Metabolic Engineering of Phenylpropanoids in Plants

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Abstract

Phenylpropanoids are diverse group of active secondary metabolites derived from the carbon backbone of amino acids phenylalanine and tyrosine. The phenylpropanoid pathway serves as the starting point for the biosynthesis of a wide range of organic compounds such as lignins, flavanols, isoflavanoids, anthocyanins, and stilbenes with an array of important functions including plant defense and structural support. Besides, they have major nutritional and pharmaceutical properties that find uses as food supplements, antioxidants, flavoring agents, insecticides, dyes, and pharmacological drugs. Major structural and regulatory genes of the phenylpropanoid pathway and associated branches have been isolated and characterized in the recent times. Consequently, the engineering of phenylpropanoid biosynthesis in plants and other ex-host systems have generated considerable scientific and economic importance to enhance their production. In this chapter, we summarize the recent advances in our knowledge of the phenylpropanoid biosynthesis. In addition, we discuss the recent strategies with respect to genetic and metabolic engineering of different phenylpropanoids in plants and microorganisms for their successful industrial production in the future.

Keywords

Phenylpropanoids • Biosynthetic pathway • Metabolic engineering • *E. coli* • *S. cereviceae* • Heterologous expression • Transcription factors

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Abbreviations

4CL	4-Hydroxycinnamate CoA-ligase
ACC	Acetyl CoA carboxylase
ACS	Acetyl CoA synthase
AMP	Adenosine mono phosphate
ANS	Anthocyanidin synthase
C3′H	p-Coumaroyl-shikimate 3' hydroxylate
C4H	Cinnamate 4-hydroxylase
CAD	Cinnamyl alcohol dehydrogenase
CCR	Cinnamoyl CoA reductase
CHI	Chalcone isolmerase
CHS	Chalcone synthase
CiED	Cipher of evolutionary design
COMT	Caffeoyl CoA 3-*O*-methyltransferase
CPR	Cytochrome P450
DFR	Dihydrofavonol reductase
DMAPP	Dimethyl allyl pyrophosphate
F3H	Flavonone 3-hydroxylase
FLS	Flavonone synthase
FPP	Farnesyl pyrophosphate
GPP	Geranyl pyrophosphate
H_2O_2	Hydrogen peroxide
HCT	Hydroxysinnamoyl-CoA shikimate:quinate hydroxylcinnamoyltransferase
HID	2-Hydroxyisoflavanone dehydratase
IFS	Isoflavonone synthase
IPP	Isopentyl pyrophosphate
LAR	Leuco anthocyanidin reductase
PA	Proanthocyanidins
PAL	Phenylalanine ammonia lyase
PAP	Production of anthocyanin pigment
PLR	Pinoresinol/lariciresinol reductase

Contents

1 Introduction

Plants synthesize a variety of organic compounds which are mainly classified as primary and secondary metabolites. Primary metabolites including nucleotides, amino acids, organic acids, acyl lipids, and phytosterols have vital roles in essential biochemical processes like photosynthesis, respiration, growth, and development [1]. In contrast, secondary metabolites are phytochemicals that are specifically accumulated through particular biosynthetic pathways in a taxonomically restricted group of organisms [2]. Although the role of secondary metabolites in plant growth and physiology is not yet clear, they have a huge structural variation and accordingly exhibit a broad series of biological activities such as protection against pathogens and abiotic stresses [3, 4], acting as signalling molecules [5] and pheromones for insects and flies for pollination [6]. Besides, they are significantly used as major source for anticancer drugs, immunosuppressant, antibacterial and antifungal agents, herbicides, insecticides, flavoring agents, essential oils, dyes, and fibers [7–9].

