-O-glycosyltransferases, and flavonoid glycoside glycosyltransferases (GGTs) (Yonekura-Sakakibara and Saito 2014). When the flavonoids have been synthesized, GGTs add monosaccharides or disaccharides, such as glucose, rhamose, neohesperidose, or rutin, to different positions on the flavonoid skeleton.

Flavonoid-specific glycosyltransferases catalyze the transformation of a glycosyl group from a nucleotide-activated sugar to a specific position (C3, C5, or C7) on the flavonoid skeleton (Lewinshon et al. 1989). The three main glycosyltransferases, 7-Glct, 1,2-Rhat, and 1,6-Rhat, can transform flavanone aglycones into glucosides with different tastes. For example, naringenin can be converted to bitter naringin, and sweet hesperetin can be converted to tasteless hesperidin and further to bitter neohesperidin. In addition to forming flavor, other glycosyltransferases, such as flavonoid 3-O-glucosyltransferase (UFGT, EC 2.4.1.91) can stabilize anthocyanidins by forming the structure of 3-O-glucosylation (Pfeiffer and Hegedus 2011) and UDP-rhamnose flavanone glucoside rhamnosyltransferase (UFGRT) sequentially convert the flavanone aglycones into their 7-O-β-D-glucosides and rhamnoglucosides (Khan, Zill-E-Huma, and Dangles 2014).

As shown above, the synthesis of citrus flavonoids occurs mainly in three major stages (synthesis of the precursor, synthesis of flavonoids, and glycosylation). The biosynthesis process involves various chemical structure transformations, including oxidation, reduction, cyclization, hydroxylation, methylation, and glycosylation. The transformation is almost entirely enzymatic, and the participation of different enzymes results in the synthesis of different flavonoids, even from the same starting substances. Therefore, enzymes are core elements during flavonoid biosynthesis. A comprehensive understanding of the biosynthesis pathway would enable targeted bioactive flavonoids to be obtained with novel biotechnologies.

Biosynthesis location and distribution of flavonoids in citrus plants

The biosynthesis pathway of flavonoids has been described above. The biosynthesis location and distribution in plants are also important, and could inform approaches to the engineering of novel metabolic pathways in living cells and improve the efficiency of flavonoid extraction from plants. Regarding the biosynthesis location in the subcellular structures, researchers have speculated that flavonoids are initially formed in the cytoplasm, and are then transferred to and accumulate in the vacuoles. In terms of their distribution, flavonoids have been found to be produced in young and rapidly growing tissues. During cell elongation and subsequent maturation of leaves and fruit, flavonoid production slows or even stops. In addition, the distribution differs significantly among citrus species and organs.

Metabolic location in citrus cell

In previous studies, the endoplasmic reticulum of the cytoplasm was found to be the location of flavonoid biosynthesis (Wagner and Hrazdina 1984). Phenylalanine ammonia-lyase exists mainly in the cell walls, secondarily thickened walls, and parenchymal cells of mesocarp tissue, and flavonoid glucosyltransferase appears to be located in the lumens of the membranes. Cinnamate 4-hydroxylase exists mainly in the chloroplasts (plastids) and nuclei of plant cells, and to a lesser extent in the cell walls and cytoplasm. 4-Coumarate: coenzyme A ligase is located primarily in the secondarily thickened walls and parenchymal cells of mesocarp vascular tissue (Chen et al. 2006). The use of immunogold electron microscopy showed that chalcone synthase exists mainly in chloroplasts, chromoplasts, and the rough endoplasmic reticulum, with small amounts in the cytoplast, at 30/70 d after full bloom. However, 100 d after full bloom in the skin tissue, chalcone synthase resides mainly in vacuoles, chloroplasts, and secondarily thickened walls (Li et al. 2008). In addition, studies of the localization of metabolic enzymes in subcellular structures have shown that enzymes participating in flavonoid biosynthesis are often in the form of multienzyme complexes. Chalcone synthase, chalcone isomerase, and dihydroflavonol reductase form a globular complex anchored to the cytoplasmic face of endoplasmic reticulum membranes. They interact with each other in an orientation-dependent manner, which could improve catalytic efficiency and end-product specificity control (Burbulis and Winkel-Shirley 1999). Considering that the distribution of enzymes in cells varies, enzymes or precursors produced by enzymes need to be transported out of the cells. Thus, precursors might be synthesized in subcellular structures that are rich in enzymes like cell walls, and then transported to the endoplasmic reticulum for the synthesis of flavonoids. Flavonoids would then be transferred into vacuoles. Therefore, the biosynthesis procedure might proceed with flavonoids initially forming in the cytoplasm, and then transferring to and accumulating in the vacuoles. The accumulation in the vacuoles plays an important role in the regulation of plant physiological conditions, especially the accumulation of anthocyanins, which determine the color of the plant.

Distribution of flavonoids in citrus plants

Flavonoid accumulation in citrus has also been widely investigated. The largest amounts of flavonoids are produced in young and rapidly growing tissues, including seedlings, young flushes of mature trees, flowers, and immature fruits (Moriguchi et al. 2001; Koca et al. 2009). During cell elongation and the subsequent maturation of leaves and fruit, the production of flavonoids has been found to slow or even stop. Consequently, flavonoid concentrations are low in these organs as a result of dilution effects (Koca et al. 2009). Hesperidin, a main flavanone in Rutaceae plants, accumulates in the peels of satsuma mandarin (*C. unshiu* Marc. cv. Aoshima). Hesperidin has been found to be more abundant in immature peels than in other tissues, and has been observed mainly in the areas surrounding vascular bundles, around the border between the flavedo and albedo layers, and just below the epidermal cells with Raman microscopy (Inoue et al. 2015). High levels of naringin are present in newly regenerated shoots, with an average concentration of 1051 ppm, which is about seven times more than in regenerated buds (148 ppm), whereas small amounts have been found in the callus (3.6 ppm) (McIntosh and Owens 2016). The distribution of flavonoids also varies seasonally, with a significant decrease during maturation.

