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The Flavonol-Anthocyanin Pathway in Blackberry and Arabidopsis: State of the Art

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Additional information is available at the end of the chapter

<http://dx.doi.org/10.5772/67902>

Abstract

Flavonols and anthocyanins are plant secondary metabolites with an increasing interest due to their beneficial effects on human health. They are present in all plants, participating in plant protection against biotic and abiotic stresses. However, only some plant species accumulate them in relevant amounts, as is the case for berries. Among the health benefits reported is prevention of metabolic syndrome, including prevention of insulin resistance associated to type 2 diabetes. Therefore, there is a big interest to improve contents on plant foods to benefit health through the diet, as well as to obtain them for functional ingredients for food supplements. In fulfillment of this objective, a deep study about their biosynthetic pathway has been carried out in model plants, where the genome is available. However, not all species that accumulate them in high amounts have their genome sequenced, as is the case for blackberry. Transcriptomic approaches have been undertaken to gain knowledge of its specific biosynthetic pathway and regulatory elements, aiming to improve bioactive contents in the edible parts. Furthermore, determining the regulatory pathways will help to improve yields and in vitro production. For this purpose, a review on elicitors used to trigger this pathway is presented.

Keywords: flavonols, anthocyanins, blackberry, Rubus, Arabidopsis, elicitors, transcription factors (MYB)

1. Introduction

Blackberry fruits are an important source of bioactive compounds, among which are flavonols, anthocyanins, and catechins. These compounds exhibit beneficial effects on human health, which is the main reason they have become so popular nowadays, especially because they are easy to include in diets, at least in developed countries where incidence of pathologies is high. Among these health benefits are their general antioxidant effects, as all these compounds can scavenge reactive oxygen species (ROS), anti-inflammatory and antimicrobial effect, glucose metabolism, and leukocyte migration (effects in both inflammation and cancer) [1, 2]. Catechins and anthocyanins have also proved their ability to improve endothelial function, hypertension, coronary heart disease, obesity, insulin resistance, as well as glucose and lipid metabolism [3, 4]. Hence, including blackberry on human diet is beneficial for health, since a high content in flavonoids has been reported, and, in addition to its beneficial effects, it lacks adverse or secondary effects. Moreover, these compounds are also responsible for major organoleptic, nutritive, and processing characteristics of feed, food, and beverages, and impact many agronomical crop traits [5, 6].

In addition to the relevance for human health, flavonols, anthocyanins, and catechins play a key role on plant physiology. As secondary metabolites, they play a wide variety of functions, mainly helping the plant to adapt to the environment, among which the following have been reported, namely (i) UV radiation and oxidative stress protection, (ii) pathogen interactions (pathogen resistance), (iii) protection from herbivore, (iv) allelopathy, (v) nodulation (symbiosis), (vi) auxin transport, and (vii) they also attract different organism for pollination because of the color of the flower [1, 7, 8].

Despite the agricultural and biological importance of the genus *Rubus*, knowledge of their genetics and genome is very limited. Hence, in order to gain knowledge about the metabolism of these compounds, different approaches need to be undertaken, including the physiological, metabolic, and transcriptomic levels. One of the most important factors affecting the quality of the fruit is its content in phenolic compounds [6]. The synthesis of these compounds mainly depends on the phenylpropanoid and its derivative pathways, which starts with the phenylalanine, as many others (**Figure 1**). Furthermore, validation of the mechanisms controlling this biosynthetic pathway needs to be referred to model plants; among which, the most commonly used is *Arabidopsis thaliana*. However, the use of *Arabidopsis* is limited since it lacks edible fruits, so for studies related to fruits, either strawberry or tomato is used as reference.

Rubus sp. is a woody plant that belongs to *Rosaceae* family, in which other plants such as strawberry (*Fragaria vesca* L.), raspberry (*Rubus idaeus* L.), cherry tree (*Prunus avium* L.), or apple (*Malus domestica*) among others are enclosed; all of them belong to a wide group of plants commonly called *berries*. They are known because of their high accumulation of secondary metabolites in the fruit, which behave as functional components, beneficial for human health. Those compounds are mainly polyphenols like flavonols, anthocyanins, and catechins, strong natural antioxidants [9–11].