Secondary metabolites are classified into one of a number of families, each having a particular structural characteristic arising from the way they have been built up. Terpenes and steroids are derived from dimethyl allyl pyrophosphate (DMAPP) and isopentyl pyrophosphate (IPP) precursors with five carbons [10]. These precursors' combines to yield geranyl pyrophosphate (GPP), leading to monoterpenes. Likewise, compounds derived from farnesyl pyrophosphate (FPP) lead to sesquiterpenes and two equivalents of FPP results in triterpenes. Polyketides and their derivatives are synthesized from acetate units derived from the acetyl coenzyme A [11].The acetate origin of these compounds leads to a predominance of even numbered carbon chains resulting in fatty acid structures. Polyketides often intersect with aromatic amino acid biosynthesis to generate phenylpropanoids [12]. They constitute a large variety of complex aromatic extractives with a phenolic backbone. Alkaloids constitute a broad range of secondary metabolites and characterized by the presence of a basic nitrogen-based heterocyclic ring within their molecules [13]. Besides, some carbohydrate and peptide molecules are also classified as secondary metabolites either forming core structures of other metabolites or act as independent modules [14, 15].

The phenylpropanoids are the most diverse group of physiologically active secondary metabolites derived from the carbon backbone of amino acids phenylalanine and tyrosine [16]. They are characterized by six carbon aromatic phenyl group and three carbon propene tail of cinnamic acid, the first product of the phenylpropanoids biosynthetic pathway [17]. They constitute a wide range of organic compounds such as lignins, flavanols, isoflavanoids, anthocyanins, and stilbenes [12] (Fig. 1). Although the phenylpropanoids are not directly required for growth and development, they are essential to plant survival by performing a wide array of important functions such as reinforcement of specialized cell walls [18], protection from ultraviolet radiation [19], protection from photo-oxidative effects [20], and symbiotic nitrogen fixation [21]. Besides, they have generated tremendous

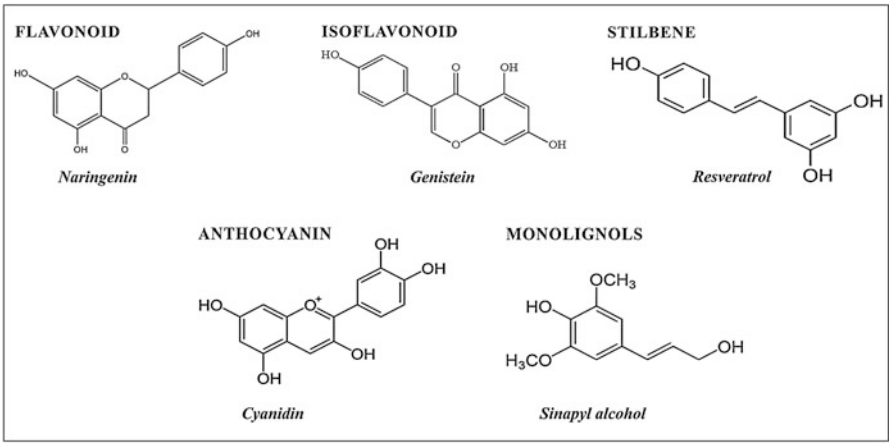

Fig. 1 Representative members of the major classes of organic compounds derived from the phenylpropanoid metabolism

commercial significance in the recent times due to their beneficial functions for human health including antimicrobial, immunomodulotary, and anticancerous attributes [22, 23]. Phenylpropanoids form an attractive system for genetic, enzymological, chemical, and molecular studies owing to their extensive availability, overwhelming diversity, and multiple functions. In recent years, the availability of large amount of information on phenylpropanoids biosynthesis and metabolism has provided the means to engineer their production in plants. Plants engineered with their phenylpropanoid profile could serve as a bridge for improving a large number of characteristics in different crop species such as improved pollination, superior commercial value of fruits and vegetables through better flavor and fragrance, enhanced resistance to diseases and abiotic stresses, and increased production of novel metabolites for medicinal and therapeutic use. Besides, alteration in the phenylpropanoid profile will also contribute to the fundamental aspects of their metabolism and regulation in plant system. Several attempts have been made to produce high levels of phenylpropanoids in transgenic plants of various species. For example, overexpression of *chalcone isolmerase* (CHI), a key enzyme of flavanoid biosynthesis, led to multiple fold increase on flavanoid levels in transgenic tomato [24]. Likewise, ectopic deposition of lignin was reported in *Populus tomentosa* through engineering the overexpression of *PtoMYB216*, a R2R3-MYB transcription factor associated with lignin biosynthesis [25].

In this chapter, we provide a concise outline of the advances in our understanding of the phenylpropanoid biosynthetic pathway in plants. We describe the recent work on the strategies of metabolic engineering of different phenylpropanoids to improve their production for different applications. Finally, we briefly overview the future prospects of engineering phenylpropanoids toward crop improvement and human health.