In addition to variation attributable to the growth period, the distribution and content of flavonoids vary dramatically among citrus species and organs (del Río et al. 2004; Moriguchi et al. 2001; Patil et al. 2009; Yáñez et al. 2012). Generally, neohesperidoside flavanones are present mainly in pummelo-related species, and rutinoside flavanones tend to accumulate in citron- and mandarin orange–related species (Moriguchi et al. 2001; Khan, Zill-E-Huma and Dangles 2014). As an example, the naringin content can account for up to 70% of the dry weight in young grapefruits (*C. paradisi*) (Koca et al. 2009), generating a bitter taste, whereas large amounts of hesperidin tend to be present in lemons, limes, oranges (*C. sinensis*), clementines (*C. reticulata*), tangerines, and tangor species of citrus fruits (Cano, Medina, and Bermejo 2008), making them taste sweet (Nogata et al. 2006; Pfeiffer and Hegedus 2011). The PMF content in *C. reticulata* is higher than that in *C. sinensis* (Xing et al. 2017). With regard to the different organs, the highest flavanone concentrations are found in peels (1.3–10.0 mg/g, fresh weight), rather than in the fleshy parts of citrus fruits (0.45–1.71 mg/g, fresh weight) (Nogata et al. 2006; Khan, Zill-E-Huma, and Dangles 2014; Zhao et al. 2017). A recent study on fruit flavonoid variation in the peels of different citrus species through UPLC-PDA system showed that hesperidin (8.0 mg/g, fresh weight) and nobiletin (1.4 mg/g, fresh weight) were the most abundant in the peels of *C. tangerina* Tanaka, while hesperidin (7.4 mg/g, fresh weight) and narirutin (0.8 mg/g, fresh weight) were the most abundant in *C. sinensis* Osbeck peels, naringin (4.5 mg/g, fresh weight) in *C. grandis* Osbeck, and eriocitrin (4.7 mg/g, fresh weight) and hesperidin (3.0 mg/g, fresh weight) in *C. limon* Burm (Zhao et al. 2017). For the distribution of each flavonoids, hesperidin exists mainly in leaves and peels, whereas narirutin is present in the flowers, albedo, juice sacs, segment epidermis, and flavedo, rather than the leaves (Moriguchi et al. 2001). In grapefruit, quantities of hesperitin (hesperidin), naringenin (naringin), and rutin are found in the fruits, whereas only hesperidin and naringin are found in the peels. In lemon, the seed contains mainly eriocitrin and hesperidin, but the peel is abundant in neoeriocitrin, naringin, and neohesperidin, which are present at similar concentrations (Bocco et al. 1998). In satsuma mandarin (*C. unshiu* Marc.), hesperidin accumulates significantly in fruits and leaves, narirutin accumulates in fruits, and rutin accumulates in leaves (Moriguchi et al. 2001). Polymethoxyflavones are distributed mainly in the peels, especially in the oil glands, while little present in pulp (Chen et al. 2015). Among them, nobiletin was the most dominant one (Zhao et al. 2017).

Factors influencing flavonoid biosynthesis

Flavonoid biosynthesis is a complicated process involving many factors, including the substances that participate in the pathway, such as precursors, enzymes, and transcription factors. Physical, chemical, and physiological conditions, such as the existing flavonoids, UV light, amino acid residues, metal mineral elements, and other stimuli, also play important roles. Elucidation of the factors influencing the biosynthetic pathway of citrus flavonoids and their corresponding roles would enable the development of strategies for targeted regulation of the biosynthesis of certain flavonoids of interest.

Influence of biosynthesis precursors

The phenylpropanoid pathway is the first stage of flavonoid biosynthesis. In this pathway, phenylalanine is the most primitive precursor. It is derived from the first metabolism of amino acids, carbohydrates, and lipids in citrus plants. It dramatically affects the downstream flavonoid synthesis of *p*-coumaroyl-CoA, as well as normal physiological functions. Malonyl-CoA is another important precursor that is also related to the metabolism of glucose, lipids, and amino acids in the synthesis of flavonoids in citrus plants (Fowler and Koffas 2009; Zill-E-Huma, and Dangles 2014). Witches' broom disease significantly increases the levels of some metabolites, including amino acids, organic acids, sugars, sugar alcohols, and fatty acids, in Mexican lime (Mollayi et al. 2015). Studies have determined that phenylalanine and malonyl-CoA are derived from some metabolism pathway of amino acid and fatty acid metabolism (carbohydrate metabolism), which indicates that flavonoid biosynthesis would be influenced in plants. In summary, the supply of direct precursor substances has a significant impact on the whole biosynthesis process.

Influence of transcription factors

Regulatory genes are involved in encoding MYB, MYC, bZIP, WD40, and Zn proteins in the flavonoid biosynthesis pathway, among which MYB is the most studied transcription factor in anthocyanin biosynthesis. MYB proteins not only activate the transcription process, but also inhibit the expression of the encoding genes. For example, R3MYB protein AtMYBL2 and R2R3-MYB protein AtMYB 60 can inhibit the synthesis of anthocyanin, and transparent testa 2 (TT2), a R2R3-MYB transcription factor, is required for proanthocyanidin biosynthesis (Dubos et al. 2008; Matsui, Umemura and Ohme-Takagi 2008; Park et al. 2008). Ectopic expression of TT2 has been shown to induce a more than two-fold increase in the expression of *UGT72L1* genes, which leads to high glucosyltransferase activity toward flavan-3-ol-(−)-epicatechin in the seed coat (Yonekura-Sakakibara and Saito 2014). The R2R3-MYB transcription factor *CsMYBF1* can regulate the accumulation of flavonols and hydroxycinnamic acids in citrus, whereas its ectopic expression results only in a high hydroxycinnamic acid content in tomato (Liu et al. 2016a). These results indicate that the same transcription factor may show different regulatory patterns in different species.

