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Chapter 8

Secondary Metabolism in *Amaranthus* spp. — A Genomic Approach to Understand Its Diversity and Responsiveness to Stress in Marginally Studied Crops with High Agronomic Potential

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Additional information is available at the end of the chapter

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Abstract

Cultivated amaranths are crops with an unrealized agronomical potential despite their high nutritional value and nutraceutic properties of their seeds and/ or leaves. They tolerate growing conditions unsuitable for cereals, and are tolerant to biotic aggressors. Several *Amaranthus* species are abundant of sources of secondary metabolites, mostly phenylpropanoids, predominantly in seeds and leaves, many of which may confer health benefits associated with their antioxidant properties. They could also act as defensive compounds against predators or pathogens. Recent biochemical and molecular approaches partly defined the mechanisms responsible for grain amaranth´s tolerance against biotic stress. However, the role played by secondary metabolites in (a)biotic stress amelioration in amaranth is practically unknown. Our group has identified several genes coding for enzymes involved in secondary metabolism pathways in A. *hypochondriacus*, in addition to related regulatory transcription factors. More than 50% of these genes involve the phenylpropanoid pathway. In this chapter, the role played by this pathway in (a)biotic stress amelioration in plants will be briefly reviewed, followed by an examination of its involvement in the conferral of nutraceutic properties to amaranth plants. A description of the progress obtained so far regarding the characterization of phenylpropanoid genes in grain amaranth will close this chapter.

Keywords: (a)biotic stress, grain amaranth, phenylpropanoids

1. Introduction

Species belonging to the Amaranthaceae compose a diverse and interesting family of plants. They can develop in highly contrasting habitats, from arid and semi-arid zones, where they can survive in sandy alkaline and/ or serpentine soils, to disturbed tropical forests. A minority are found in aquatic, semi-aquatic or marine environments. Their high affinity to saline conditions stems, in part, from to the weedy nature of most of the species that constitute the *Alternanthera*, *Amaranthus*, *Celosia, Chamissoa, Froelichia, Gomphrena, Guilleminea,* and *Iresine* genera. These plants are widely distributed in tropical and subtropical zones due to their ability to colonize or invade diverse habitats [1].

The *Amaranthus* genus is highly diverse, including approximately 70 species. A fraction of these may grow in saline soils; in this regard, *A. greggii* is considered to be a marker of saliferous soils. This genus includes grain-producing species, the most important being *A. cruentus*, *A. caudatus,* and *A. hypochondriacus*. Grain amaranths are valued for the high protein content and multiple nutraceutic properties of their seeds [2,3]. Moreover, they possess an inherent tolerance to high temperatures and drought, traits which have been associated with their C4 physiology, indeterminate flowering habit and superior water use efficiency due to their ability to grow long tap roots and develop an extensive lateral root system [4–6]. In concordance with related species within the Amaranthaceae, grain amaranths can tolerate poor and saline soils conditions and erratic rains unsuitable for the cultivation of other grain crops. Besides, they are not particularly susceptible to major diseases or insect pests [5–8]. Their high tolerance to severe defoliation, produced by mechanical means [9,10] or by insect folivory [6,11], is believed to contribute to this trait. In addition, they can readily respond to chemical elicitors of defense responses, such as jasmonic acid (JA) [12-14] or benzothiadiazole (BTH) [15], to increase their resistance against highly damaging phloem-feeding insect pests, such as the tarnished bug *Lygus lineolaris*, or against potentially lethal pathogenic bacteria. However, the contribution of secondary metabolites to (a)biotic stress tolerance in grain amaranth has been barely explored.

In this chapter, we shall first review the role played by phenylpropanoid pathway in the amelioration of both biotic and abiotic stress in plants. Then, we shall proceed to describe the state of the art with regard to the phenylpropanoid pathway in *Amaranthus*, mostly in relation to stress, but also in relation to the their role in the biosynthesis of compounds with proposed nutraceutic properties. Finally, a thorough description of the progress we have achieved so far with respect to the genomic characterization of this particular secondary metabolic pathway in grain amaranth, mostly in terms of stress tolerance and/ or resistance will be made. The information gathered as a result of these efforts will expand the knowledge, perhaps into unknown territory, about the chemical diversity in plants, particularly in grain amaranth and related species, many of which can thrive in extreme habitats.

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2. Reactive Oxygen Species (ROS)

2.1. Brief description of their ubiquitous and malignant role in plant stress and the antioxidant defense mechanisms induced for their control — What is known regarding amelioration of ROS-related damage in amaranth plants during stress?

The abundance of reactive oxygen species (ROS) tends to increase in the tissues of plants exposed to diverse environmental challenges including contact with heavy metal contaminants in soil, water or salinity stress, which is often accompanied by high light and temperature, and nutrient deficiency [16]. Most stress conditions in plants cause an accumulation of ROS, such as superoxide ion, hydrogen peroxide, oxygen-containing radicals, and others. These chemical entities can produce extensive oxidative damage in the apoplastic compartment and may also harm cellular membranes by lipid peroxidation. Additionally, they can have an impact on ion homeostasis mechanisms, which are crucial for many stress tolerance mechanisms, by interfering with ion fluxes [17]. ROS detoxification frequently involves the combined action of both antioxidant metabolites such as ascorbate, glutathione, tocopherols, and ROS-detoxifying enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), and catalase (CAT) [18,19]. In this sense, overproduction of antioxidants in response to drought-induced oxidative stress has been frequently found to be associated with the drought stress tolerance of different plant species [20,21]. Also, enhanced drought tolerance has been generated in several different transgenic plants transformed with genes encoding diverse types of antioxidant-related enzymes or metabolites (e.g., SOD, APX, monodehydroascorbate reductase, and tocopherol cyclase, a key enzyme of tocopherol biosynthesis, [22]). Regarding grain amaranth, a series of proteomic studies performed in plants exposed to drought and saline stress detected the accumulation of various antioxidant enzymes, similar to those mentioned above [23,24]. In addition, the participation of antioxidant genes, such as *AhCAT*, *AhAPX*, and *AhSOD*, was implied by findings showing their induced-up-regulation in grain amaranth plants primed to resist infection by pathogenic bacteria [15]. However, a further characterization of the additional six *CAT*, four *APX*, and three *SOD* genes identified in grain amaranth [12] remains to be performed.

Glycine betalaine (GB) is a quaternary ammonium compound that acts as an osmolite with protective functions in plants subjected to the osmotic stress normally produced under drought, high temperature and/ or excessive salinity conditions. GB accumulation in the cytoplasm reduces ion toxicity, and ameliorates the highly damaging effects caused by the usually simultaneous presence of dehydration, salinity, and extreme temperature stresses. The protective effect is proposed to be exerted by the stabilization of macromolecular structures, and/ or by the protection of chloroplasts, particularly the photosystem II complex. The latter is believed to involve the thermodynamic stabilization of the indirect interaction of extrinsic photosystem II complex proteins with membrane phosphatidylcholine moieties [25]. GB is synthesized by the two-step oxidation of choline. The first step is catalyzed by choline monooxygenase, followed by the action of betaine aldehyde dehydrogenase. Both genes have

been described in different amaranth species [12,26,27], whereas the presence of GB has been reported in all Amaranthaceae species examined so far, with the exception of *Chenopodium quinoa* and *Noaea mucronata*. However, the GB concentration needed to exert a protective effect in plants of this family has been found to vary widely in a species-specific manner [25]. On the other hand, GB-accumulating transgenic plants usually show a significant improvement in their tolerance to different abiotic stress conditions [28].

2.2. *Betacyanins* in amaranth: More than pigments?

Betalains are water-soluble, nitrogen-containing pigments that are found only in one group of angiosperms, the Caryophyllales. For reasons that remain unresolved, they have never been found jointly with anthocyanins in the same plant [29,30]. This particular trait has been employed for chemo-taxonomical purposes. These pigments can be divided into the red-violet betacyanins and the yellow betaxanthins. Both are immonium conjugates of betalamic acid covalently bonded with cyclo-dihydroxyphenylalanine (cDOPA) glucosides, which can undergo further acylations [31–33]. These pigments are possibly needed for the optical attraction of pollinators and seed dispersers. Regarding stress, a protective role against accumulating ROS has been inferred from a number of studies [34–36] whereas the betacyanin amaranthine has also been proposed to exert protective effects against photo-oxidative damage in *A. tricolor* [36].

Recently, an analysis of key genes/ enzymes of the betacyanin biosynthetic pathway in *A. hypochondriacus* was performed. The study included genes coding for cyclo-DOPA glycosyl-transferase (*AhcDOPA5-GT*), two 4, 5-DOPA-extradiol-dioxygenase isoforms (*AhDODA-1* and *AhDODA-2*, respectively), a betanidin 5-glycosyl transferase (*AhB5-GT*), and an ortholog of the cytochrome P-450 R gene (*CYP76AD1*). The expression pattern of these genes, together with DOPA oxidase tyrosinase assays, was determined in both green and red tissues. The results obtained suggested that two apparently independent glycosylation pathways leading to betanins could be operating in *A. hypochondriacus* to synthesize amaranthine. In addition, these genes/ enzymes were shown to be induced differentially in a tissue-specific and genotype-specific manner in response to different stimuli, including water- and salt-stress and insect herbivory. The results obtained from the abiotic stress assays suggested that genes other than those examined in this study were probably contributing significantly to pigment content in tissues of stressed *A. hypochondriacus* plants. They also offered the possibility that these genes, due to their high induction by insect herbivory, particularly in acyanic plants, could function in defense responses against chewing insect pests by still undetermined mechanisms [37].

In addition to pigments, diverse phytochemical studies have shown that amaranth plants are capable of synthetizing a notable diversity of secondary metabolites [3,38,39]. Although many of these compounds are not considered to be essential for the primary needs of the plant, they are certainly required for survival in and/ or adaptation to challenging environmental conditions. Many of these compounds may be employed in amaranth and other plants as signaling compounds, in defense and/ or for communication with other organisms, such as pollinators [40–44].

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The most commonly found secondary metabolite families found in amaranth and related species are phenylpropanoids, including flavonoids, phenolic acids, and their related amides, followed by alkaloids and terpenoids. From an anthropocentric perspective, some of these chemicals, including betacyanins, flavonoids polyphenols, and phenolic acids, are responsible for conferring amaranth and quinoa tissues with the bioactive antioxidant activity associated with their well-documented health benefit effects [3,38,45–49].