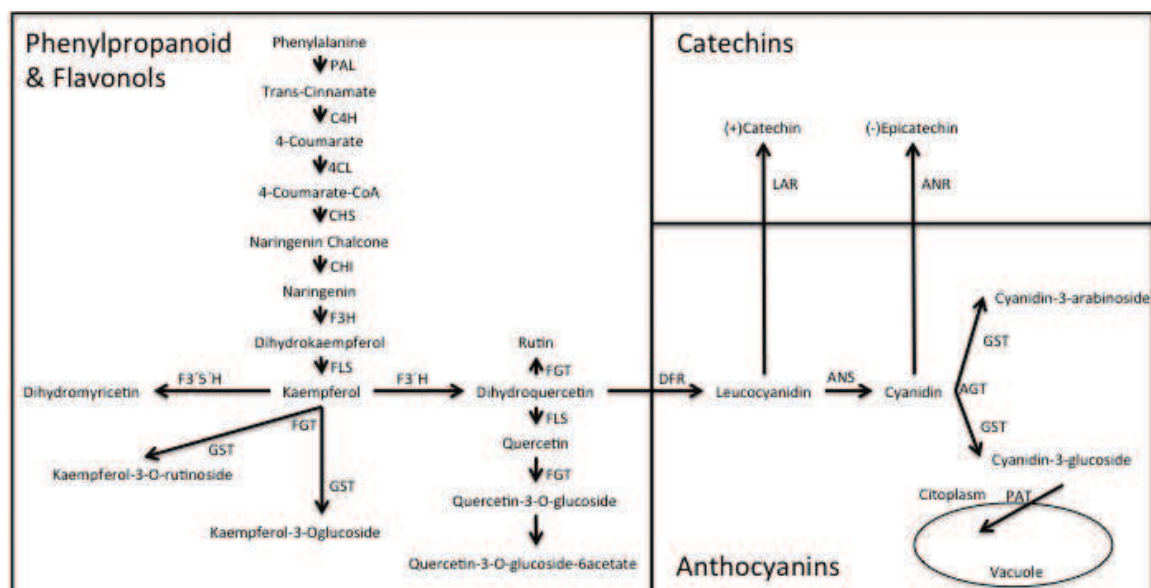


Figure 1. Phenylpropanoid pathway.

Rubus L. genus is composed of 600–800 species approximately, distributed all around the world in very different habitats, and classified into 12 different subgenus that are in turn divided into different groups. The most abundant species in Europe is the *Rubus* spp. subgenus (Eubatus Focke) that is divided in more than 130 species; their natural habitat and distribution is in the woods (**Table 1**).

Subgenus	Number of species
<i>Anoplobatus</i> (Focke)	6
<i>Chamaebatus</i> (Focke)	5
<i>Chamaemorus</i> (Hill)	1
<i>Comaropsis</i> (Rich.)	2
<i>Cylactis</i> (Raf.)	14 (4 series)
<i>Dalibarda</i> (L.)	5
<i>Dalibardastrum</i> (Focke)	4
<i>Idaeobatus</i> (Focke)	117 (9 sections)
<i>Lampobatus</i> (Focke)	10
<i>Malachobatus</i> (Focke)	115 (7 sections)
<i>Orobatus</i> (Focke)	19
<i>Rubus</i> L. (=Eubatus Focke)	132 (6 sections)

Table 1. *Rubus* subgenus.

Plants that belong to the subgenus *Rubus* spp. are typical wild species and are usually hand-picked in the season. However, since it became an important plant for agriculture, there has been an increasing interest in improving the size of the fruit, the organoleptic properties, fruit yield, and get rid of the thorns, since they constitute a nuisance for harvest. To achieve these objectives, classic crossbreeding has resulted in development of many commercial varieties to favor a given trait that benefits production in each geographical location. Among these cultivars are “Ashton Cross” that is vigorous and thorny, “Bedford Giant” that in addition to these two traits shows a good yield; “Black satin,” also vigorous but thornless; “thornless evergreen” that provides a thornless plant, high yield and high quality fruits; “Fantasia” that produces very large fruits and finally in this shortlisted group is “Loch Ness,” that is a thornless cultivar with very large fruits and semierect canes, which is the cultivar used in this study (Table 2) [12].

Rubus spp. Var. Loch Ness is a high yielding thornless tetraploid ($4n = 28$) blackberry, and one of the most widely cultivated varieties. However, despite its high-added economic value and as a source of bioactive compounds, its genome has not been sequenced yet. Therefore, other strategies need to be used to gain knowledge of the production and health-related benefits.

The aim of this chapter is to review the literature about blackberry and report the state of the arts about this plant species. As the genome is not reported, data about the core genes in the biosynthetic pathway as well as regulatory genes are referred to as the model plant *A. thaliana*. Also, structure of the bioactives which is responsible for health benefits as well

	Variety	Anthocyanins (mg eq cyanidin/100g FW)	Flavonols (mg eq catechin/100g FW)
Thorny	Darrow	99.33 ± 48.32	4.23 ± 0.48
	Early Wilson	64.76 ± 23.68	3.21 ± 0.38
	Gazda	27.97 ± 13.71	6.52 ± 0.82
	Lesniczanka	96.63 ± 32.18	5.36 ± 0.45
	Zagroda	143.66 ± 52.59	4.98 ± 0.52
Thornless	Black Beaty	179.46 ± 57.84	3.06 ± 0.40
	Black Satin	175.52 ± 53.97	1.58 ± 0.31
	Chester Thornless	200.34 ± 65.58	3.68 ± 0.46
	Hull Thornless	105.39 ± 31.08	0.82 ± 0.35
	Loch Ness	220.11 ± 81.07	2.39 ± 0.28
	Orkan	142.42 ± 44.01	2.26 ± 0.25
	Smoothstern	186.55 ± 58.94	2.32 ± 0.33
	Tayberry	177.84 ± 56.20	1.7 ± 0.32
	Thornless	147.46 ± 44.02	1.07 ± 0.33

Table 2. Phenolic compound contents of thorny and thornless blackberries (mg/100g FW) [13].

as their qualitative and quantitative composition in berry fruit and in model plant are presented, so a relationship between composition and core and regulatory genes description is envisaged and vice versa. Finally, the physiological role of these secondary metabolites for plants is also presented, and finally highlights the relevance of this pathway of plant secondary metabolism, as well as its biotechnological potential.