Influence of biosynthesis-related enzymes

Flavonoid biosynthesis is an almost entirely enzymatic process. Enzymatic reactions possess significant substrate specificity and regioselectivity for different enzymes. The products depend on the substrate and the enzyme. Two O-methyltransferases, CrOMT2 and CrOMT4, are obtained from

Catharanthus roseus, while only the former has sequential methylation ability at the 3′- and 5′-positions of B ring (Cacacea et al. 2003). In a systematic analysis of the O-methyltransferase gene in citrus, 27 OMT genes were identified as being associated with the O-methylation of flavonoids, and 18 and 11 of them had higher expression levels in peel and pulp, respectively, during fruit development (Liu et al. 2016b). These results also indicate the different methylated functions of OMTs. Specially, CdFOMT5, an O-methyltransferase from *C. depressa*, has relatively broad substrate specificity and regioselectivity, which could catalyze the methylation of the 3-, 5-, 6-, and 7-hydroxyl groups of flavones. Polymethylated products could not be detected from naringenin, (-)-epicatechin, and equol under the catalysis of CdFOMT5 (Itoh, Iwata, and Toda 2016). In addition, the functions of different enzymes usually differ, which could lead to the production of different compounds even with the same substrate. As mentioned above, intermediates (e.g., naringenin, apigenin, and dihydroflavonols) in the biosynthesis pathways could be converted to various flavonoids with different enzymes. Therefore, the biosynthesis products depend on the type, substrate specificity, and regioselectivity of different enzymes.

Factors influencing enzyme activity

Enzyme activity is often the main factor directly influencing the synthesis of flavonoids, and it is dramatically affected by the physiological condition of plants and the existing environment (e.g., existing flavonoids, UV light, amino acid residues, and metal mineral elements). Due to the similarity of their chemical structures, the activity of enzymes may be suppressed by existing flavonoids. As an example, 4′-O-methyltransferase was thought to be inactivated because an increase in eriocitrin levels coincided with a decrease in hesperidin and diosmin levels (del Río et al. 2004). If the flavonols myricetin and quercetin bind to the catalytic site of DFR-NADP in a stacking arrangement, the geometry of the enzyme would be altered and it would be inactivated. In some conditions, substrates can enhance the activity of enzymes (Lo Piero 2015). External environmental conditions also have dramatic effects on the activity of enzymes. For example, PAL has been reported to be promoted in plants primarily by UV wavelengths, particularly those in the UV-B region (Fowler and Koffas 2009). Enzymes associated with anthocyanin, such as DFR, LAR, and ANS, can be stimulated by different wavelengths of light, especially visible, ultraviolet, and infrared light. Furthermore, the catalytic activity of enzymes is associated strongly with amino acid residues at active sites, with amino acid residues able to cause enzyme inactivation. His20 deprotonates the flavonoids in the O3 hydroxyl group, and Asp119 can support this function of histidine. The mutagenesis of H20A and/or D119A can remove any detectable glycosylase activity (Yonekura-Sakakibara and Saito 2014). Amino acid residues, such as cysteine, histidine, arginine, tryptophan, and tyrosine, can also affect enzyme activity (Owens and McIntosh 2009). Other studies have demonstrated that the *C. paradisi* flavonol-3-O-glucosyltransferase and UDPare inhibited by Cu^{2+}, Fe^{2+}, and Zn^{2+}.

Other factors

The flavonoid biosynthesis pathway could also be affected by various other stimuli in the growth environment, such as hormones, water stress, and pathogen attacks (Moriguchi et al. 2001). The accumulation of anthocyanins can be induced by pathogen infection, fungal elicitors, and methyl jasmonate. Auxins and/or cytokines have been shown to induce anthocyanins in cell cultures and whole plant systems, and gibberellins have been linked to anthocyanin production in pigmented orange. In a study of the influence of external stress on flavonoid biosynthesis, the content of 21 flavonoids was relatively low in citrumelo under flooding pressure, whereas only 12 flavonoids were present at relatively low levels in Carrizo. In addition, leaf concentrations of most compounds, including quercetin, kaempferol, and eriodictyol as aglycones, were increased under soil flooding, whereas endogenous levels of apigenin-based flavonoids were reduced in citrus leaves under stress (Djoukeng et al. 2008). A recent study found that salicylic acid (SA) could act against the devastating pathogen *Penicillium digitatum*, the causal agent of green mold disease in blood oranges. These findings demonstrate that an increase in anthocyanin levels may be closely related to the antifungal activity triggered by SA in blood oranges (Lo Piero 2015). Considering the importance of enzymes, the above mentioned environmental factors may influence flavonoid biosynthesis through interactions with biosynthesis-associated enzymes, although no direct evidence supports this assertion.

The biological significance of flavonoid biosynthesis in citrus plants

Flavonoids are biosynthesized in some specialized plant cells at different growing or disease stages, or under special conditions (Yáñez et al. 2012). Changes in chemical structure during the biosynthesis process would lead to different flavonoid functions, including pigmentation/co-pigmentation, signaling, pollinator guidance, seed dispersal roles, phytotoxicity, defense against toxins, UV resistance, and plant–microbe and –fungal interactions. These features would have dramatic effects on the physiological conditions of citrus plants (del Río et al. 2004; Lucheta et al. 2007; Massenti et al. 2016; Nebo et al. 2014) (Figure 3).