2 Biosynthesis of Phenylpropanoids

An intricate string of branching biochemical reactions constitutes the biochemical pathway of phenylpropanoids [26]. The plant shikimate pathway is the way in to the biosynthesis of phenylpropanoids through its end product, the aromatic amino acid phenylalanine. The difference in structures of plant phenylpropanoids is the result of various enzyme(s) complex actions involving methylation, acylation, hydroxylation, glycosylation, sulfation, aromatization, cyclization, and condensation reactions [27]. Phenylalanine ammonia lyase (PAL) catalyzes the oxidative deamination of phenylalanine to trans-cinnamic acid and initiates the carbon course from shikimate pathway to phenylpropanoids metabolism. Cinnamate 4-hydroxylase (C4H) mediates the hydroxylation of trans-cinnamate to p-coumaric acid which in turn is subjected to hydroxycinnamate CoA-ligase (4CL)-mediated thio-esterization to form p-coumaroyl-CoA. These three initial steps of the phenylpropanoids pathway catalyzed by PAL, C4H, and 4CL form the basis for all subsequent branches and resulting organic compounds (Fig. 2).

PAL is the first enzyme involved in the phenylpropanoid metabolism and has been extensively studied. PAL isoforms constitute a multigene family in various plant species such as four genes in *Arabidopsis thaliana*, five in *Populus trichocarpa*, and nine in *Oryza sativa* [28, 29]. Each of the individual PAL gene exhibits differential response to biotic and abiotic stresses, and their expression is highly variable across spatial regions and developmental stages [30]. In *Arabidopsis*, *PAL1*, *PAL2*, and *PAL4* are heterologously expressed at a higher level in the mature stems, while *PAL3* demonstrated a very low activity [31]. Considering the presence of specific promoter element in *PAL1* and *PAL2* genes, the enzyme encoded by them are considered to be the prominent one associated with *Arabidopsis* phenylpropanoid metabolism [16, 28, 29]. Usually PAL is a soluble enzyme residing in cytoplasm; however both cytoplasmic and membrane-bound localizations of several PAL isoforms have been reported [28, 32, 33]. Similarly, organ-specific expression analysis of poplar PAL genes revealed that three out of the five are active with the first one involved in lignin formation, the second one is specifically targeted for tannin formation, while the third one is associated with flowering [29, 34]. In contrast, as many as 20 *PAL* genes are associated with the phenylpropanoid biosynthesis in *Lycopersicon esculentum* although only one has been found to be constitutively expressed in all tissues [35]. The molecular mechanisms associated with the inactiveness of such a large number of PAL gene in tomato is still unclear. Additionally, a quadruple mutant *pal1pal1pal3pal4* in *Arabidopsis* demonstrated a marginal PAL activity suggesting that unidentified PAL-like genes are also linked to phenylpropanoid biosynthesis [36].

The subcellular association of PAL with the endoplasmic reticulum bound C4H has been hypothesized, enabling an effective conduit of metabolites through the phenylpropanoid pathway [37, 38]. C4H belongs to the oxidoreductase family of enzymes and one of the most studied plant cytochrome P450 monooxygenase involved in plant metabolism [39]. It is the only member of the *CYP73A* subfamily in *Arabidopsis* although it exists as multigene families in many species [40]. Heterologously expressed