2. Chemical structure

Flavonols, anthocyanins, and catechins are molecules belonging to a wider group of secondary metabolites, the flavonoids. Flavonoids represent a large subgroup of a phenolic class of plant specialized metabolites, which are found in almost every plant in the nature. The basic flavan skeleton that forms all flavonoids is a 15-carbon phenylpropanoid core (C6-C3-C6 system), which is arranged into two aromatic rings (A and B) linked by a heterocyclic pyran ring (C). They are characterized by the presence of a double bond between C-2 and C-3, and the attachment of the B ring to C-2. According to the oxidation status and saturation of the heterocyclic ring, flavonoids are categorized into flavonols, flavones, catechins, flavanones, anthocyanins, and isoflavonoids [1]. The most abundant compounds present in blackberry (also in berries) are flavonols, anthocyanins, and catechins (**Figure 2**).

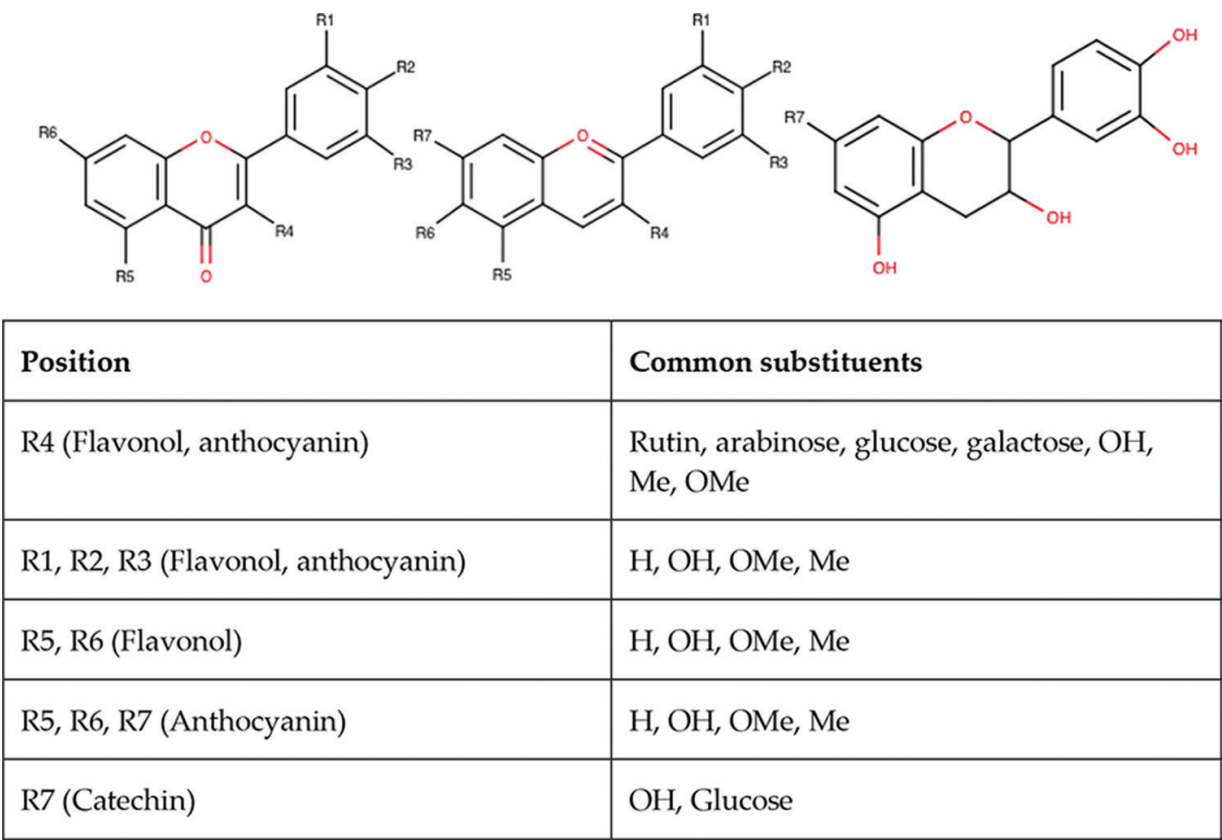


Figure 2. Flavonol, anthocyanin, and catechin molecular structures and common substituents.

Flavonols have a 3-hydroxyflavone (IUPAC name: 3-hydroxy-2-phenylchromen-4-one) as the main structure. The diversity of these compounds is derived from the different positions of the hydroxyl groups of the phenolic ring that are usually glycosylated and can undergo further modifications like acylations; in this group, the three main families are derived from kaempferol (4'OH), quercetin (3', 4', 5'OH) and rutin (3', 4'OH).