Hydroxylation

Hydroxylation is an important step for flavonoid biosynthesis in plants. The first hydroxylation in the process of flavonoid synthesis is the introduction of a hydroxyl group from cinnamic acid to A ring at C4 position to form *p*-coumaroyl acid (Winkel-Shirley 2001). This process is crucial for flavonoid synthesis when forming the important precursor *p*-coumaroyl-CoA in the middle of the phenylalanine pathway. It not only affects the downstream of the biosynthesis pathway

or substance transportation, but also promotes the use of the upper precursor and the metabolic cycle of the whole pathway. The hydroxylation of flavonoids plays an important role in the regulation of plant physiological status. Flavonoids with 7-hydroxyl group, such as eriodictyol, homoeriodictyol, and luteolin, not only endow plants with a specific taste like bitterness, but also repel animals and insects (Dreyer and Jones 1981). When the hydroxyl group is replaced by glucosides, those bioactivities disappear, indicating the importance of the hydroxyl group for the bioactivities (Duffey and Isman 1981). Hydroxyl groups adjacent to O-diphenols were found to be sensitive to oxidation, producing o-diquinones or even o-diphenol dimers. Therefore, cyanidin, delphinidin, and petunidin, which contain the o-diphenol structure on the B ring, are more sensitive than other flavonoids to oxidation. In contrast, malvidin and peonidin do not possess the ortho-positioned hydroxyl groups, which results in their comparatively greater resistance to oxidation (Yáñez et al. 2012). At the same time, some polyhydroxyl flavonoids can form colored structures with anthocyanidins, and endow plants with a colorful appearance, which could attract insects to spread pollen and seeds, encouraging the survival and reproduction of the plants. Hydroxylation in the B ring of the anthocyanidins directly affects hue and color stability. Hydroxylation and methylation can result in direct damage to bacterial or fungal cell structures, providing some protection from those microbes.

Glycosylation

Flavonoids are usually modified by glycosylation, including the addition to –OH groups (O-glycosides), C groups (C-glycosides), or N groups (N-glycosides) (Gattuso, Barreca, Gargiulli, Leuzzi, and Caristi 2007; Gentili and Horowitz 1968; McIntosh and Owens 2016; Peterson et al. 2006). O-glycosides are the predominant form of modified flavonoid in citrus plants. Such modification can result in changes of physical properties, such as solubility, transportability, and stability (Yáñez et al. 2012). Glycosylation is thought to play vital roles in the modification of biological activities and the accumulation of flavonoids in citrus. For example, the flavanone diglycoside naringin is a major compound associated with a bitter taste that is found in grapefruit and pummelo (Berhow et al. 1998; Owens and McIntosh 2011). The bitter taste of naringin is due to its glucose moiety, which could protect the plants from herbivores or insects (McIntosh and Owens 2016). In oranges, the non-bitter flavanone diglycoside hesperidin accumulates to high levels and can result in the production of 'cloudiness' in juice products, which can affect consumer acceptance (McIntosh and Owens 2016). In addition, in pigmented citrus varieties, the glucose molecule can be linked to anthocyanins through glycoside bonds at the C3 position to form the 3-Omonoglycoside anthocyanins. Junctions can also occur at C3 and C5 positions to produce 3,5-O-diglycoside anthocyanins, which seem to be more stable than their mono-glucosidated counterparts. Glycosylation at the C-3 position is necessary for the accumulation of flavonoids (Yáñez et al. 2012).

Methylation

Methyl groups have also been shown to have multiple functions in various studies. Methoxylation could significantly influence the physical properties of flavonoids, including decreasing solubility and improving stability and transportability (Yáñez et al. 2012). Based on their physical properties, methoxylated flavonoids exert excellent physiological regulation in plants. Notably, polymethoxyflavones, such as nobiletin and tangeretin, display antifungal activity against the known plant pathogens *Fusarium moniliforme*, *Sclerotium rolfsii*, and *Verticillium albo-atrum*. The methylation pattern of the B ring of anthocyanidins can directly affect hue and color stability, with an increase in methylation causing redness (Yáñez et al. 2012).

Health-promoting functions of citrus-derived flavonoids

Citrus-derived flavonoids not only play vital roles in the regulation of plant physiological conditions, but also have various health-promoting functions, such as the prevention of cancer, inflammation, atherosclerosis, and neurodegenerative diseases, in human body. They have become important resources for the development of bioactive compounds in functional food. The functions of food components depend on their chemical structures. Herein, we present the health-promoting functions of different types of flavonoids (flavanones, flavones, and flavonols).

Health-promoting functions of flavanones

Flavanones are present in glycoside and aglycone forms in citrus plants. Naringenin and hesperetin are the most important aglycone forms, and they can be glycosylated with neohesperidose and rutinose to form naringin, neohesperidin, hesperidin, and narirutin. In recent decades, many studies have focused on the protective effects of citrus flavanones against diseases (Table 1). Among the various reported beneficial effects, citrus flavanones have been shown to exhibit antioxidant activity, a foundation of many other bioactivities, by scavenging free radicals (Gulcin 2012). The antioxidant activity of flavanones extremely dependents on the number and spatial arrangement of hydroxyl groups, including the catechol on the B ring, the presence of glycoside (glycosides or aglycones), and the form of hydroxyls (free or substituted) (Di Majo et al. 2005; Khan, Zill-E-Huma, and Dangles 2014). Generally, the more the hydroxyls is, the more potent the antioxidant activity, and catechol on the B ring confers higher antioxidant activity. The glycosylation of hesperetin on the C7-OH group by neohesperidose affects antioxidant activity, whereas glycosylation by rutinose has no effect, suggesting that antioxidant activity varies among glycosyl moieties (Di Majo et al. 2005). Specially, hesperidin shows more potent antioxidant activity than hesperetin in different antioxidative models (DPPH radical scavenging, superoxide anion scavenging, reducing power and metal chelating effect) (Yang et al. 2012).