3. Phenylpropanoid secondary metabolites

Plants have accumulated a great diversity of phenolic compounds as a result of their long process of evolutionary adaptation. Approximately 40% of these compounds are derived from the highly diverse phenylpropanoid metabolism. The phenylpropanoid compounds constitute a highly diverse assortment of phenylalanine-derived secondary metabolites. These include flavonoids, which are generally sub classified into the anthocyanins, proanthocyanidins, flavonols, isoflavonoids, phlobaphenes, flavanones, and flavones subgroups, which are found in the majority of higher plants, in addition to the aurone subgroup, which is widespread, but not ubiquitous. Also included are monolignols, lignans, coumarins, phenolic acids, quinines, stilbenoids, and xanthones. Other phenolics include alkylmethoxyphenols, alkylphenols, curcuminoids, furacoumarins, hydroxybenzaldehydes, hydroxybenzoketones, hydroxycinnamaldehydes, hydroxycoumarins, hydroxyphenylpropenes, methoxyphenols, naphthoquinones, phenolic terpenes, and tyrosols.

Most of the flowers and fruits pigments employed for pollinator attraction and seed dispersal are water-soluble anthocyanins [50]. Proanthocyanidins, or condensed tannins, are colorless flavonoid polymers produced by the condensation of flavan-3-ol units [51]. Similarly colorless are the abundant flavonols which are usually found in the form of mono-, di-, or triglycosides [52]. Isoflavonoids and phlobaphenes are groups of flavonoids characterized by being predominantly found in the Papilionoideae family [53] or by their red pigmentation which results from the polymerization of flavan-4-ols [54], respectively. Based on their carbon skeleton, the ubiquitous phenolic acids can belong to the hydroxycinnamic acid type (chlorogenic, ferulic, rosmarinic, and sinapic acids) or to the hydroxybenzoic acid type (p-hydroxybenzoic, vanillic, and protocatechuic acids). Finally, the stilbenes represent a small family of phenylpropanoid metabolites dispersed in over 70 unrelated plant species [55–57]. Interestingly, the latter compounds are induced in response to several biotic and abiotic stimuli or by functionally related elicitors, such as methyl jasmonate (MeJA), and ethylene. Flavonoids are also involved in the regulation of auxin transport [58–60] and participate in the chemical dialog established between the plant roots and nitrogen-fixing bacteria and in signaling pathways designed to modulate ROS levels in plant tissues [61,62]. These compounds are also determining factors of male fertility and precursors for the synthesis of lignin [63–65]. The latter is an aromatic heteropolymer that confers mechanical strength to the cell wall and rigidity to plant stems, whose synthesis involves the assembly of p-coumaryl, coniferyl, and sinapyl alcohol monolignols. Lignin is also a waterproof insulator for cell walls and, as such, facilitates

the transport and assimilation of water through the vascular system. It also provides protection against wounding, UV light, and pathogen attack [66].

As noted, phenylpropanoids have notable structural and biological function diversity. In terms of defense in plants, phenylpropanoids can be classified into three broad categories according to their function. Those having signaling activity, those known as phytoanticipins, which are part of the basal defensive arsenal of the plant and constitutively accumulate in certain plants tissues, and those whose *de novo* accumulation in plants, as phytoalexins, is induced in response to a biotic aggressor [67,68]. Phenylpropanoids also contribute to human diet and health. Their recognized bioactive properties have acquired medicinal and nutritional importance, mostly due to their antioxidant, antibacterial, antiviral and other reported activities or as effective therapeutic agents against certain types of cancer, cardiovascular pathologies, diabetes, osteoporosis, and neurodegenerative illnesses associated with oxidative stress [43,69,70].

3.1. Phenylpropanoid biosynthesis: A profusely branched pathway

Phenylpropanoids contain at least one aromatic ring with one or more hydroxyl groups, and are synthesized via the shikimate pathway alone or in combination with the mevalonate pathway. The first three steps in the synthesis of phenylpropanoid-derived compounds are catalyzed by phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H), and 4-coumarate coenzyme A ligase (4CL), collectively referred to as the general phenylpropanoid pathway (GPP). GPP products then serve as precursors for phenylpropanoid-derived compounds [71,72].

The deamination of phenylalanine to cinnamic acid catalyzed by phenylalanine ammonia lyase (PAL, EC 4-3.1-5) is the initial step shared by all phenylpropanoid secondary metabolites. PAL is a conserved homotetrameric protein that is a key enzyme in the phenylpropanoid pathway of higher plants [73–76]. PAL enzymes are grouped as families having many isoforms that are responsive to different developmental and environmental stimuli [77,78].

Cinnamate is the basic structure from which simple phenylpropanoids with the basic C6–C3 carbon skeleton of phenylalanine are produced, via a series of hydroxylation, methylation, and dehydration reactions. This group includes compounds such as p-coumaric, caffeic, ferulic, and sinapic acids and simple coumarins, which rarely accumulate as free acids inside plant cells, being usually conjugated to sugars, cell wall carbohydrates, or organic acids. Salicylic, benzoic, and other acids are uncharacteristic phenylpropanoids that lack the three-carbon side chain, even though they originate from cinnamate and p-coumarate, whereas a large number of stress-induced phenylpropanoids are derived from the C15 flavonoid skeleton, which is synthesized via the chalcone synthase (CHS)-catalyzed condensation of p-coumaroyl-coenzyme A (COA) and three molecules of malonyl-COA. The tetrahydroxychalcone product resulting from the CHS-catalyzed step in most plants is further converted to other flavonoids, such as flavones, flavanones, flavanols, anthocyanins, and 3-deoxyanthocyanidins (Figure 1) [67,68]. Lignin and suberin represent an increased level of complexity, since they are large polymers, constructed from monolignol phenylpropanoid precursors, whose composition varies from species to species.

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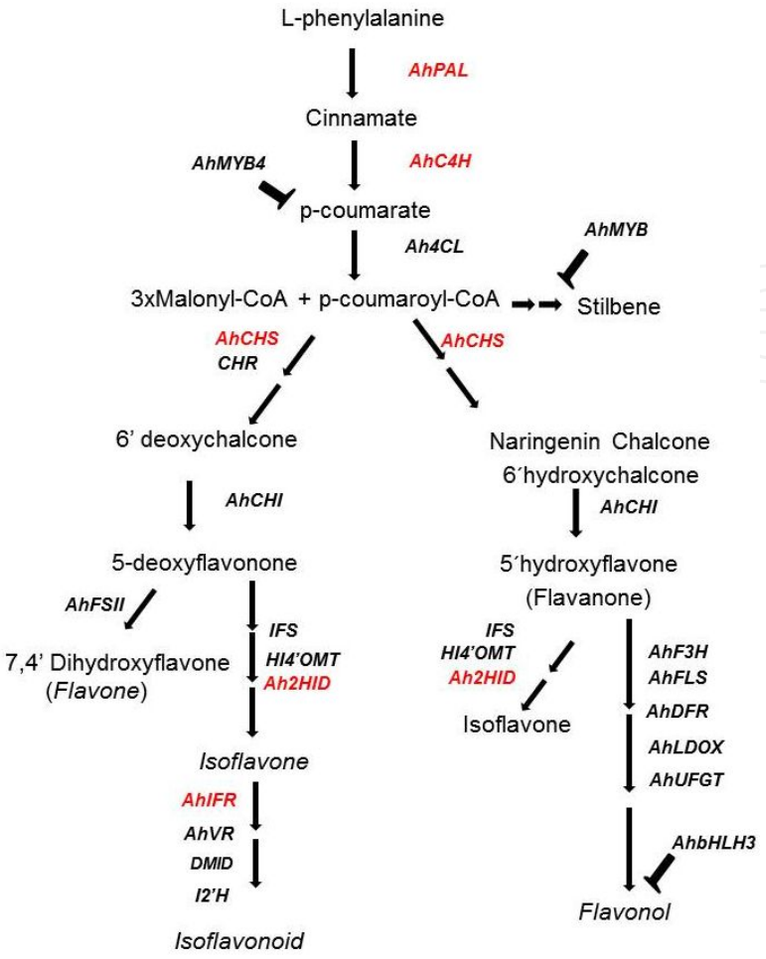

Figure 1. Schematic representation of phenylpropanoid biosynthesis. The image is modified from the original version in [68]. C4H, cinnamate 4-hydroxylase; CHI, chalcone isomerase; CHR, chalcone reductase; CHS, chalcone synthase; 4CL, 4-coumarate:CoA ligase; DMID, 7,2′-dihydroxy-4′-methoxy-isoflavonol dehydratase; FSII, flavone synthase II; 2HID, 2-hydroxyisoflavanone dehydratase; HI4′OMT, 2-hydroxyisoflavanone 4′-*O*-methyltransferase; IFR, isoflavone reductase; IFS, isoflavone synthase; I2′H, isoflavone 2′-hydroxylase; PAL, L-phenylalanine ammonia-lyase; VR, vestitone reductase; F3H, flavanone 3-hydroxylase; UFGT, UDP-glucose flavonol 3-O-glucosyl transferase; FLS, flavonol synthase; DFR, dihydroflavonol 4-reductase; LDOX, leucoanthocyanidin dioxygenase. The genes in red text represent those found to be induced by stress conditions in the grain amaranth transcriptomic analysis [14].

3.2. Regulation of phenylpropanoid biosynthesis: A complex scenario

The biosynthesis and accumulation of secondary metabolites, including phenylpropanoids, are usually tissue- and developmental-stage-specific. As mentioned above, phenylpropanoids can be present as pigments in leaves, flowers, fruits, and seeds or participate in the establishment of mutualistic or detrimental interactions either with beneficial fungi or bacteria or with pathogenic oomycetes [79]. They can participate in the synthesis of lignins and related fibrous materials associated with changes in the cell wall occurring concomitantly with development, in response to stress [66], or in the determination of pollen function. The latter involves the conjugation of polyamines with hydroxycinnamic acid [63–65,80–82]. Numerous factors mediate the expression of phenylpropanoid genes, including sugar levels, transcription factor

(TF) regulation, and diverse types of stress. Sucrose, for instance, has a dual function, first by providing carbon for phenylpropanoid metabolism, and second, by modulating transcriptional and post-translational regulation of many pigment-related genes [83,84]. Recently, a sugar-related regulatory loop was described in which the induction, by sucrose, of AN1, a MYB TF that activates the phenylpropanoid biosynthetic pathway, was self-regulated by the increased sucrolytic activity induced in parallel by the action of AN1 [85]. Structural variability in secondary metabolism is also determined by post-translational chemical modifications of the primary chemical structure by diverse reactions. This is a mechanism that profoundly alters the biological activity of phenylpropanoid compounds via its ability to modify various critical biochemical parameters, including stability, solubility, and/or localization within the cell. For instance, the glycosylation of hydroxycinnamic acids was found to have an important participation during N-limiting stress conditions in *Populus*, which is a plant known for the great diversity of its phenylpropanoid compounds [86]. Similarly, the glycosylation of diverse secondary metabolites has been shown to be needed to regulate oxidative stress responses in various plant species [87,88]. Moreover, methylation and acylation, for example, can favor the volatilization of secondary metabolites by the generation of esters or ethers. A well-known example is salicylic acid (SA), a phenolic acid which is temporarily transformed to its methyl ester to facilitate its transport to distal tissues in order to regulate systemic signaling during the systemic acquired resistance response to biotrophic pathogens [89,90]. Volatile phenylpropanoid esters can also be found as components of the aroma of fruits such as banana and strawberries [91].