Anthocyanins are mainly glycosylated as well, being the aglycon the anthocyanin molecule. The chemical structure of this aglycone is the flavylum ion (2-phenyl-benzopyrilo) that has a benzopyran aromatic ring, and a phenolic ring. There are six different families within this group, namely cyanidin, pelargonidin, delphinidin, malvinidin, peonidin, and petunidin. As in the case of flavonoids, the greatest source of chemical diversity is the number and position of sugars for glycosylation. Acylation is another main biochemical mechanism leading to diverse anthocyanin molecules in *Arabidopsis* [14, 15]. Up to date, several enzymes have been characterized to catalyze these acylation reactions, using either malonyl-CoA or *p*-coumaroyl-CoA as substrates to transfer the malonyl or *p*-coumaroyl groups to cyanin structures [16]. Diversity can be further increased transferring sinapoyl groups to cyanins to form sinapoylated cyanins [17].

Catechins have two benzene rings (A-, B-) and a dihydropyran heterocyclic ring (C) with a hydroxyl group over carbon 3. As a result of this structure, catechins have four diastereoisomers, two with *trans* configuration called catechin ((+)-catechin and (-)-catechin), and two with *cis* configuration called epicatechin ((+)-epicatechin and (-)-epicatechin). These catechins can further polymerize to form proanthocyanins, in which the diversity of structures relies on the number of monomers that polymerize and the type of bonds that stabilize them.

3. Biosynthetic pathway and regulation

Biosynthesis of the flavone backbone is originated from the phenylpropanoid pathway followed by the flavonoid biosynthetic branch (**Figure 1**). The phenylalanine ammonia-lyase (PAL) deaminates the phenylalanine, being converted into *trans*-cinnamate, after that a hydroxyl group is introduced on the phenyl ring by cinnamic acid 4-hydroxylase (C4H), being the *trans*-cinnamate converted into 4-coumarate. The carboxyl group of *p*-4-coumarate is then activated to form 4-coumarate-CoA (by a thioester bond), catalyzed by 4-coumarate-CoA ligase (4CL). This product, 4-coumarate-CoA, is substrate for different enzymes, so it represents a branching point of the pathway to either stilbenes or flavonoids. In this case, 4-coumarate-CoA is then condensed with three units of malonyl-CoA by the chalcone synthase (CHS, first enzyme of the flavonoid pathway), forming the naringenin chalcone (flavonone), which is transformed into naringenin by the chalcone isomerase (CHI). Naringenin is hydroxylated by flavonone-3-hydroxylase (F3H) being converted in dihydrokaempferol, which is then hydroxylated by flavonoid-3'-hydroxylase (F3'H) and transformed in dihydroquercetin or by flavonoid-3'-5'-hydroxylase (F3'5'H) to form dihydromyricetin. Flavonols are synthesized at this point by the flavonol synthase (FLS), which introduces a double bond between C2 and C3 in either of the three above-mentioned molecules forming kaempferol, quercetin, or myricetin, respectively. Dihydroquercetin is reduced by dihydroflavonol reductase (DFR) to obtain leucocyanidin; similarly, dihydrokaempferol is transformed in leucopelargonidin

and dihydromyricetin in leucodelphinidin. Anthocyanins are synthesized at this point by the anthocyanidin synthase (ANS) obtaining cyanidin, pelargonidin, or delphinidin, respectively. Catechins include (+)-catechin and (–)-epicatechin; (+)-catechin is obtained when leucocyanidin reductase (LAR) reduces leucocyanidin, and (–)-epicatechin is obtained when anthocyanidin reductase (ANR) reduces cyanidin [1, 7, 8].

All these aglycons are highly apolar, so they are immediately glycosylated to increase polarity, in order to be stored in vacuoles or translocated throughout the plant, hence glycosyltransferases are very important for glycosylation as well as transport mechanisms. In *Arabidopsis*, three genes, *TT12*, *TT19*, and *AHA10*, have been functionally characterized to be associated with the transport of anthocyanins. However, these enzymes show different levels of specificity for flavonoids [18]. Anthocyanins are stored in the central vacuole of cells, so they need to be transported from the cytosol to the vacuole. Two major hypotheses have been proposed to solve this transport: either transporter-mediated or vesicle-mediated transport [19–21].

Plants have depicted a system in which all these enzymes are extremely well organized in the different compartments within cells, in order to improve efficiency of these natural products' synthesis. Successive enzymes are arranged in imaginary units termed metabolons, anchored to the ER membrane, ensuring channeling of the intermediate precursors in the complex without diffusing to the cytosol, avoiding metabolic interferences [18, 22].

A. thaliana is a good model species for the identification of genes controlling flavonoid metabolism (**Table 3**) [23], because all pathway core genes of anthocyanins have been molecularly, genetically, and biochemically characterized in this plant. On the other hand, it is amenable to both molecular and classical genetic analysis [24, 25].

On the other hand, homologues to all core genes in the flavonol-anthocyanin pathway have been identified in *Rubus* sp. Var Loch Ness [26]. Also, most genes corresponding to the MYB transcription factors have also been identified with similar functions. Interestingly, MYB12 [26] that was originally identified as a key flavonol-specific transcriptional activator in *A. thaliana* [27] and in

Enzyme	Regulator
CHS (chalcone synthase)	MYB12, MYB11, MYB111
CHI (chalcone isomerase)	MYB12, MYB11, MYB111
F3H (flavonol 3 hydroxylase)	MYB12, MYB11, MYB111
F3'H (flavonol 3'hydroxylase)	MYBL2
FLS (flavonol synthase)	MYB12, MYB111, MYB11
DFR (dehydroflavonol reductase)	TT2, TT8, TTG1, MYBL2
ANS (anthocyanidin reductase)	TT1, TT2, TT8, TTG1, TT16
ANR (anthocyanidin synthase)	TT2, TTG1
LAR (leucoanthocyanidin reductase)	TT2, TT8, TTG1

Table 3. List of the flavonol-anthocyanin pathway core and regulatory genes in *A. thaliana* [23].

other plant species such as tomato [28] has not been found in *Rubus* [26, 29] suggesting a different control mechanism of the flavonol-anthocyanin pathway in this plant species.