Table 1. Health promoting effects and corresponding mechanisms of citrus flavanones.

Flavanones	Anti-oxidant	Anti-inflammation	Anticancer	Anti-atherosclerotic effects	Vasodilatation effects
Naringenin	Inhibiting ROS production.	1. Inhibition of COX-2 and iNOS expression 2. Inhibition of NF-κB, MAPK and TLR4/ NF-κB pathways	1. Inducing cell cycle arrest in G0/G1 phase and apoptosis 2. Inhibiting cell proliferation 3. Inhibiting or down-regulating NF-κB, MMPs, Bcl-2, VEGF, CYP1A1, PI3K, MEK, TGF-β, PKC, PCNA, Akt, ROS, and β-catenin/ Tcf signaling pathway 4. Increasing the expression of Bax, P53, caspase-3 and ASK1	1. Activating Ras/Raf/ERK signaling pathway, hemeoxygenase-1 2. Inhibiting VSMCs proliferation, invasion and migration 3. Inhibiting or down-regulating expression of ICAM-1 and MMP-9, PI3K/ Akt signaling pathway, platelet-derived growth factor, angiotensin II, NF-κB, Ap-1, NADPH oxidase and ACAT activities, ROS production, phosphorylation of $ERK^{1/2}$, neointimal macrophage infiltration, fatty streak formation, and VCAM-1 and MCP-1 genes 4. Reducing cholesterol levels via HMG-CoA reductase, ACAT activities and atherogenic index and apoB secretion	1. Activating BK_{Ca} channels 2. Improving endothelial dysfunction by regulating ROS/ caspase-3 and NO pathway 3. Inhibiting phosphodiesterase isoenzymes
Hesperetin	1. Inhibiting ROS production 2. Inhibiting xanthine oxidase		1. Inducing cell cycle arrest at G1 phase 2. Inhibiting or down-regulating MAPKs, cyclins, GLUT1, GLUT4, IR-β, Akt, cyclin D1, CDK4, Ncl-xL and HER2 3. Activating caspase-8, caspase-9, Bax, p21, p57Kip2 and (ASK1)/JNK 4. Increasing or up-regulating intracellular ROS, ATP and Ca^{2+}	1. Stimulating LDLr gene expression 2. Increasing phosphorylation of PI3K and $ERK^{1/2}$ 3. Inhibiting platelet-derived growth factor and angiotensin II 4. Lowing LDL-cholesterol 5. Reducing apoB secretion 6. Inhibiting platelet aggregation	1. Activating K^+ channels 2. Enhancing eNOS expression and Kv channels function 3. Inducing Akt, AMPK and ER-α 4. Inhibiting or down-regulating thrombogenic plasminogen activator inhibitor-1 and phosphodiesterase isoenzymes
Naringin	1. Inhibition ROS production 2. Increase the gene expressions of antioxidant enzymes, and enhancing SOD and catalase		1. Inhibiting cell proliferation, inducing cell apoptosis and G1 cycle arrest 2. Suppressing DNA damage 3. Activating or up-regulating caspase-8, caspase-9, MAPKs and p21 4. Inhibiting MAPKs, AP-1, NF-κB, IKKs, HER2, IκB, (GSK)-3β, APC/β-catenin, PI3K/Akt/mTOR cascade, cyclin D1/CDK4, cyclin E-CDK2, Akt and MMP-2 5. Alterating glycolipids by suppressing EGFR	1. Activating Ras/Raf/ERK signaling pathway 2. Inhibiting VSMCs proliferation, invasion and migration 3. Inhibiting MMP-9 expression, PI3K/Akt signaling pathway 4. Suppressing NF-κB and Ap-1 binding activities 5. Reducing cholesterol levels via inhibiting HMG-CoA reductase and ACAT activities 6. Reducing secretion of apoB and expression of VCAM-1, and cell adhesion molecules and ICAM-1 7. Inhibiting platelet aggregation	Modulating inflammation, oxidative stress, apoptosis and MAPK pathway
Hesperidin	Inhibiting ROS production	Inhibiting overexpression of COX-2, iNOS, PGE2, TNF-α and IL-1β	1. Inducing growth arrest and apoptosis 2. Inhibiting $ERK^{1/2}$, MMP-9, AP-1, NF-κB, JNK, HGF, ACF, p38, Bcl-2, PI3K, mTOR, GSK-3β and Akt 3. Activating or up-regulating Bax, caspase-3 and mitochondrial pathway	Inhibiting ICAM-1 expression	1. Enhancing K_v channels function and NO production 2. Inhibiting ET-1 secretion

Flavanones effectively reduce inflammation by inhibiting several proinflammatory mediators. Hesperidin has been proven to inhibit the overexpression of COX-2 and iNOS, overproduction of PGE2 and NO in macrophage cells, and expression of TNF-α, IL-1β, and *i*NOS in the lung (Sakata et al. 2003; Yeh et al. 2007). *In vivo* studies have demonstrated that hesperetin metabolites (hesperetin sulfates/glucuronides) inhibit the LPS-induced expression of COX-2

and iNOS through the suppression of nuclear factor κB (NF-κB) activation in macrophages or smooth muscle cells, and are more effective than hesperetin and hesperidin (Yang et al. 2012). Naringenin and naringin have similar anti-inflammatory effects. Naringenin can inhibit the production of NO and proinflammatory cytokines (TNF-α and IL-1β), as well as the expression of *i*NOS and COX-1, which are mediated by suppression of cytokine signaling (SOCS)-3 activation through the adenosine monophosphate activated protein kinase (AMPK) and protein kinase C (PKC) signaling pathways (Chao et al. 2010; Vafeiadou et al. 2009; Wu et al. 2016). *In vivo* studies have shown that naringenin has stronger inhibitory effects towards jejunum contraction in Sprague/Dawley (SD) rats with TNBS-induced colitis than hesperetin, which demonstrates 4′-hydroxy is the essential group. Similar effects can be also found towards LPS-induced pro-inflammatory cytokines in RAW264.7 Cells (He et al. 2018).