Controlled transcription of biosynthetic genes is one major mechanism regulating secondary metabolite production in plant cells. Several TFs involved in the regulation of metabolic pathway genes have been isolated and studied. Synthesis of more than one class of phenylpropanoid-derived compounds is predominantly under the control of V-myb myeloblastosis viral oncogene homolog (MYB) proteins of the R2R3-MYB class that can act both as transcriptional activators and repressors [92]. The participation of these TFs in many phenylpropanoid-related processes has been extensively recorded in various plant species. In Arabidopsis, several R2R3-MYB members have been implicated as positive regulators of lignin synthesis. For instance, the secondary cell wall-associated AtSND1 protein, in association with related proteins, starts a cascade of events that regulate secondary cell wall formation by inducing the expression of the *AtMYB83* and *AtMYB46* R2R3-MYB genes that then trigger the expression of *AtMYB58*, *AtMYB63*, and *AtMYB85*. These, in turn, upregulate various lignin synthesis genes by interacting with their promoter AC elements [93-96]. Conversely, other R2R3-MYB TFs have been shown to act as repressors of the monolignol pathway in both mono and dicot plant species, thereby leading to the suppression of lignin biosynthesis [97–102]. Anthocyanin pigment synthesis is regulated predominantly by a transcriptional complex consisting of three proteins: (i) R2R3-MYB, (ii) basic-helix-loop-helix (bHLH), and (iii) WD-repeat (WDR), which is known as the MBW complex. The first report describing the formation of a MYB bHLH complex for the activation of anthocyanin biosynthesis was made in maize [103]. Subsequent studies recognized the multiple dependence between MYB TFs (i.e., transparent testa2, TT2), WD40, and bHLH (i.e., TT8) for the regulation of the *BANYULS* gene coding for an anthocyanidin reductase required for proanthocyanidin biosynthesis [104].

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Complex formation, initiated by the activation of R2R3-MYB genes can be induced by environmental stress conditions or in response to developmental cues. Known targets of this complex are genes encoding dihydroflavonol 4-reductase (DFR), bHLH2, and, curiously, two MYB repressors whose activation leads to a self-regulatory feedback repression loop [105]. The AtDOF4-2, BrMYB4, and AtMYB4 TFs have been reported also as negative regulators of flavonoid and lignan biosynthesis [80,106,107]. In Arabidopsis, anthocyanin biosynthesis via the MBW complex has been demonstrated to be stimulated in response to light, sucrose, nitrogen, and JA [83,108–110].

In addition, anthocyanin patterning and spatial localization are mainly determined by R2R3-three subgroups of MYB activators, many of which have been identified in plants [92]. The MYB, bHLH, and WDR transcription factors have also been shown to be prevalent in the regulation of proanthocyanidin genes [105,111]. Conversely, the regulation of the flavonol pathway is species-specific, and diverges from the above regulatory mechanisms by its diversity, which may require the action of either a single MYB TF, the formation of an MYB-bHLH dimer or an MBW complex. Additionally, augmented flavonol content in Arabidopsis has been found to result from the association of members of the plant-specific teosinte branched1, cycloidea, and proliferating cell nuclear antigen factor, or TCP TF family that interact with AtMYB12 and AtMYB111 [112]. Additionally, the expression of *AtMYB12* in response to both visible and UV-B light is regulated by the basic leucine zipper transcription factor HY5 [113]. Experimental evidence gathered to date clearly indicates that MYB regulation pattern is dependent on cell and tissue type, developmental stage, and environmental conditions. However, information regarding the mechanistic basis of MYB protein responses to biotic and abiotic stimuli remains limited.

3.3. Phenylpropanoid and other less abundant secondary metabolites in amaranth: Nutraceutical properties and suggested defensive roles

Several chemical analyses of diverse tissues of *Amaranthus* species indicate that they differentially accumulate diverse types of secondary metabolites (Table 1). A particular interest in the study of phenolic acids, flavonoids, and other polyphenolics in diverse amaranth species consumed as vegetables and grain has been generated by their high antioxidant activity. As mentioned above, this is mostly because this property has been associated epidemiologically with a decreased risk of diseases associated with oxidative stress, such as cancer and cardiovascular disorder [114]. Consequently, several phenolic acids, flavonoids, and their glycosides have been identified in various *Amaranthus* species (Table 2). These compounds have been isolated using various solvent combinations in various fresh plant tissues obtained from plants at different developmental stages and/ or grown under diverse ambient conditions. They have also been isolated from tissues subjected to diverse processing, from milling to cooking. The concentration of many of these compounds has been observed to vary widely between species and varieties within a species, tissue type, processing and/ or growing conditions, including exposure to (a)biotic stress [3]. A number of selected examples will be described next to illustrate this point.

Metabolic group	[1]cru	*hyp*	*hyb*	*cau*	*pan*	*spi*	*ret*	*liv*	*gan*	*tri*
Flavonoids	[2]+	+	+	+		+				
Betacyanins	+	+	+							+
Phenolic acids	+	+	+	+		+				
Tannins	+	+	+	+		+				
Steroids and triterpenoids	+	+	+	+		+				
Saponins	+	+	+	+		+				
Coumarins					+					
Alkaloids		+				+				
Amarantholidosides				+			+			
Polyhydroxylated nerolidols							+			
Carotenoids	+			+				+	+	
Chlorophylls	+									+
Phytate	+	+		+						
Resinols		+								
Tocopherols and tocotrienols								+		

[1]Amaranthus species included are: **cru**= *cruentus;***hyp**= *hypochondriacus;***hyb**= *hybridus;***cau**= *caudatus;***pan** = *paniculatus*; **spi** = *spinosus;* **ret** = *retroflexus;* **liv** = *lividus;* **gan** = *gangeticus*, and **tri** = *tricolor*.

[2]The + sign represents species in which these compounds have been detected.

Table 1. The diversity of secondary metabolites in amaranth.

Compound	[1]hyp	cru	cau	hyb	man	spi	pan	ret	tri	gan
Total phenolic acids	[2]S, F	S,V	S							
Gallic acid	F, L	S, Sp		L			S			
Vanilic acid	F, S, L	S		L	S					
Cinnamic acid		S								
Syringic acid	Fl, S	Sp								
p-Coumaric acid	S, L	S, Sp, L	S	L	S		S			
Ferulic acid	Fl, L	L, S, Sp	S, IF	L			S			
Protocatechuic acid	S	S	S, Sp		S		S			
p-Hydroxybenzoic acid	Fl, S, L	S	S	L	S		S			
Caffeic acid	L	L, S	S	L			S			
Chlorogenic acid	L			L						

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Compound	[1]hyp	cru	cau	hyb	man	spi	pan	ret	tri	gan
Sinapic acid	**L**			**L**			**S**			
Salicylic acid	**L**		**S**	**L**			**S**			
Caffeoylquinic acids						**St**				
Cumaroylquinic acids:						**St**				
Feruoylquinic acids						**St**				
Hydroxycinnamates (quinic acid)	**L**			**L**		**St, L**				
Flavonoids	**Fl, S**	**L**		**L**						
Rutin	**S, L, St, Fl**	**S, L, St, Fl, Sp**	**S, L, St, Fl**	**S, L, St, Fl**		**WP, St**		**S, L, St, Fl**	**S, L, St, Fl**	
Isoquercetin	**S, L**								**L**	
Quercetin: released from rutin	**S, L, St, Fl**	**S, L, St, Fl**	**S, L, St, Fl**	**S, L, St, Fl**		**L**		**S, L, St, Fl**		**L**
Quercetin diglycoside						**St**				
Quercetin-3-O-glucoside						**St**				**St**
Nicotiflorin	**S**	**S**			**S**					
Vitexin		**S**								
Isovitexin		**S**				**St**				
Orientin		**Sp**								
Morin		**Sp**								
neohesperidin and hesperidin		**Sp**								
Kaempferol diglycoside					**L**	**St**				
Tannins	**S, L**	**S, L**								
Polyphenolics	**S, L**	**S, L**		**S, L**						
Anthocyanins	**S**									
Betains, betacyanins	**L**	**L**			**L**				**L**	
Phenolic Amides	**L**				**L**					**L**

[1]Amaranthus species included are: **hyp** = hypochondriacus; **cru** = cruentus; **cau** = caudatus; **hyb** = hybridus; **man** = mantegazzianus; **spi** = spinosus; **pan** = paniculatus; **ret** = retroflexus; **tri** = tricolor, and **gan** = gangeticus.

[2]Tissues or plant developmental stage examined include: **F** = flower; **Fl** = seed flour; **L** = leaf; **S** = seed; **Sp** = sprout; **St** = stem, and **WP** = whole plant.

Table 2. Diversity of phenylpropanoid metabolites reported in different *Amaranthus* species (modified and amended from [3])

A recent study reported a significantly variable content of bioactive substances and phenolic contents in leaves of various cultivars of *A. tricolor* and *A. hypochondriacus* [49]. For instance, leaf color attributes and betacyanins varied widely among the cultivars. Also, the hyperoside flavonoid was found only in one *A. hypochondriacus* cultivar, in contrast to isoqercetin and rutin, which were abundantly found in all amaranth cultivars examined. SA, syringic, gallic, vanilic, ferulic, p-coumaric, and sinapic acids were also common phenolic acids detected in all amaranth cultivars, whereas significant amounts of ellagic and sinapic acids were only detected in *A. hypochondriacus* cultivars. Lastly, total phenol content was found to be strikingly greater than total phenol index in *A. tricolor*. The free phenolic acid profile in seed ethanol extracts isolated from *A. caudatus* and *A. paniculatus* was also found to be significantly different [115]. However, the differences observed had only a slight influence on their antioxidant activity. A subsequent study performed with seeds of *A. cruentus* showed that processing (i.e., popping or flaking) and cultivation area had significant effects on their total phenolic acid content, whereas differences in individual phenolic acids (e.g., ferulic acid in processed seeds) were highly variable and were found not to have statistical significance [116]. A similar influence of climatic and agro-technical factors on the polyphenol content of amaranth seeds was described prior to this report [117].