Currently, dihydrokaempferol and dihydroquercetin are the only two dihydroflavonol molecules identified in *Arabidopsis* [18]. Flavonoids have been analyzed using liquid chromatography-mass spectrometry (LC-MS) and/or nuclear magnetic resonance (NMR). Briefly, anthocyanins and glycosylated kaempferol flavonols are mostly found in leaves [17], whereas seeds contain epicatechin, PAs, and larger amounts of glycosylated quercetin flavonols [30, 31]. Interestingly, arabidopsis seeds contain large amounts of PAs similar to those present in other crop seeds or fruits (**Table 4**) [32–34].

Group	Compound
Flavonols	Quercetin-rhamnoside-hexoside
	Quercetin-hexoside-rhamnoside
	Quercetin-3-O-rhamnoside
	Quercetin-rhamnoside dimer 1
	Quercetin-rhamnoside dimer 2
	Quercetin-rhamnoside dimer 3
	Quercetin-rhamnoside dimer 4
	Quercetin-di-rhamnoside
	Quercetin-3-O-glucoside
	Kaempferol
	Kaempferol-rhamnoside
	Kaempferol-rhamnoside-hexoside
	Kaempferol-3, 7-di-O-rhamnoside
	Kaempferol-3-O-glucoside-7-O-rhamnoside
	Isorhamnetin-hexoside-rhamnoside
	Isorhamnetin-di-rhamnoside
Anthocyanins	Isorhamnetin-rhamnoside
	Procyanidin dimer
	Procyanidin trymer
	Procyanidin tetramer
	Procyanidin pentamer
	Procyanidin hexamer
Catechins	Procyanidin heptamer
	Epicatechin

Table 4. Compounds identified by LC-MS-MS in seed extracts of wild type *A. thaliana* [31].

The metabolic profile of flavonols and anthocyanins in blackberry fruits is formed by the flavonols kempferol and quercetin and their respective derivatives, while cyanidin derivatives are the unique anthocyanidins present. Interestingly, catechins and epicatechins are also present, especially upon fruit ripening [26, 35]. The specific composition for blackberries obtained from *Rubus* spp. Var Loch Ness appears in **Table 5**.

Group	Compound
Flavonols	Rutin
	Kaempferol-glucoside
	Quercetin-glucoside
	Kaempferol-rutinoside
	Quercetin-3-O-glucoside-6"-acetate
Anthocyanins	Cyanidin-3-glucoside
	Cyanidin-3-arabinoside
Catechins	(+)-Catechin
	(-)-Epicatechin
	Epicatechin isomer

Table 5. Compounds identified in *Rubus* spp. Var. Loch Ness fruit by LC-MS-IT-ToF [26].

4. Flavonol, anthocyanin, and catechin functions in plants

Plants are sessile organisms, due to this fact they have developed different methods for protection against the stressful conditions of the surrounding, including abiotic and biotic stimuli. The most important mechanism is the production of secondary metabolites, like flavonols, anthocyanins, and catechins [36, 37].

There is an increase in the production of these compounds under adverse or stressing conditions, such as intense UV radiation, heat, drought, and salt stress, presence of heavy metals, herbivores, insects, nematodes, etc., because reactive oxygen species (ROS), the natural products obtained from metabolic reactions, play a relevant role in cell signaling and homeostasis. In certain situations, as previously described, ROS levels can undergo a mild increase, triggering defensive responses as SAR or ISR [38], or suffer a dramatic increase that results in cell damage (lipids, DNA, and protein structures) if not controlled.

Living beings have different methods to get rid of these ROS, enzymatic and nonenzymatic; but if the amount is too high the organism cannot transform all of them, causing the damages described before. Phenylpropanoids and flavonoids, in which flavonols, anthocyanins, and catechins are enclosed, are the nonenzymatic antioxidants known to have high antioxidant activities, because of their capacity to directly quench ROS, thanks to the hydroxyl group

present in rings A and B; they also interfere over the enzymatic systems composed of cyclooxygenase (in animals only), lipoxygenase, glutathione S-transferase, and xanthine oxidase, which is the other system in charge of ROS removal, together with the SOD-APX and the ascorbate-glutathione cycle enzymes that contribute to ROS control [39]. Hence, these compounds are involved in fine tuning of defensive and adaptive metabolisms, integrating all the external information, to optimize plant energetic resources for survival.

Flavonoids are known to be nonessential regulators auxin transport, modulating different transporters such as PIN proteins, and the transporter superfamily (ABCB) [40–42] proteins involved in their transport along the plant. It has been demonstrated that changes in flavonols accumulation lead to changes in auxin transport, therefore changes in auxin distribution [43], and the corresponding changes in plant physiology.