Cancer is a leading cause of death worldwide, and the development of efficient anticancer drugs is an important task. Numerous studies have provided evidence for the anti-cancer bioactivities of citrus flavonoids, especially flavanones and PMFs (Cirmi et al. 2016). Hesperetin induces apoptosis by regulating MAPKs and cyclins in A431 human skin cancer cells, and naringenin exerts the same anticancer effect by inducing the generation of reactive oxygen species (ROS) and G0/G1 phase arrest (Smina et al. 2015; Ahamad et al. 2014). Naringenin inhibits HepG2 cells by inhibiting or down-regulating NF-κB, vascular endothelial growth factor (VEGF), and matrix metalloproteinases (MMPs), activating apoptosis-related proteins (e.g., p53, Bcl-2, Bax, and caspase-3), or inducing G0/G1 and G2/M phase arrest (Subramanian and Arul 2013; Arul and Subramanian 2013). Naringenin also displays many other anti-cancer bioactivities, such as lung, liver, breast, gastric, colon, and prostate cancers (Yi, Ma, and Ren 2017). Although the molecular mechanisms may differ, naringin, hesperidin, and hesperitin have also been shown to inhibit various cancers (Yi, Ma, and Ren 2017).

Epidemiological studies have demonstrated the association between the intake of flavonoid-containing food and decrease in cardiovascular disease. Numerous studies have documented the anti-atherosclerotic, antihypertensive, and vasoprotective effects of citrus flavonoids, flavanones (hesperidin, hesperetin, naringin, and naringenin), and PMFs (Mulvihill, Burke, and Huff 2016; Li and Schluesener 2017; Yi, Ma, and Ren 2017). The anti-atherosclerotic effects can be attributed to the protection of endothelial cells, inhibition of vascular smooth muscle cells (VSMC) proliferation and migration, lipid metabolism regulation, low-density lipoprotein oxidation suppression, and platelet aggregation reduction. Among citrus flavanones, hesperidin has been most frequently reported to have antihypertensive effects. The hypotensive effect of hesperetin has been suggested to be associated with NO-mediated vasodilation (Yamamoto et al. 2008b). Hesperidin exerts a hypotensive effect through the inhibition of phosphodiesterase isoenzymes in the vasculature (Yamamoto et al. 2008a). Further investigations have shown that it can also alter the gene expression of vascular regulatory molecules (e.g., NADPH oxidase and thromboxane A2 synthase) (Yamamoto et al. 2013).

Health-promoting functions of flavones

PMFs exist almost exclusively in citrus, especially in the fruit peels. They exhibit a wide range of bioactivities and have attracted particular interest. In contrast to other flavonoids with free hydroxyl groups, PMFs have multiple methoxyl groups, which gives them a less polar and approximately planar structure, and consequently high degree of biological membrane permeability and greater membrane transport ability. Therefore, PMFs exhibit more potent bioactivities (Gao et al. 2018). For example, tangeretin exerts a greater antiproliferative effect than baicalein (Benaventegarcía and Castillo 2008). However, the presence of methoxyl groups also leads to low water solubility, causing slower dissolution and dramatically limiting absorption in the small intestine. Hydroxylated PMFs (OH-PMFs) have similar permeability and membrane transport ability to those of PMFs. Because hydroxyl groups improve solubility, OH-PMFs are more water soluble than their fully methoxylated counterparts. Generally, the bioactivities of flavonoids, especially their antioxidative activity, anti-inflammatory and cytotoxic effects, depend significantly on hydroxyl groups, whereas methoxyl groups are associated with interactions with proteins, cellular membranes, and enzymes. OH-PMFs combine the advantages of methoxyl and hydroxyl groups, and various studies have shown that OH-PMFs are more potent for disease prevention than are their permethoxylated counterparts (Li et al. 2007; Wu et al. 2015a, b; Zheng at al. 2013).

The health-promoting functions (e.g., antioxidant, anti-inflammatory, anti-atherosclerotic, and anti-diabetic effects) of PMFs have been studied extensively in recent years, as have the corresponding mechanisms (Table 2). PMFs have been shown to inhibit ROS production (Yoon et al. 2011; Zhang et al. 2018) and improve the activity of antioxidant enzymes, including superoxide dismutase, catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR) (Sundaram, Shanthi, and Sachdanandam 2015; Gao et al. 2018). Some OH-PMFs, such as 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone and 5-demethylnobelitin, exhibit 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging activity at levels more potent than those of nobiletin (Hamdan et al. 2011). This activity might be attributable to the hydrogen-donating ability of OH-PMFs. The anti-inflammatory mechanisms of PMFs include inhibition of the production of inflammatory mediators (e.g., COX-2, iNOS, IL-6, IL-1α, IL-1β, and TNF-α) and PGE_2 (Pan, Lai, and Ho 2010; Arab et al. 2016; Chen, Tait, and Kitts 2017; Wu et al. 2017), suppression of the enzymes involved in mitogen-activated protein kinase (MAPK) pathways (e.g., proMMP-1, proMMP-3, and MMPs) (Lai et al. 2008), and regulation of the NF-κB level (Shin et al. 2012) . Various studies, including *in vivo* and *in vitro* models, have demonstrated nobiletin and its demethylated metabolites exhibit anti-inflammatory potency in the order of 3′-

Table 2. Health promoting effects and corresponding mechanisms of citrus polymethoxyflavones.