In this study, the levels of 11 different polyphenols, including three flavonoids, i.e. rutin, isoquercitrin, and nicotiflorin, and 8 phenolic acids, i.e., protocatechuic, vanillic, 4-hydroxybenzoic, p-coumaric, syringic, caffeic, ferulic, and salicylic acids, were analyzed in mature seeds of 18 *Amaranthus* genotypes, including *A. cruentus*, *A. hypochondriacus*, *A. mantegazzianus*, and one grain amaranth hybrid. All were cultivated in three different countries in two continents and in two different locations within the same country. Interestingly, the results derived from principal component analysis (PCA) showed that varying environmental conditions had a contrasting effect on the rutin and nicotiflorin flavonoid levels in the seeds, which largely affected the former. In contrast to the above study, individual phenolic acids in seed samples, such as p-coumaric and protocatechuic acids, were found to be descriptors of climatic and other variations between the different locations studied. Besides, genotype-dependent effects were also observed, since polyphenols content in *A. hypochondriacus* displayed the lowest variation between cultivation sites and the highest content of flavonoids. Comparable results were obtained from the analysis of the aerial tissues of *A. mantegazzianus* and the grain amaranth hybrid plants grown in the same locations. However, this study included the determination of additional compounds such as hydroxycinnamyl amides (N-trans-feruloyltyramine and N-transferuloyl- 4-O-methyldopamine) and betaines (glycinebetaine and trigonelline) [118].

Once more, PCA clearly identified that samples from one location (i.e., in Argentina) differed from all other experimental sites by having a higher content of most compounds analyzed. Phenolic acids were, once again, a key group of compounds since their analysis permitted the separation of the different experimental groups, while separation of both amaranth genotypes could be performed primarily by the higher contents of trigonelline and the two hydroxycinnamyl amides present in *A. mantegazzianus*. Additionally, the contents of polyphenols and betaines in the aerial parts of grain amaranth were found to be very dependent on growing

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conditions. Further analysis revealed that trigonelline and the two hydroxycinnamyl amides could be tentatively used for chemo-taxonomic classification. Mention must be made of the presence of these and other cinnamoylphenethylamines (i.e., caffeoyltyramine, feruloyldopamine, sinapoyltyramine, p-coumaroyltyramine, and feruloyl-4-O-methyldopamine), which had been just previously reported in the Amaranthaceae for the first time [119]. Feruloyldopamine was different from the other cinnamoylphenethylamines detected by its seemingly ubiquitous presence in the genus *Amaranthus* and to its moderate antifungal activity. In addition to these effects, cinnamoylphenethylamines were previously associated with various other biological activities, such as the potentiation of antibiotics and inhibition of prostaglandin biosynthesis. In the same context, a recent study also determined that leaves and flowers of *A. hybridus*, as well as their extracts, possessed higher antioxidant activities compared to stems and seeds. An on-line HPLC- 2, 2-diphenyl-1-picrylhydrazyl radical assay determined that rutin was the main radical scavenger in these amaranth tissues/extracts, compared to other phenolic compounds detected, such as nicotiflorin, isoquercitrin, 4-hydroxybenzoic, and p-coumaric acids [38].

The influence of other experimental effects on polyphenol levels, such as tissue type, ripeness, or time of harvest was demonstrated by results obtained from a two-year field study performed with various grain and foliar amaranth species, in addition to two amaranth hybrids [120]. The tissue-type effect was clearly demonstrated by results that showed a more than 300-fold difference in rutin content between seeds and leaves. This study also showed that rutin was predominantly found in mature amaranth leaves, in accordance with previous reports describing a progressive accumulation of rutin in maturing amaranth and other rutin-accumulating plants, such as common buckwheat. Genotype-dependent effects were again observed, since noticeable variations between the species and even between the varieties belonging to the same species were detected. For instance, the highest rutin contents found in *A. retroflexus* leaves examined just before harvest contrasted with those measured in *A. tricolor*, which had approximately 12-fold and 67-fold lower rutin contents in leaves and flowers of plants sampled at the same developmental stage, respectively.

Other environmental factors, such as light, or the lack of it, have been also found to selectively influence the level of certain phenolic compounds. Thus, growth of *A. cruentus* sprouts in plain daylight had no effect on gallic acid content but increased the amount of rutin, whereas growth in darkness led to the accumulation of vitexin and isovitexin [121]. In related studies, the antioxidant activity and related color parameters were analyzed in *A. tricolor* and other pigmented leafy vegetables when grown under different photoperiods and light intensities. Betacyanin, chlorophyll, total polyphenol, and antioxidant activity peaked in leaves of *A. tricolor* plants maintained under a 12 h photoperiod but were severely reduced when exposed to constant light for 24 h. The quality and intensity of the light were also found to be significant factors, since shading by blue polyethylene sheets increased betacyanins, polyphenols, and antioxidant activity [122–124]. Sample preparation and the subsequent extraction and measurement protocols are factors that are also known to significantly affect the analyses of polyphenols content in amaranth species. Several studies, for example, have reported a wide range of tannin content in seeds and leaves of various grain and vegetable amaranth species [125,126].

Aside from expected genotype- and tissue-related variations, the differences observed were also suggested to be caused by the analytical methods employed for tannin content determination. Posterior processing procedures (e.g., roasting of seeds, cooking, or blanching of leaves, etc.) were also found to be significant factors affecting tannin content in amaranth seeds or leaves. Subsequently, a pertinent study assessed that the nutraceutical value of leaves of *A. cruentus* and *A. hybridus*, measured in terms of *in vitro* antioxidant (provided mostly by the presence of polyphenols, tannins, flavonoids, and betalains) and xanthine oxidase inhibitory activities, was highly dependent on the type of solvents used for extraction [47]. Similar variations were obtained in another study in which the combined effect of the extraction method and type of solvent on the antioxidant capacity and total phenolic content of extracts from seeds or leaves of *A. hypochondriacus* was evaluated [127]. In this respect, the extraction of phenolic compounds from seeds is believed to be affected by the complex interaction existing between phenolic acids and their cell wall constituents. For example, an investigation of the association of ferulic acid, an alkali-extractable phenolic acid, with the dietary insoluble fiber and non-starch polysaccharides of seeds of *A. caudatus* led to the identification of three complex compounds: O-(6-O-trans-feruloyl-β-D-galactopyranosyl)-(1 → 4)-D-galactopyranose, O-(2-O-transferuloyl-α-L-arabinofurano-syl)-(1 → 5)-L-arabinofuranose, and O-α-L-arabinofuranosyl-(1 → 3)-O-(2-O-trans-feruloyl-α-Larabinofuranosyl)-(1 → 5)- L arabinofuranose. This study also demonstrated that ferulic acid in amaranth seed cell walls is predominantly bound to pectic arabinans and galactans [128]. Additionally, the presence of ferulate associations with polysaccharides of dietary fibers could be considered to have taxonomic potential based on a previous study in which ferulate cross-links in the cell walls of dietary fibers were found to be restricted to species belonging to families of the Caryophyllales [129].

Two recent reports focused on direct or indirect changes in polyphenolic content in grain amaranth plants exposed to different stress conditions. The first one determined changes in the abundance of 3 flavonoid glucosides (rutin, nicotiflorin, and isoquercitin), 9 phenolic compounds (coumaric, vanillic, caffeic, syringic, ferulic, sinapic, protocatechuic, salicylic, and 4-hydroxybenzoic acid) and 3 betalains (amaranthine, iso-amaranthine, and betanin) in leaves of five varieties of three grain amaranth species subjected to insect folivory, in a one-year field trial [44]. Multivariate regression analysis revealed significant and predictable differences in the chemical composition of the leaves between grain amaranth genotypes. A similar analytical approach indicated that 8 of the 15 compounds analyzed in the plants, including all 3 flavonoid glucosides, 2 betalains, and 3 phenolic acids, had significant linear relationships with insect herbivory in the field. However, the experiment was not designed to determine biological relevance of the herbivory-induced accumulation of some of these metabolites in amaranth leaves. Thus, the possibility that phenolics could have been acting as feeding deterrents, phagostimulants, digestion inhibitors, digestion stimulants, toxins, toxicity reducers, signal inhibitors, and/or signal transducers in damaged grain amaranth remained unanswered [130]. In potato (*Solanum tuberosum*), for instance, rutin is known to accumulate to high levels only in varieties which are resistant to *Pectobacterium atrosepticum*, a very destructive necrotrophic bacterial pathogen. On the other hand, rutin is considered to be a susceptibility factor with respect to *Phytophthora infestans* infections [131].

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In a related proteomic study, the upregulation of transcription factors (i.e., DOF and MIF) was found to be coupled with the downregulation of caffeic acid O-methyltransferase, an isoflavone reductase-like protein, and two different S-adenosylmethionine synthetases, which are enzymes related to secondary metabolism associated with flavonoid and lignin synthesis [23]. Based on these results, the authors suggested that repressed root growth in grain amaranth plants subjected to severe drought is an adaptive response occurring in response to decreased root lignification. This proposal is in accordance to other reports showing that roots of plants exposed to different stresses may change their lignin content and composition [132]. One possible advantage derived from reduced lignification of the roots, particularly in the elongation zone, is that it may facilitate growth recovery once drought stress has been alleviated [133].

3.4. Secondary metabolism biosynthetic pathways and related genes in amaranth: Wandering into unknown territory?

The information provided above indicates that a potentially high nutritive and medicinal benefit may be derived from the consumption of amaranth seeds and foliage, which are high in antioxidant phenolic compounds, among other health-enhancing constituents. Until recently, information about the biosynthesis of these bioactive compounds and of the genes coding for the respective biosynthetic enzymes was practically null in amaranth. However, a recent transcriptomic study of grain amaranth leaves subjected to various stress treatments [14] revealed the presence of several genes involved in secondary metabolism, mostly in the phenylpropanoid biosynthetic pathway. The rest of this chapter will concentrate on the description of their characteristics and possible functions.

The transcriptomic study permitted the identification of 95 genes that code for the enzymes that most probably form part of the secondary metabolite biosynthetic flow in *A. hypochondriacus*. These are shown in Table 3. The transcriptomic data also uncovered the presence of several TFs that could be involved in the regulation of distinct branches of the secondary metabolism biosynthetic processes in grain amaranth, including the phenylpropanoid pathway.