4.1. Abiotic stress

Abiotic stress is defined as the negative impact caused by the nonliving factors in the plant. Under adverse conditions, like intense UV radiation, heat, drought, and salt stress, presence of heavy metals, etc., there is a high increase of the reactive oxygen species (ROS) that lead to signal transduction to activate plant defense or to oxidative damage, as described above.

The UV radiation causes a stressful situation for plants [44], which is handled in two ways. First of all flavonoids and other pigments present mainly in the outer parts of the plant (epidermis and mesophyll tissues) absorb and considerably reduce the amount of radiation; the second one would consist in decreasing the effect of ROS caused by the radiation by scavenging of ROS [7]. Among flavonols, the main compound related to light absorption is kaempferol 3-O-glucoside because of its monohydroxy B-ring, and the flavonol with the greatest antioxidant properties is quercetin 3-O-glucoside, because of its dihydroxy B-ring. It has been shown that upon different UV exposure, synthesis of phenolic compounds is increased [45]. This may be the primary mechanism of response, which can be followed by others such as accumulation of pigments or lignification processes. Hence, flavonoids and anthocyanins are involved in protection against oxidative stress due to high UV radiation.

The impact of drought and salt stress on flavonoid biosynthesis has been studied in *A. thaliana* [46, 47]. An increase of glycosides of quercetin, cyanidin, and kaempferol during drought stress has been reported, being kaempferol glycosides the most significantly increased [47]. Although the behavior of flavonoids during these types of stresses is still not well documented, this evidence their role against salt and drought stress.

These studies carried out in *A. thaliana* are very convenient to elucidate the mechanism of action of these flavonoids and to see the flavonoid profile. However they cannot be directly extrapolated to evaluate behavior in fruit production, fruit quality, or fruit endurance, as *A. thaliana* does not have edible fruits. For these purposes, other model plants are used, such as tomato or strawberry among the berries.

Concerning fruit quality, there is a great concern in the endurance of the fruit after harvest; it is one of the most important traits for commercial value and economic profit. The relationship

between the overripening and the antioxidant properties has been evaluated [48]. A study on tomato overexpressing AtMYB12, the transcription factor activating the flavonol anthocyanin pathway, showed a notable increase in flavonoid biosynthesis, as well as its antioxidant capacity. The high anthocyanin and high flavonol profiles resulted in a longer, more durable shelf life, comparing with control plants, indicating that the endurance is directly correlated with this profile. Based on this data, it seems that the overripening time is determined by the oxidative damage of the fruit under changing conditions [49]. Therefore, an increase in flavonoids and anthocyanins is related to better fruit quality during the postharvest period.

Another stress factor is the levels of heavy metals. As a consequence of industrial development, pollution with heavy metals has dramatically increased. Heavy metals toxicity can result from different mechanisms, the first one is the generation of ROS by Fenton reaction and autoxidation [7], blocking of essential functional groups in biomolecules, and displacement of essential metal ions from biomolecules. Cadmium and other metals provoke a depletion of GSH and inhibit mainly the glutathione reductase (among other enzymes implicated in the ROS cycle) [45]; in consequence, the plant has to increase dramatically other antioxidants such as flavonoids in order to keep a normal the normal homeostasis of the plant cells. Flavonoids are known to form specific union with heavy metals, providing a great adaptation method to heavy metals toxicity autoxidation [7]. Based on these characteristics, some applications derived from these studies have been proposed to improve survival of plants in hostile environments, for example, increasing flavonoid synthesis to allow plant growth in the presence of heavy metals, so soil detoxification can be achieved by phytoremediation [50].

4.2. Biotic stress

Flavonoids are important molecules for plant adaptation under adverse conditions, among which defense to biotic stress is included. These molecules have a nonspecific mechanism of action; their effect is partly derived from their antioxidant properties, because of the ROS generated by plants when they are attacked by some pathogen. Flavonoids are involved in the earliest defense mechanism and the programmed cell death, and they have been found in necrotic and adjacent cells to pathogen invasion in the hypersensitive response [46, 51].

Their role in defense is not limited to the hypersensitive response, since consistent with their ability to chelate metals, they are able to inhibit some pathogen enzymes, mainly those involved in digesting the cell wall by chelating metals, blocking, or retarding pathogen invasion [52]. Different studies have shown that there are different mechanisms of action against pathogen infection, inhibition of cellulases, pectinases, and xylanases, chelation of metal ions that belongs to cell membranes and enzymes, and more general detoxifying cells of ROS [45].

They can also affect bacterial DNA synthesis, by interacting with DNA gyrases, as the B ring of flavonoids can form hydrogen bonds with nucleic acid bases, or by direct interaction with the ATP binding site of the gyrase, leading to an inhibition of the synthesis of new DNA. This may be their method of protection against virus [45].

Antifungal properties have also been proved for flavonoids; these properties depend on their structure, for example dihydroquercetin has proved to be much more active against

Fusarium sp. infections than other types of flavonols, and it is believed that is due to the hydroxyl groups [53]. In addition to the antifungal effects reported in plant, some have shown that certain compounds like phenols, phenolic acids, flavonoids, and isoflavonoids inhibit pathogen proliferation in the rhizosphere, preventing root infections.