Polymethoxyflavones	Anti-oxidant	Anti-inflammation	Anticancer	Anti-atherosclerotic effects	Anti-diabetic effects
Nobiletin		1. Inhibiting NO production 2. Suppression of proMMP-1, proMMP-3, COX-2, iNOS, IL-6, IL-1α, IL-1β, TNF-α, PGE2, and PGE3	1. Inhibiting HGF-induced cell invasion, angiogenesis and migration through inhibiting ERK^2 and Akt 2. Activating apoptotic process, and cell cycle arrest at G0/G1 and G2 phase 3. Inhibiting EMT, CXCR4, MMP-9, MMP-2, TMK-1, PI3K, Bcl-2, Akt, and NF-κB 4. Activating Bax	1. Inhibiting platelet-derived growth factor, angiotensin II, and platelet aggregation 2. Reducing MTP activity, diacylglycerol acyltransferase expression, VLDL-triglyceride secretion and acetylated LDL uptake 3. Reducing apoB100 by activating MAPKerk, enhancing LDLr expression 4. Inhibiting proliferation and migration by suppressing PI3K/Akt signaling pathway	1. Improving hyperglycemia and insulin resistance 2. Regulating Glut1, Glut4 and adipokines 3. Impairing lipid homeostasis by activating MAPK, ERK signaling and decreasing DGAT1/2 mRNA expression
Tangeretin	1. Inhibiting ROS production 2. Improving SOD, CAT, GR and GPx	1. Inhibiting NO production 2. Suppression of MMPs, PGE3 and COX-2 3. Inhibiting IL-6, IL-1α, IL-1β and TNF-α.	1. Inhibiting invasion and inducing cell cycle arrest at G1 phase 2. Inhibiting PI3K, EMT, notch-1, CDK2, CDK4, and Akt 3. Activating caspase-3, miR-410 p21, p27 and p53	Inhibiting proliferation and migration by suppressing PI3K/Akt signaling pathway	1. Stimulating glucose uptake by regulating AMPK pathways 2. Reverting enzymes of carbohydrate metabolism to normal levels
Sinensetin		Inhibiting inflammation genes expression by regulating NF- κB	1. Anti-angiogenesis by cell cycle arrest at G0/G1 phase 2. Down-regulating mRNA expression of flt1, kdrl and hras		
3,5,6,7,8,3′,4′-hepta-methoxyflavone		Inhibiting IL-6, IL-1α, IL-1β and TNF-α		1. Reducing SR-A expression and ox-LDL uptake 2. Blocking foam cell formation	
5-hydroxynobiletin	Free radical scavenging	Inhibiting IL-6, IL-1α, IL-1β and TNF-α	1. Inducing G2/M cell cycle arrest and apoptosis 2. Decreasing IL-1, IL-6, iNOS and COX-2 3. Regulating caspase cascade, p21, p53, CDK2, CDK4, JNK and Rb	Inhibiting expression of CD36, SR-A and scavenger receptors	
5-hydroxytangeretin			1. Inducing G2/M cell cycle arrest and apoptosis 2. Down-regulating expression of CDK2 3. Regulating p53, p21 and Bax		
5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone	Free radical scavenging	Suppressing MAPKs, PI3K/Akt and NF-κB.	1. Inducing cell cycle arrest and apoptosis by p53, Bax and p21 2. Reducing iNOS, Mcl-1, COX-2 and K-Ras	1. Reducing SR-A expression and ox-LDL uptake 2. Blocking foam cell formation	

demethylnobiletin >3′,4′-demethylnobiletin >4′-demethylnobiletin > nobiletin (Li et al. 2007; Wu et al. 2017). Among their health-promoting properties, the anticancer activities of PMFs have been studied most extensively (Song et al. 2016; Qiu et al. 2017; Song et al. 2017; Wu et al. 2015c; Zheng et al. 2013; Charoensinphon et al. 2013). Extensive studies demonstrate similar structure-activity relationship towards anticancer to anti-inflammatory effect of PMFs (Zheng et al. 2013; Wu et al. 2015c; Qiu et al. 2011). PMFs exhibit anticancer effects though various mechanisms (Gao et al. 2018). Most commonly, they can induce cell cycle arrestat different phases (G0/G1, G1, G1/S, and G2/M) and apoptosis (Wu et al. 2018; Lien et al. 2016; Arivazhagan and Pillai 2014; Ma et al. 2014; Morley, Ferguson, and Koropatnick 2007). Further investigations have demonstrated that they are associated with the regulation of some key signal kinases, including the up-regulation of p53/p21 and down-regulation of cyclin D1, CDK2, CDK4, Akt, and