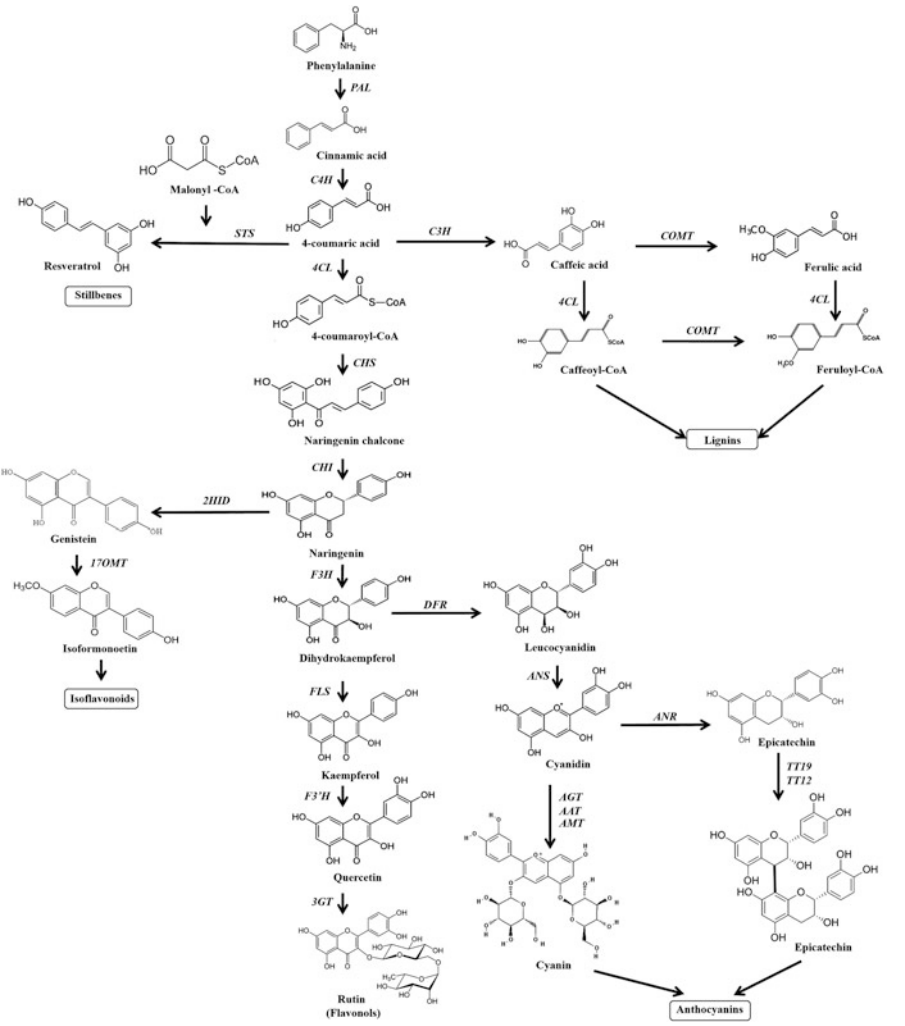

Fig. 2 Phenylpropanoid biosynthetic pathway with extended branching for various organic compounds. Enzymes are as follows: *PAL* phenylalanine ammonia lyase, *C4H* cinnamate-4 hydroxylase, *4CL* 4-hydroxycinnamate CoA-ligase, *CHS*- chalcone synthase, *CHI* chalcone isomerase, *F3H* flavonone 3-hydroxylase, *FLS* flavonone synthase, *3GT* flavonoid 3-*O*-glucosyltransferase, *HID* 2-hydroxy isoflavanone dehydratase, *I7OMT* isoflavone 7-*O*-methyltransferase, *DFR* dihydrofavonolreductase, *ANS* anthocyanidin synthase, *ANR* anthocyanidin reductase, *AGT* anthocyanin glycosyltransferase, *AAT* anthocyanin acetyltransferase, *AMT* anthocyanin methyltransferase, *TT19* transparent testa 19, *TT12* transparent testa 12, *STS* stilbene synthase, *C3H* p-coumaroyl-shikimate 3′ hydroxylate, *COMT* caffeoyl CoA 3-*O*-methyltransferase

C4H as well as the protein directly isolated from the plant exhibit high substrate specificity with t-cinnamic acid. Overexpression of C4H transcripts is often associated with high lignifications and enhanced production of phenolic compounds, while the

downregulation of C4H results in altered lignin content [40, 41]. Allelic mutant of *Arabidopsis* C4H gene, *ref3*, has reported a reduced deposition of lignin and tannins, impaired water transport due to collapsed xylem, and reduced apical dominance and male sterility [42]. Taken together, these results suggest that C4H is a significant component of physiological processes that are critical to the survival of the plants besides phenylpropanoid metabolism. The subsequent step downstream of C4H is mediated by AMP forming small gene family of 4CL ligase resulting in the formation of the CoA thioester 4-coumaroyl CoA [43]. The importance of 4CL genes and proteins is evident from the fact that the activated 4-coumaroyl CoA represents the starting point of not only the phenylpropanoid biosynthesis, but the metabolism of a wide variety of secondary metabolites including flavonoids, isoflavonoids, and stilbenes [12, 16].