Metabolic Group	Putative gene	[4]No	Function
ID[1]	Indole 3-glycerol phosphate lyase (*AhIGL*)	1	Emission of volatile compounds related to defense [169].
ID[1]	Anthranilate synthase (*AhANS*)	2	Production of tryptophan pathway metabolites as part of an Arabidopsis defense response (e.g., camalexin) [170, 171].
ID[1]	*AhWRKY33*, transcription factor	1	Pathogen-induced transcription factor; camalexin biosynthesis [172].
A[2]	Berberine bridge enzymes (*AhBBE*)	3	Upregulation of these genes was observed at specific stages of development and in response to osmotic stress and pathogens attack [173,174].
A[2]	Strictosidine synthase like (*AhSSL*)		The four SSL-coding genes in Arabidopsis are regulated individually, suggesting specific roles in

Metabolic Group	Putative gene	[4]No	Function
			basal (SSL4) and inducible (SSL5-7) plant defense mechanisms [175].
A[2]	Tropinone reductase (*AhTR*)	4	The *TR-I* gene expression was found to be upregulated on exposure to signal molecules (MeJA and SA) and in response to mechanical injury [176].
A[2]	Hyoscyamine 6-hydroxylase (*AhH6H*)	2	The overexpression of *H6H* increases scopolamine production [177].
T[3]	Acetoacetyl-CoA thiolase (*AhAACT*)	2	The AACT is a regulatory enzyme in isoprenoid biosynthesis involved in abiotic stress adaptation [178].
T[3]	3-Hydroxy-3-methyl-glutaryl-CoA reductase (*AhHMGR*)	3	HMGR activity induced by wounding, elicitor, or pathogen challenge is correlated with increased *HMGR* mRNA. The mutant *hmgr1* shows early senescence and sterility, as well as a dwarf phenotype [179, 180].
T[3]	1-Deoxy-xylulose 5-phosphate synthase (*AhDXS*)	1	*DXS* genes type II been proposed to be primarily involved in defense responses and secondary metabolism [181, 182].
T[3]	Sesquiterpene synthase (*AhSS*)	2	Involved in indirect defense of maize against herbivore attack [183].
T[3]	Squalene synthase (*AhSqS*)	1	The downregulation of *SqS* reduced stomatal conductance and conserved more moisture; it also led to increased grain yield and improved drought tolerance [184].
T[3]	Squalene epoxidase (*AhSqE*) y β-amyrin synthase (*AhβAS*)	4	The synthesis of saponins and expression of respective genes (*squalene synthase*, *squalene epoxidase*, and *β-amyrin synthase*) is induced in response to elicitors or MeJA treatment [185,186].
T[3]	*AhWRKY1*, transcription factor	1	The orthologous genes, *GaWRK1*, regulates the expression of δ-cadinene synthase needed for the synthesis for phytoalexin gossypol. This gene is also part of the defensive responses to pathogens [187,188].

[1] ID: Indole derivates

[2] A: alkaloids

[3] T: terpenes

[4] Number of genes detected in the grain amaranth transcriptome [14]

Table 3. Secondary metabolism genes identified in grain amaranth

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In this respect, the phenylpropanoid pathway is the best represented, with more than 69.5% of the above 95 biosynthesis-related genes coding for enzymes related to their biosynthesis [14]. Their position in the intricate phenylpropanoid biosynthetic reaction pathway is shown in Figure 1. Many of these genes have been amply characterized in other plant species. However, those described below code for enzymes that should be highly active, considering that, as mentioned in previous sections of this chapter, amaranth plants have been frequently found to be unusually rich in these compounds (Table 4). Thus, these particular grain amaranth enzymes and/or genes could have attractive biochemical and/or regulatory properties that could offer potentially important biotechnological applications. In addition, four TFs similar to those described above as key regulators of this biosynthetic pathway are described.

Flavonoids (μg/mg)	***Zea mays***	***Sorghum sp.***	***Amaranthus sp.***
Catequin	0.15-0.23	0.07-0.22	-
Rutin	0.38-0.47	0.17-0.30	4.0-10 (4798-15531)*
Quercetin/isoquercetin	-	0.1-0.65	0.3-0.5 (27.8-279.5)*
Nicotiflorin	-	-	4.8-7.2 (141-1281)*

*Numbers in parentheses indicate the quantity of these compounds detected in leaves

Table 4. Flavonoid content in amaranth, compared to other grain-producing C4 species

As already mentioned, PAL catalyzes the first committed step of the phenylpropanoid pathway, which is shared by all compounds produced by downstream ramifications of the pathway (Figure 1). It also represents a bifurcation point between primary and secondary metabolism. Diverse environmental stimuli and developmental programs regulate PAL. It is induced by lignin demands for cell wall fortification and by both biotic (e.g., pathogen and insect damage) and abiotic stresses (e.g., UV irradiation, low temperatures, and nutrient deficiency) [134,135]. PAL activity has been found in all the higher plants analyzed so far, and in some fungi and a few bacteria, but not in animals. In all species studied, *PAL* is part of a multigenic family. For instance, four *PAL* genes have been described in Arabidopsis, five in *Populus tricocharpa* and tomato (*Solanum lycopersicum*) and sixteen in potato (*S. tuberosum*). Two genes have been identified so far in *A. hypochondriacus* [14]. In Arabidopsis, the induction of *PAL* genes is closely related to a defense-related accumulation of flavonoids [136]. Similarly, the induced expression of *AhPAL1* in *A. hypochondriacus* and *A. cruentus* plantlets pre-treated with the BTH, a priming agent, and subsequently infected by *Clavibacter michiganensis* or various *Pseudomonas syringae* pathovars, all of which are opportunistic but highly damaging aggressors of grain amaranths, could contribute the increased resistance observed [15]. However, the exact relationship of this gene with defense-related accumulation of phenylpropanoid compounds and/ or cell wall fortification via lignin incrustations has not been evaluated.

A number of R2R3 MYB transcription factors are known to be able to transactivate *PAL* promoters to control the tissue-specific expression of this gene. An *in silico* analysis of the 64

MYB TF identified in grain amaranth revealed, however, that only five of them are candidates to participate in the phenylpropanoid pathway. In common with orthologs identified in other plants species (Figure 2), these amaranth TFs may also activate the phenylpropanoid pathway via the activation of *PAL* expression [135]. Available transcriptomic data support their role in the regulation of stress responses. Thus, AhMYB5 (Figure 2A) was found to be induced by bacterial infection, which coincided with the participation of AtMYB44, its homolog in Arabidopsis, in the regulation of resistance responses against pathogens and/or water and salt stress [14,137,138]. However, nothing is known in amaranth regarding the interaction of these MYB TFs with other regulatory components, such as certain bHLH and WD40 proteins. Further *in silico* analysis of amaranth transcriptomic data revealed the presence of 50 genes belonging to either of the two gene families. However, only AhbHLH3, whose nomenclature is indicative of its similitude with a similarly named ortholog TF present in *Beta vulgaris* (Figure 2B), was found to have the potential to participate in the regulation of the phenylpropanoid pathway in addition to a possible role in the control of the JA-dependent wound response, via its potential interaction with JAZ proteins [139].

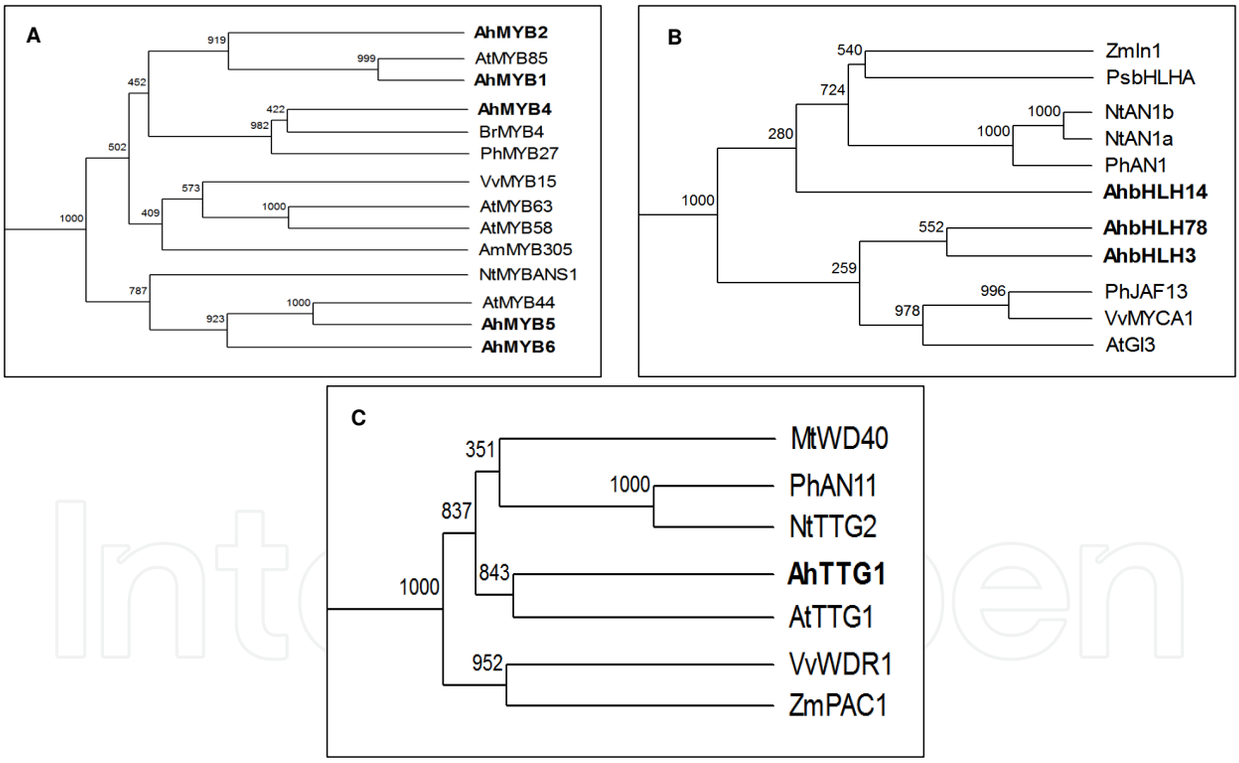

Figure 2. Phylogenetic trees of transcription factors from amaranth and other species: MYB (A), bHLH (B), and WD40 (C). *Amaranthus hypochondriacus*: AhMyb1, AhMyb2, AhMyb4, AhMyb5, and AhMyb6; AhbHLH3, AhbHLH14, and AhbHLH78, and AhTTG1. *Arabidopsis thaliana*: AtMYB85 = AEE84639, AtMYB63 = NP_178039.1.1, AtMYB58 = EFH69169.1, AtMYB44 = NP_201531, AtGL3: NP_680372, AtTTG1: AED93321.1; *Brassica rapa*: BrMYB4 = ADZ98868.1; *Petunia hybrida*: PhMYB27 = AHX24372.1, PhAN11: AAC18914, PhAn1: AAG25927, PhJAF13: AAC39455; *Vitis vinifera:* VvMYB15 = AHA83524.1, VvMYCA1 = ABM92332, VvWDR1 = ABF66625; *Zea mays*: ZmIn1 = AAB03841, ZmPAC1: AAM76742; *Pisum sativum*: PsbHLHA = ADO13282; *Nicotiana tabacum*: NtAN1a = AEE99257; NtAN1b = AEE99258, NtTTG2 = ACN87316; *Medicago truncatula*: MtWD40: ABW08112

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Correspondingly, the *AhTTG1* gene, which codifies for a protein having a conserved WD40 domain, shared 77% homology with the Arabidopsis *AtTTG1* gene (Figure 2C). Based on the observed similarity, it could be hypothesized that AhTTG1 could participate in flavonol (quercetin)-dependent developmental processes, such as the control of root growth under abiotic stress conditions [140].