MAPK. Many other mechanisms, such as the reduction of IL-1, IL-6, iNOS, and COX-2 (Song et al. 2014) levels; activation or up-regulation of caspase-3, miR-410 p21, p27, p53, and Bax (Dong et al. 2014; Qiu et al. 2010, 2011); and inhibition or down-regulation of MMP-1, MMP-7, MMP-9, and NF-κB (Walle 2007; Chien et al. 2015), have also been identified. The anti-atherosclerotic effects of citrus PMFs have also been reported widely. The mechanisms are associated with the inhibition of platelet-derived growth factor, angiotensin II, and platelet aggregation (Manthey et al. 1999); suppression of the expression of diacylglycerol acyltransferase, LOX-1, CD36, SR-A, and scavenger receptor (Eguchi et al. 2006); reduction of very-low-density lipoprotein (VLDL)-triglyceride secretion (Whitman et al. 2005); and inhibition of SR-A mRNA expression and ox-LDL uptake (Eguchi et al. 2007; Kou et al. 2013). In addition, PMFs have excellent anti-diabetic effects through the improvement of hyperglycemia and insulin resistance; stimulation of glucose uptake by the regulation of AMPK signaling pathways (Kim et al. 2012); regulation of Glut1, Glut4, and adipokines (Lee et al. 2010); reduction of enzymes involved in carbohydrate metabolism to normal levels (Sundaram, Shanthi, and Sachdanandam 2015); impairment of lipid homeostasis by activation of MAPK; extracellular signal-regulated kinase (ERK) signaling (Mulvihill et al. 2011); and reduction of DGAT1/2 mRNA expression (Mulvihill et al. 2011).

Health-promoting functions of flavonols

Although the content is significantly less than flavanones and flavones, flavonols (e.g., quercetin, kaempferol, and myricetin) are beneficial functional components of citrus due to their multiple health-promoting functions, including antioxidant, anti-inflammation, anti-cancer, cardiovascular disease preventive (vasoprotective, antihypertensive, and anti-platelet aggregation effects), antidiabetic, neurodegenerative disorder preventive, and gastroprotective effects (Survay et al. 2011; Heiss, Keen, and Kelm 2010). The mechanisms of anti-cancer effects include free radical scavenging (Lakhanpal and Rai 2007); down-regulation of the expression of MMP-9, NF-κB, and COX-2 (Sivaramakrishnan and Devaraj 2009; Jung et al. 2010) ; upregulation of metalloproteinase-9 (Hwang et al. 2009); and arrest of the G2/M cell cycle (Zhang et al. 2009). Citrus-derived flavonols exert anti-inflammatory effects by suppressing GRP78 expression (Natsume et al. 2009), regulating arachidonic acid metabolism (Hernández et al. 2007), and modulating prostanoid synthesis and cytokine production (TNF-α, PGE2, MIP-2, and RANTES) (Survay et al. 2011). These functions of flavonols are due mainly to their antioxidant properties, which depend on the number and spatial arrangement of hydroxyl moieties (Survay et al. 2011).

In summary, citrus-derived flavonoids play important roles in the prevention of various diseases. Their functions dramatically depend on their chemical structures, especially the presence of hydroxyl groups, including their number, spatial arrangement, free or substituted status, presence or absence of glycoside, and full methoxylation. The order of potency for vasoprotective effects is flavonols > flavones > flavanones (Ajay et al. 2003), which demonstrates the structure of the nuclear skeleton also significantly influences the health-promoting functions. These effects might be attributable to the 2,3-double bond and 3-hydroxyl group on B ring. Investigation of the biosynthesis of citrus-derived flavonoids is of critical importance to enable specific flavonoids, with excellent disease prevention functions, to be obtained.

Conclusion and prospective

Due to their various significant health-promoting properties for humans and their regulation of physiological conditions of plants, citrus-derived flavonoids have been subjected to extensive research. The functions of citrus flavonoids strongly depend on their chemical structures that are determined by their biosynthesis pathway. Flavonoids in citrus are biosynthesized through the phenylpropanoid pathway. Generally, the synthesis occurs in three major stages: synthesis of a precursor (*p*-coumaroyl-CoA), synthesis of flavonoids (flavanones, flavonols, flavones, and anthocyanins), and glycosylation. The entire biosynthesis process is influenced by many factors, including precursors, enzymes (content and activities), and environmental factors (e.g., UV light, amino acid residues, metal mineral elements, hormones, water stress, and pathogen attack). The biosynthesis process is almost entirely enzymatic, and enzymes are thus the most important factors, as they significantly determine the chemical structures and accumulation of flavonoids in the plants. Flavonoid biosynthesis in citrus plants involves various chemical reactions, including hydroxylation, glycosylation, and methylation. These reactions significantly influence the physicochemical properties, and thus affect the physiological functions of flavonoids in plants, including color changes in flower and fruit, flavor development, and anti-stress physiology. More importantly, the changes in the chemical structure are of special significance for health-promoting functions in humans. A comprehensive understanding of the biosynthesis (the whole process, intracellular location, distribution, influencing factors, and corresponding significance) and health effects of flavonoids would provide scientific guidance for the development of healthy citrus foods and health-promoting products containing citrus flavonoids. Recent breakthroughs in biotechnologies, especially sequencing technology, greatly facilitate biosynthesis studies of phytochemicals. Along with the development of leading-edge multi-omics data, integration of multiple omics, including genome, transcriptome, metabolome and proteomic techniques, has been efficient strategy to discover key genes participating in the important biosynthesis processes, such as synthesis, regulation and transport. With better understanding of the biosynthesis pathway, metabolic locations, and influencing factors, the content of certain bioactive flavonoids can be improved by modulating their biosynthesis with biotechnology or changing the environmental conditions of the plants for enrichment of bioactive flavonoids. More importantly, these would provide scientific guidance for the production of high-value metabolites and their

development of industrial applications in pharmaceuticals, food, health products, dyes, and cosmetics.

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ORCID

Chengying Zhao http://orcid.org/0000-0002-9862-5089
Hang Xiao https://orcid.org/0000-0001-6194-2796
Jinkai Zheng http://orcid.org/0000-0002-8166-0048

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