4.3. Other functions

Flavonoids play a very important role in symbiotic bacteria relations. Bacteria belonging to the family Rhizobiaceae include several genera, each of them specific to a legume species. Rhizobiaceae are capable of fixing nitrogen for the plant; in exchange they obtain photosynthates. First, they need to establish the symbiotic relationship and form the nodule; in this process, flavonoids are key since these bacteria are attracted by these flavonoids that are specific signals for each rhizobia-legume couple. There are studies of different plants growing in soils with low nitrogen concentration that induces the accumulation of flavonoids [54]. Based on this fact, knowledge of the specific flavonoids that enhance symbiosis establishment could be applied to field production of legumes, in low productivity soils, to enhance nodulation, which in turn, will enhance yield in developing areas. This goal could be achieved at a low cost and easily implemented in local areas therefore contributing to food security, as marked by the FAO.

Connecting with this improvement in production and also with their natural physiological role, flavonoids provide color, taste, and fragrance to the fruit and seeds, and also play an important role in pollination, because these characteristics attract insects [45]. Although these characteristics may attract some organisms can also deter some others, in the cases of herbivores and some nematodes, avoiding to be eaten by these living beings.

5. Flavonols', anthocyanins', and catechins' health properties

Flavonoids are known because of their beneficial effect on human health. It has been known for long time and mainly attributed to its antioxidant potential. Compounds able to scavenge free radicals are in general beneficial for health. As all living beings, humans also produce ROS and there is also a system to get rid of these free radicals to prevent damage of the cells (DNA, lipids, and proteins). These damages are called oxidative damages which have been related to carcinogenesis, neurodegeneration, atherosclerosis, diabetes, and aging; however, the precise underlying mechanisms for these health benefits are starting to be unraveled.

Flavonoids have received increasing attention due to their anti-inflammatory, antimicrobial, and anticancer activities. Structural-functional relationship analyses identified luteolin as one of the most potent inhibitors of xanthine oxidase, a key enzyme in ROS production. Reduction of ROS by apigenin prevents endothelial damage during acute inflammation and restores mitochondrial function. Most of the anti-inflammatory and antimicrobial activities attributed to flavones seem to be centered on their ability to regulate the Toll receptor (TLR)/NF κ B axis. This is a central pathway in the host-pathogen interplay in mammals, responsible for the expression of inflammatory mediators, including tumor necrosis factor (TNF α), interleukin-1 (IL-1 β) and cyclooxygenase-2 (COX-2), an enzyme mediating the conversion

of arachidonic acid to prostaglandins. Notably, great similarities are found between the mammalian TLR/NF κ B and plant pathogen defense pathways, suggesting that flavones may regulate evolutionary conserved targets [55]. It has also been reported that in animal models, apigenin reduces the phosphorylation of the NF κ B p65 subunit, required for its transcriptional activity. Inhibition of p65 phosphorylation reduces the expression of inflammatory cytokines, limiting the cell damage characteristic of acute inflammation [56]. Other flavones inhibit COX-2 by halting NF κ B nuclear localization [57]. Overall, glycosides show less anti-inflammatory activity than aglycones, probably a consequence of their reduced cellular absorption [58]. Recent studies identified additional mechanisms responsible of the anti-inflammatory activity of flavones, including the regulation of noncoding RNAs. Large microRNA screenings showed that apigenin reduces microRNA155 (miR155) expression, a main inflammatory regulator miR155 binds to 3'-UTR regions of several inflammatory cytokines, suggesting an additional mechanism by which flavones can restore homeostasis during acute inflammation, independent of their anti-oxidant activity [1].

Consistent with the ability of flavones to regulate inflammation, interventions with the Mediterranean diet, which is rich in flavonoids, showed improved cardiac function, reduced hypertension and obesity [59, 60]. Flavones also affect leukocyte migration, with very specific targets, deeply affecting cancer and inflammation [61, 62]. Flavones ability to reduce cell migration has great impact on cancer, suggesting alternative therapeutic approaches to reduce metastasis. The anticarcinogenic effect of flavones is given in part by their ability to induce DNA damage, and is accompanied by cell cycle arrest at G1 or G2, depending on the particular cell type. Interestingly, the ability of apigenin to induce cell death in cancer cells is independent of ROS production [63] supporting a beneficial role of flavones independent of their anti-oxidant activity.

Identification of the direct targets will highly contribute to understand the molecular mechanism related to flavones and health. The use of PD-Seq (phage display high-throughput sequencing), a novel approach for small target identification, identified several targets, suggesting that dietary compounds, unlike pharmaceuticals, may target several molecules [64]. This statement encourages the use of healthy plant-based foods or extracts, rich in polyphenols but with a complex mixture of compounds that will contribute to prevent the onset of disease by reaching many small targets simultaneously. Under this rationale, a study of naturally healthy fruits or plant materials is seriously encouraged to prevent the onset of disease.

As flavonoids, anthocyanins' health-promoting effects have been frequently linked to their high antioxidant activities. However, there is increasing evidence reporting that some of their biological effects may be related to their ability to modulate mammalian cell signaling pathways [65, 66]. Anthocyanins also offer protection against certain age-related degenerative diseases cancers, cardiovascular disease [9, 55, 67, 68]; anti-inflammatory activity [69], promotion of visual acuity [70], and hindering obesity and diabetes [71, 72] have also been reported as beneficial effects of these compounds.