Cinnamate 4-hydroxylase (C4H) is the second key enzyme in the phenylpropanoid pathway and catalyzes the hydroxylation of *trans*-cinnamic acid to *p*-coumaric acid, which is the biosynthetic precursor of flavonoids, phytoalexins, lignin, pigments, and many other defense-related compounds. C4H belongs to the large CYP73 family of cytochrome P450 monooxygenases (P450s). In addition to its central role in the phenylpropanoid biosynthetic pathway, it is also involved in the biosynthesis of various other compounds (e.g., fatty acids, alkaloids, and terpenoids) and may participate in the detoxification of herbicides and pesticides. In accordance with many key biosynthetic genes, *C4H* genes form extensive multi-gene families in various plant species, including *Populus*, orange, and pea [141-143]. Several studies have shown that *C4H* genes have a tissue-specific expression pattern, and that similar to *PAL* genes, they may be also induced by wounding, pathogen infection, and nutrient deficiency [144–147]. Four genes, *AhC4H1-4*, have been identified in grain amaranth. Based on transcriptomic and other data, only the *AhC4H2* is induced by (a)biotic stress [14,134]. However, nothing is known regarding its participation in the synthesis of phenylpropanoids, and perhaps other compounds, in grain amaranth.

The enzyme 4-coumaric acid CoA ligase (4CL) plays an important role in the biosynthesis of lignin precursors such as hydroxycinnamate-CoA thioesters. The *4CL* gene is differentially expressed during development in various tissues and presents multiple isoforms with different substrate specificities. In Arabidopsis, four 4CL isozymes have been identified. Of these, 4CL1 and 4CL2 are known to be involved in the lignin biosynthesis, while 4CL3 participates in flavonoid and other non-lignin biosynthesis pathways. In *P. trichocarpa*, 17 genes showing sequence similarity with known *4CL*s were identified, whereas a similar 4CL1 enzyme was detected in developing xylem tissues of *P. tremuloides*. In addition, *Ptr4CL2* was proposed be involved in flavonoids biosynthesis. Their role in lignin biosynthesis was demonstrated by reports showing that the downregulation of 4CL1 in Arabidopsis, 4CL1 in poplar, and 4CL3 in rice resulted in reduced lignin content [134]. The *4CL* gene family is divided into two main groups. Group II contains those genes which are associated with flavonoid biosynthesis, including two that were identified in grain amaranth: *Ah4CL2-3* (Figure 3). Conversely, the *Ah4CL1* gene could be part of group I and, thereby, participate in lignin biosynthesis (Figure 3). Numerous studies have reported the induction of *4CL* genes by wounding, UV radiation, and pathogen infection in potato, soybean, Arabidopsis, and rice [148–153].

Chalcone synthase (CHS) is another key enzyme of the flavonoid/ isoflavonoid biosynthesis pathway. Besides being part of the plant developmental program, *CHS* gene expression is induced in plants exposed to diverse stress conditions such as UV light, excess salinity, insect herbivory, and bacterial or fungal infection. *CHS* expression leads to the accumulation of flavonoid and isoflavonoid phytoalexins and is involved in the SA defense pathway. Multiple

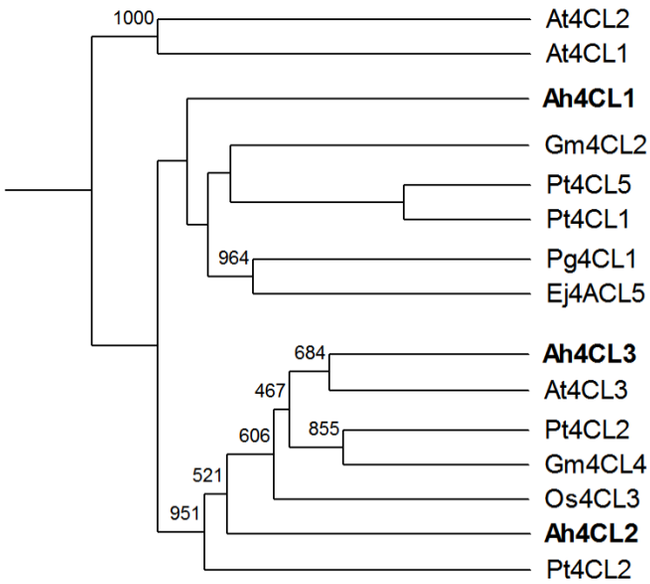

Figure 3. Phylogenetic tree of genes encoding 4-coumarate CoA ligase from amaranth and other species. *Amaranthus hypochondriacus*: AhCL1, AhCL2, and AhCL3. *Arabidopsis thaliana*: At4CL1 = U18675, At4CL2 = B1GUZ3, At4CL3 = AY376730; *Glycine max:* Gm4CL2 = X69954, Gm4CL4 = X69955; *Populus tremuloides:* Pt4CL1 = AF041049, Pt4CL2 = AF041050, *Populus* trichocarpa: Pt4CL5 = EU603299; *Populus generosa*: Pg4CL1 = AF008184; Ej4ACL5 = EF685345; *Oryza sativa:* Os4CL3 = L43362; *Pinus taeda*: Pt4CL2 = U12013.

copies of the CHS gene have been detected in several plants including *Gerbera hybrida*, *Petunia hybrida*, *Ipomoea sp.*, *Cannabis sativa*, and *Pisum sativum*. In contrast to grain amaranth, in which two CHS genes (*AhCHS1-2*) have been identified, only single copies of the *CHS* gene have been found in Arabidopsis, parsley, and snapdragon. The structural and catalytic domains present in the hypothetical AhCHS1-2 enzymes are shown in Figure 4. Chalcone isomerase (EC 5.5.1.6), CHI, is one of the most important intermediate enzymes in the flavonoid pathway whose activity involves the modification of substrates previously synthesized by CHS. Although these modifications can occur spontaneously, the efficiency of the reactions are 10^7-fold higher if catalyzed by CHI. A consequence of CHI activity is the typical lack of chalcones and naringenin chalcone in plants, due to their rapid isomerization to naringenin by this enzyme. However, other reactions such as the isomerization of 6-deoxychalcone to 5-deoxyflavanone are rather slow because of the intramolecular hydrogen bond in the substrate molecule. CHIs are classified into two types, and their distribution is highly family-specific. CHIs generally found in non-legumes exclusively catalyze the isomerization 6-hydroxychalcone to 5-hydroxyflavanone. CHIs with this catalytic function are referred to as type I CHIs. On the other hand, most, if not all, of the CHIs found so far in leguminous plants (referred to as type II CHIs) recognize both 6-deoxychalcone and 6-hydroxychalcone as substrates, yielding 5-deoxyflavanone and 5-hydroxyflavanone, respectively. Although more additional information is needed, available data indicate that each of the two *AhCHI* genes (i.e., *AhCHI1-2*) identified in grain amaranth may belong to the different types described, as shown in Figure 5. This characteristic could allow the synthesis of an increased diversity of phenylpropanoid metabolites in amaranth and in related species [134].

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AhCHS1          LKFLDWVKKLLLRPSRSGANLSQKITHVIMCTTSGVDMPGADYQLTKLLGLRPSVRRFML
AhCHS2          ------------------------------------PGADYQLTKLLGLRPSVRRFML
MsCHS           ----------------------ITHLIVCTTS-VDMPGADYQLTKLLGLRPYVKRYMM

AhCHS1          YQQGCFAGGTVLRLAKDLAENNRGARVLVVCSEITAICFRGPTETHLDSMVGQALFGDGA
AhCHS2          YQQGCFAGGTVLRLAKDLAENNRGARVLVVCSEITAICFRGPTETHLDSMVGQALFGDGA
MsCHS           YQQGCFAGGTVLRLAKDLAENNKGARVLVVCSEVTAVTFRGPSDTHLDSLVGQALFGDGA

AhCHS1          GALIVGADPDELIERPLFKMVWTAQTILPDSEGAIDGHLREVGLTFHLLKDVPGLISKNI
AhCHS2          GALIVGADPNEPIERPLFKMVWTAQTIIPDSEGAIDGHLREVGLTFHLLKDVPGLISKNI
MsCHS           AALIVGSDPVPEIEKPIFEMVWTAQTIAPDSEGAIDGHLREAGLTFHLLKDVPGIVSKNI

AhCHS1          NKALEDAFLPLGINDWNSIFWIAHPGGPAILDQVEQKLGLNEEKLTATRNVLRDYGNMSS
AhCHS2          NKALEDAFNPFGINDWNSIFWIAHPGGPAILDQVEEKLGLDKEKLKATRNVLRDYGNMSS
MsCHS           NKALVEAFEPLGISDYNSIFWIAHPGGPAILDQVEQKLALKPEKMKATREVLSEYGNMSS

AhCHS1          ACVLFILDEMRKKSSEEGKATTGEGLDWGVLFGFGPGLTVETVVLHSVPLNY-
AhCHS2          ACVLFILDEMRKKSCEDGKSTTGEGLHWGVLFGFGPGLTVETVVLQSVPIAET
MsCHS           ACVLFILDEMRKKSAQDGLKTTGEGLEWGVLFGFGPGLTIETVVLRSVTI---
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Figure 4. Alignment of chalcone synthase enzymes identified in grain amaranth (AhCHS1-2) and *Medicago sativa* (MsCHS). The amino acid residues found in the catalytic site are shown in red, whereas those that compose the structural domains are highlighted in yellow [203].

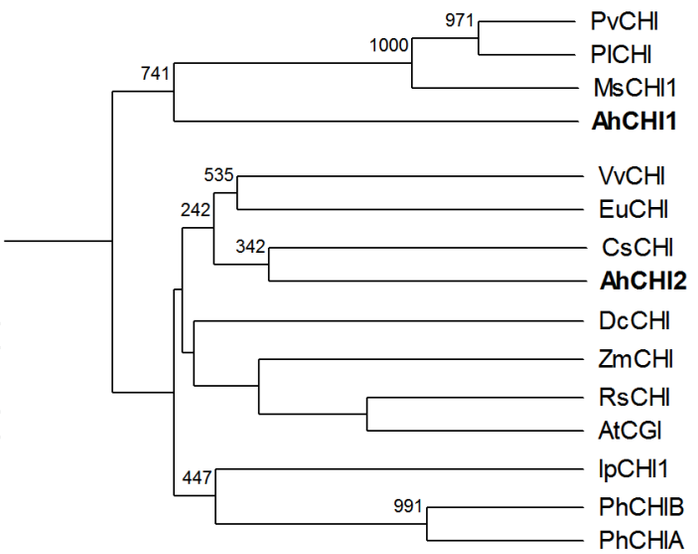

Figure 5. Phylogenetic tree of genes encoding chalcone isomerase from amaranth and other species. *Amaranthus hypochondriacus*: AhCHI1 and AhCHI2. *Zea mays:* ZmCHI = Z22760.1; *Medicago sativa*: MsCHI1 = M31079.1; *Pueraria lobata:* PlCHI = D63577.1; *Phaseolus vulgaris*: PvCHI = XM_00712628.1; *Arabidopsis thaliana*: AtCGI = M86358; *Raphanus sativus*: RsCHI-AF031921.1; *Petunia* hybrida: PhCHIA = AF233637.1, PhCHIB = X14590.1; *Ipomoea purpurea*: IpCHI1 = AF028238.1; *Dianthus* caryophyllus: DcCHI = Z67989.1; *Elaeagnus umbellata*: EuCHI = AF061808.1; *Citrus sinensis* CsCHI = AB011794.1; *Vitis vinífera:* VvCHI = X75963.1.

Flavanone 3-hydroxylase (EC 1.14.11.9), F3H, is a key enzyme in the flavonoid biosynthetic pathway, catalyzing the 3-hydroxylation of (2S)-flavanones, such as naringenin to dihydroflavonols. In soybean seeds the downregulation of *F3H* is accompanied by increased accumulation of isoflavonoids [154]. Seven copies of the F3H gene have been identified in wheat, barley, and rye. F3H activity has been detected in young flower petals and its expression is associated with disease resistance in plants [155]. The antisense repression of the *F3H* gene in carnation flowers (*Dianthus caryophyllus*) and strawberries reduced anthocyanin levels while compounds such as methyl benzoate and 2-hydroxymethyl benzoate (responsible for the flower's fragrance) or polyphenols, including p-coumaroyl-glucose and pcoumaroyl-1-acetate among many others, were found to accumulate [156,157].

Tolerance to UV radiation and severe water deprivation in the extremophyte *Reaumuria soongorica* was associated with increased expression and enzymatic activity of F3H. This increase was also correlated with flavonoid accumulation in consequent antioxidant activity [155]. In grain amaranth, the *AhF3H1* gene, sharing a 77% of homology with the *F3H* gene in *R. soongorica*, was identified. This finding suggests that this gene could be an important factor in the proposed role played by the phenylpropanoid pathway in the tolerance mechanisms used by extremophytes, many of which belong the Caryophyllales order, to thrive in the highly adverse environmental they inhabit [1,158-160]. The use of this gene, and perhaps others related to the phenylpropanoid biosynthetic pathway, offers the attractive possibility of their application for biotechnological purposes in commercial crops.

Flavonol synthase (EC 1.14.11.23), FLS, is another relevant enzyme due to its crucial participation in the conversion of several precursors needed for different branches of the flavonoid biosynthesis. For instance, the biosynthesis of flavonols from dihydroflavonols is catalyzed by FLS, a soluble 2-oxoglutarate-dependent dioxygenase (2-ODD) (Figure 6). The expression is tissue- and organ-specific organ and is regulated by various light intensities, pathogen infection, and herbivore attack [134,161–163]. Silencing of the *FLS* gene in tobacco led to suppression of flavonol content through a decrease in transcript level of flavonol synthase. Moreover, silencing of flavonol synthase diverted the pathway toward catechin and epicatechin production through enhanced expression of genes encoding dihydroflavonol reductase (DFR) and anthocyanidin synthase (ANS). Curiously, it also increased the activity of ROS-detoxifying enzymes such as glutatione reductase (GR), APX, and CAT. Grain amaranth transcriptomic data showed that a related gene, *AhFLS2*, was induced by both drought and salinity stress. The above information suggests that this gene is a promising candidate for crop improvement, acting as a controller of stress-related responses, perhaps by its participation in the biosynthesis of cathequin and the regulation of other protective biochemical processes that could help ameliorate oxidative stress in plants [164].

Dihydroflavonol 4-reductase (DFR, EC 1.1.1.219), DFR, is a pivotal enzyme in the flavonoid biosynthetic pathway that plays a crucial role in producing simple and condensed anthocyanins. This enzyme catalyzes the production of flavan-3, 4-diols (leucoanthocyanidins) via the reduction of three colorless dihydroflavonols, i.e., dihydrokaempferol, dihydroquercetin, and dihydromyricetin. These compounds are also intermediates of flavonol biosynthesis, occurring through the flavonol synthase reaction. These leucoanthocyanidins are subsequently

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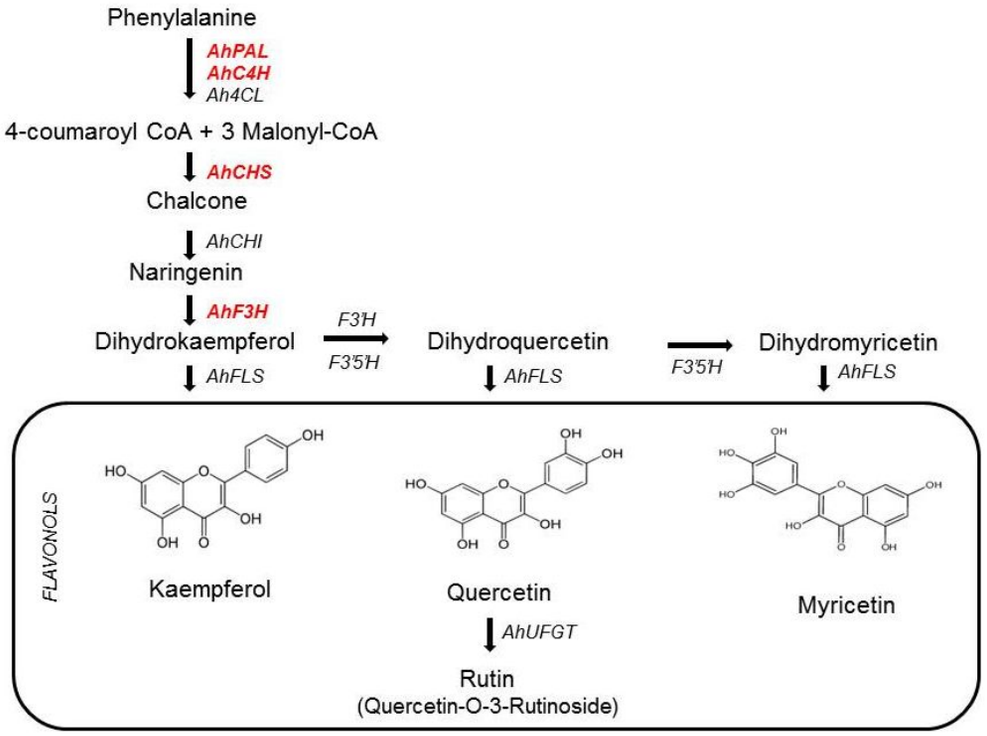

Figure 6. Flavonol biosynthesis in plants. The figure is modified from the original version in [136]. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; FLS, flavonol synthase. The genes in red text represent those found to be induced by stress conditions in the grain amaranth transcriptomic analysis [14].

converted to pelargonidin, cyanidin, and delphinidin, respectively. DFR can accept wide range of substrates, although substrate specificity of DFR has been shown to vary depending on the specific types of anthocyanins that accumulate in a given plant species. In plants, DFR can be either present as a single gene or as a multicopy gene family. Single DFR genes have been found in *Arabidopsis*, grape, tomato, rice, snapdragon, rose, barley, buckwheat, and grain amaranth (*AhDFR*) while multicopy DFR genes have been found in *Vitis vinifera*, *Ipomoea purpurea*, *Populus*, lotus, and *M. truncatula*. Increased flower pigmentation has been observed by transformation of petunia with a heterologous DFR. Also, the expression of *DFR* has been shown to be spatially and developmentally regulated, may be organ-specific, and its induction leads to the accumulation of anthocyanins in different plant tissues. Furthermore, tobacco plants overexpressing *CsDFR* showed early flowering and significantly higher seed yields, in addition to increased resistance to insect herbivory and antioxidant potential [165]. Other external factors can modulate the expression of *DFR*, including light and UV radiation, exogenous sucrose or JA, and cold or freezing stress [134,166].

Leucoanthocyanidin dioxygenase (LDOX: 1.14.11.19), also called 2-oxoglutarate iron-dependent dioxygenase (2-ODD) or anthocyanidin synthase (ANS), is also involved in anthocyanin biosynthesis and catalyzes the conversion of colorless to colored leucoanthocyanidin. Expression of *LDOX* has been detected in different organs of Shiraz grapevine, such as leaves, roots, seeds, flowers, berry skin, and flesh. An *LDOX* cDNA has been cloned from *Arabidopsis* and

the *transparent testa18* and *transparent testa19* mutants were subsequently shown to be *ldox* mutants. In developing *V. vinifera* grapes, the expression of *LDOX* was detected both before and after the ripening stage [134]. The detection of two *LDOX* genes in grain amaranth, one of which (*AhLDOX1*) was responsive to bacterial infection, was intriguing, considering the taxonomic restriction of anthocyanin synthesis in the Caryophyllales.

As mentioned, lignin is the generic term for a large group of aromatic polymers that result from the oxidative combinatorial coupling of 4-hydroxyphenylpropanoids (Figure 7). These polymers are deposited predominantly in the secondary walls of thickened cells, making them rigid and impermeable. In addition to developmentally programmed deposition of lignin, its biosynthesis can also be induced upon various biotic and abiotic stress conditions, such as wounding, pathogen infection, metabolic stress, and perturbations in cell wall structure. Activation of the monolignol precursor biosynthesis in the apoplast requires the combined activity of enzymes such as peroxidases (POX), laccases (LAC), or other polyphenol oxidases that transfer electrons from monolignols to electron receptors. These apoplastic enzymes interact with ROS such as hydrogen peroxide or superoxide, which act as electron receptors or modulators of POX and LAC enzymes through their signaling function. Once oxidized, monolignol radicals can bind to other similarly formed radicals to form the three-dimensionally cross-linked structures that characterize lignin. This polymerization process constitutes the final step of lignin biosynthesis.