In addition to the many target-specific effects of each compound detailed above, effects are more complex to evaluate when any of these phenolics are delivered through the diet in a complex food matrix. The variability of effects relays in two points: on the one hand, natural variability

in composition and on the other hand, variability in absorption at the individual level. It has been estimated that only 5–10% of the total polyphenol intake is absorbed in the small intestine. Currently, it is estimated that 500–1000 different microbial species inhabit the gastrointestinal tract. However, they do not seem to be ubiquitous but reflect the interpersonal differences in the gut microbial community [73]. Consequently, apart from the interindividual variation in daily intake of polyphenols, interindividual differences in the composition of the gut microbiota may lead to differences in bioavailability and bioefficacy of polyphenols and their metabolites [74, 75].

The other factor that will condition effects on health is intimately associated with the sessile nature of plants. Plants have to overcome environmental changes by changing their chemical composition, synthesizing metabolites that will contribute to a better adaptation to changes in abiotic factors or to fight back biotic challenges. Since environmental conditions are variable along the year, and flavonols and anthocyanins play a role in adaptation to UV stress, it may be anticipated that concentration in plant will be higher in spring and summer when light hours and intensity are higher. Hence, fruits produced in winter or in summer will presumably have different concentrations, as has been demonstrated in blackberries [76]. Moreover, given their role in plant defense, their levels may also fluctuate depending on disease prevalence along a given season, and therefore, health benefits will be different, since the dose is different. Consequently, any attempt to modulate the amplitude of these fluctuations will result in enhanced fruit quality, more reliable in terms of health benefits.

In order to achieve this goal, understanding the metabolic pathway and its regulation is a milestone on the way to develop varieties in which the main regulators are overexpressed to ensure a high and constant, or low variability, fruit bioactive contents. This goal may be achieved through crossbreeding or by the means of metabolic engineering in plants [77] or through elicitation of secondary metabolism with external agents such as beneficial bacteria or derived molecules [76, 78] or even other chemical molecules such as salicylic acid.

6. Elicitation

An alternative to new varieties is using beneficial bacteria or parts of the same as a tool to trigger plant metabolism in field production in order to cause a mild-biotic stress in the plant that smoothly and constantly triggers secondary metabolism to achieve constant concentrations of bioactives in the edible fruits [77, 78]. The ability of many beneficial bacteria to trigger plant metabolism in different species has been reported and there is increasing evidence of bacterial derived elicitors with the same effect [79]. These bacterial derived molecules are termed as MAMPs (microbe-associated molecular patterns) and can be either structural molecules from the bacterial cell wall, or derived metabolites that bacteria release to trigger the plant in their intimate relationship. Each MAMP or strain has to be evaluated for each plant species, since genome-genome specificity has been occasionally reported.

To support the value of this strategy in the study of blackberry metabolism, a transcriptome analysis from field grown blackberry fruits was performed to study this plant species; contigs were obtained and blasted to the genome of *Fragaria*, the model plant within the Rosaceae

finding 73.5% similarity with *Fragaria vesca* subspecies *vesca* [80]. Once the gene information was available, field grown blackberry plants were inoculated with a beneficial bacteria strain (*Pseudomonas fluorescens* N21.4) as a biostimulant, aiming to trigger flavonoid biosynthesis as part of an induced systemic response (ISR). Fruits were studied in three states along maturation, carrying on a bioactive characterization and studying core and regulatory gene expression. As a result, the concentration of flavonoids increased in the fruit along with maturation over the noninoculated controls; hence, core and regulatory genes were characterized and their expression was studied demonstrating genes likely involved in controlling the activity of pathway branches, associated with enhanced accumulation of anthocyanins, catechins, and flavonols in developing fruits of blackberry [81].

These results prove that elicitation is a very interesting tool to achieve better fruit quality in terms of higher amounts of bioactives, as flavonoid biosynthesis can be modulated without genetic manipulation. Using this method, we can achieve two different goals at the same time: first we increase plant defense by a natural way without using any chemicals, which are harmful for the environment and humans also, therefore contributing to environmentally friendly agricultural practices. The second one would be obtaining a food with reliable beneficial effects for human health, since delivering the bacteria through the roots will attenuate fluctuations in bioactive compounds. This second statement is nowadays one of the world's great concerns, since achieving food security refers to both ends of society: those that are hungry should have enough amounts of healthy and nutritious foods, as well as those that have a lot of food, but unbalanced and low quality. Therefore, healthy eating is becoming really popular as population is trying to have a good diet to prevent development of disease rather than healing, so to decrease the intake of different drugs in a long time period. Both this two goals can be achieved by the application of these rhizobacterias that will increase these secondary metabolites by a natural method. Beyond health-related issues in food production, these elicitors and elicitation technology [80] can be used to unravel metabolic pathways and their regulation for further application in metabolic engineering and cell cultures.

Acknowledgements

This Project was funded by Ministerio de Economía y Competitividad: AGL-2013-45189-R. Grant reference: BES-2014-069990.

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