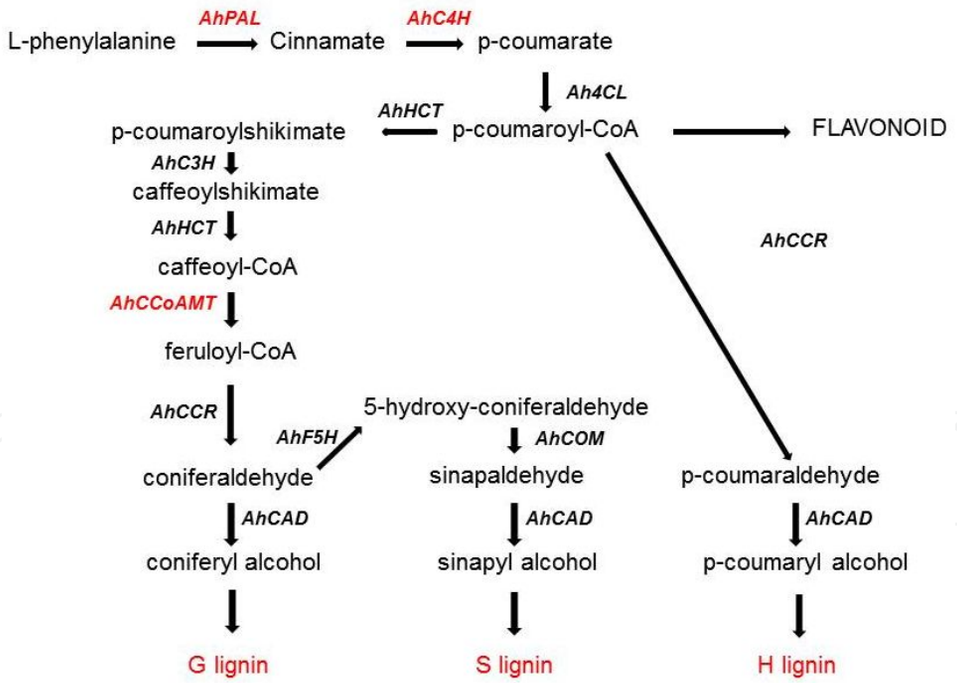

Figure 7. The main biosynthetic route toward the monolignols p-coumaryl, coniferyl, and sinapyl alcohol (the figure is modified from the original version [66]. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; C3H, p-coumarate- 3-hydroxylase; HCT, p-hydroxycinnamoyl-CoA: quinate/shikimate p-hydroxycinnamoyltransferase; CCoAOMT, caffeoyl-CoA o-methyltransferase; CCR, cinnamoyl-CoA reductase; F5H, ferulate 5-hydroxylase; COMT, caffeic acid O-ethyltransferase; CAD, cinnamyl alcohol dehydrogenase. The genes in red text represent those found to be induced by stress conditions in the grain amaranth transcriptomic analysis [14].

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Contrary to lignin, lignans represent a structurally diverse class of plant-specialized metabolites that are ubiquitously distributed in all land plants. They are presumed to have a predominantly defensive role *in planta*, considering several reports that have described their antibacterial and/ or antifungal activities. As such, lignans can act as both phytoanticipins and phytoalexins. In many tree species, the constitutive deposition of lignans in the heartwood is believed to reinforce durability, longevity, and resistance to many wood-rotting fungi. On the other hand, *de novo* formation of lignans in response to fungal attack has been reported in both woody and non-woody species [68]. Our transcriptomic-derived data indicate the possible presence of lignans biosynthetic genes in grain amaranth (Figure 8). This finding is in accordance with a recent report describing the deposition of these metabolites in seeds of *Amaranthus* species. The presence of lignans in amaranth seeds has been linked with some of its medicinal properties, considering the presumed antioxidant, antiviral, antitumoral, antibacterial, antifungic, insecticidal, estrogenic, and anti-estrogenic properties assigned to these particular secondary metabolites [3]. The flux of carbon to lignin biosynthesis can be seriously affected by changes in the expression levels of diverse other genes. Examples of a number of these genes, together with their respective homologs identified in grain amaranth, is described in Table 5.

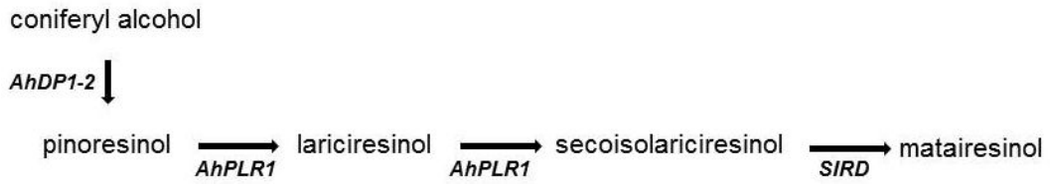

Figure 8. Lignan biosynthetic pathway. DP, dirigent protein; PLR, pinoresinol/lariciresinol reductase; SIRD, secoisolariciresinol dehydrogenase. Two lignan-biosynthesis-related genes were found in grain amaranth; two of them code for *DP* and one for PLR.

Coumarins may be subclassified as simple coumarins (benzo-α-pyrones syn 1, 2-benzopyrone), 7-oxygenated coumarins (furanocoumarins syn. furobenzo-α-pyrones or furocoumarins) or pyranocoumarins (benzodipyran-2-ones). Simple coumarins, furanocoumarins, and pyranocoumarins share the same biosynthetic pathway, whereas the most common phenylcoumarins (i.e., coumestans) originate from isoflavone. Coumarin is characterized for its pleasant vanilla-like odor. The presence of this metabolite has been reported in a diversity of plants, including members of the Fabaceae, Lauraceae, Lamiacea, Apiaceae, Asteracea, Rutaceae, and Amaranthaceae (*Spinoside*, isoflavone). However, the transcriptomic data yielded no gene related to the biosynthesis of these interesting compounds, probably because they were not inducible under the stress conditions employed. Nevertheless, search for these genes in amaranth should continue due to the great interest invested in coumarins due to their potent physiological, bacteriostatic, and anti-tumor activity [167, 168]. Moreover, it has been proposed that coumarins may be engineered to have novel therapeutic properties by further derivatization of their backbone structure.

Gene	[1]No. Seq.	Function in stress	Reference(s)
Cinnamoyl-CoA-reductase (*AhCCR*)	1	In wheat, this gene is involved in pathogen defense signaling. In tomato and other plants, the repression of this gene produced severe development-related phenotypes.	[189,190]
Cinnamyl alcohol dehydrogenase (*AhCAD*)	16	The silencing *CAD* led to changes in the lignin structure, fiber quality, and/or improved digestibility. It also led to development related phenotypes during vegetative and reproductive stages. Pathogen resistance was also modified. Phylogenetic analysis suggests that the *AhCAD2* gene could be involved in stress tolerance responses.	[189,191,192]
Caffeoyl CoA 3-O-methyltransferase (*AhCCoAOMT*)	3	This gene can be highly induced by drought and cold stress, suggesting a possible role in plant abiotic stress resistance. In maize and wheat, *CCoAOMT* is involved in the mechanism of resistance/susceptibility against fusariosis and powdery mildew, respectively.	[193–195]
4-Coumaroyl shikimate 3-hydroxylase/ p-coumarate 3-hydroxylase (*AhC3H*)	1	C3H downregulation led to a dramatic accumulation of several glucosides of *p*-coumaric acid, including phenylglucosides (populoside, grandidentatin, and trichocarposide). This modification may be an acceptable strategy to increase the secondary metabolite diversity against pathogens.	[196]
Caffeic acid O-methyltransferase (*AhCOMT*)	3	In wheat, silencing of this gene is linked to increased susceptibility to fungal pathogens, such as *Blumeria graminis*. Conversely, it led to an augmented resistance to *Hyaloperonospora arabidopsidis* in Arabidopsis.	[193,197]
Ferulic acid hydroxylase (*AhF5H*)	2	*F5H* transcripts accumulated in the leaves in response to mechanical wounding or the application of related elicitors, such as ethylene, ABA, and hydrogen peroxide (H_2O_2). *f5h* mutants were unable to develop normally under UV light, probably because of a lack of UV-radiation protective synapate esters. This gene is essential for expression of anthocyanin biosynthesis-associated genes and anthocyanin accumulation under photo-oxidative stress.	[198–201]
Hydroxycinnamoyl CoA: shikimate hydroxycinnamoyl transferase (*AhHCT*).	2	Downregulation of the HCT gene in alfalfa plants exhibited constitutive activation of defense responses, triggered by release of bioactive cell wall fragments and production of hydrogen peroxide generated as a result of impaired secondary cell wall integrity.	[202]

[1] Number of genes detected in the grain amaranth transcriptome [14].

Table 5. Lignin biosynthetic pathway genes identified in grain amaranth

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Finally, many stress-induced phenylpropanoids are classified as phytoalexins. These are antimicrobial compounds synthesized in response to pathogen attack. They include pterocarpans (e.g., glyceollin), isoflavans, prenylated isoflavonoids (e.g., kievitone), stilbenes, psoralens, coumarins, 3-deoxyanthocyanidins, flavonols (e.g., quercetin, kaempferol), and aurones. Most of these compounds have trivial names, such as the coumarins umbelliferone (7-hydroxycoumarin), esculetin (6, 7-dihydroxycoumarin), scopoletin (6-methoxy-7-hydroxycoumarin), and others. No phenylpropanoid phytoalexins have been reported in amaranth. Findings from a recent report [15] suggested, however, that they could be participating as part of the induced defense responses against bacterial pathogens produced by the application of defense-related inductors, such as BTH, JA, or the exposure to avirulent pathogens. This proposal is supported by the induced expression of *AhPAL* in addition to several other known defensive genes, including those coding for ROS-detoxifying enzymes and pathogenesis-related proteins.

4. Conclusion

The several hypotheses raised by the discovery of the numerous stress-related phenylpropanoid genes in grain amaranth represents a strong incentive for the initiation and subsequent deepening of secondary metabolite studies in *Amaranthus* plants, which may yield promising results in various areas of interest, including food science and nutrition, medicine and stress plant physiology, among others.

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