2.1 Biosynthesis of Monolignols, Lignin, and Lignan

A major downstream reaction in the phenylpropanoid pathway involves the biosynthesis of monolignols. These are the hydroxyl cinnamyl alcohol monomers that act as source materials for the biosynthesis of lignin and lignan. Following the synthesis of p-coumaroyl-CoA by 4CL, the p-coumaroyl group is transferred to shikimate by the enzyme hydroxylsinnamoyl-CoA shikimate to quinate hydroxylcinnamoyl-transferase (HCT) [44] (Fig. 2). The p-coumaroyl-shikimate is then hydroxylated at the 3′ end by p-coumaroyl-shikimate 3′ hydroxylate (C3′H) to form caffeoyl shikimate [45]. HCT undergoes a reverse reaction by transferring the caffeoyl part back into the CoA forming the caffeoyl CoA [44]. The first methyl transfer reaction is the phenylpropanoid metabolism catalyzed by caffeoyl CoA 3-*O*-methyltransferase (CCoAOMT) resulting in the formation of feruloyl CoA from caffeoyl CoA [12, 46]. The hydroxyl cimmamoyl CoA thioesters are subsequently reduced to hydroxyl cinnamaldehydes by the NADPH-dependent cinnamoyl CoA reductase (CCR) [47]. Consequently, cinnamyl alcohol dehydrogenase (CAD) catalyzes the next step of the reaction, reducing cinnamaldehydes to cinnamyl alcohols using NADPH [47]. CAD plays an extensive role in lignin biosynthesis and formation of other plant defense metabolites [48, 49].

Lignin is a prominent phenylpropanoid with vital role in plant structure and defense. It is the second most abundant organic complex after cellulose evenly deposited on the secondary walls of tracheary elements, sclereids cells, and to some extent in the middle lamella and primary cell walls of plants. Lignin is generated by oxidative polymerization of two hydroxy cinnamyl alcohols, namely, coniferyl alcohol and sinapyl alcohol to yield an intermediate radical [50] (Fig. 2). Constituents of lignin differ in angiosperm plants from that of the gymnosperms. In angiosperms, lignin consists of coniferyl and sinapyl alcohols [guaiacyl (G)-syringyl (S)], whereas in gymnosperms it consists mostly of coniferyl alcohol (G type) with small amounts of p-hydroxyphenyl (H)-type lignin [50]. Initial characterization suggested that the formation of lignin by monolignol polymerization was believed to be a random process mediated by H_2O_2-dependent peroxidases, H_2O_2-independent laccases, and polyphenol oxidases having phenoxy radicals as by-products

[50]. Conversely, later studies have shown that the dimerization of monolignols is a stereoselective to enantio-selective mechanism in which the bimolecular phenoxy radical coupling is guided by an auxiliary dirigent protein [51]. Further, lignin depositions at cell wall might be a result of the endwise polymerization of monolignols and its elongation via a template mechanism [52]. Additionally, other nonspecific oxidases are also involved in generating the free radical species from monolignols [53].

Lignans are phenylpropanoid dimeric metabolites where monomers are linked by the central carbon (C8) atoms [54]. Based upon the incorporation of oxygen into the carbon skeleton and subsequent cyclization pattern, lignans can be divided into eight subgroups such as furofuran, furan, dibenzylbutane, dibenzyl butyrolactone, aryltetralin, arylnaphthalene, dibenzo cyclooctadiene, and dibenzyl butyrolactol [54]. The level of oxidation of aromatic ring and the side chains vary along the subgroups of lignans. Lignan is formed by the enantio-selective dimerization of two coniferyl alcohol units along with dirigent protein to form pinoresinol or furofuran [55]. This reaction is catalyzed by the enzyme pinoresinol synthase (PS). Pinoresinol undergoes two consecutive stereo-specific reactions catalyzed by the DADPH-dependent pinoresinol-lariciresinol reductase (PLR) to synthesize (+)-lariciresinol (furan) which is further reduced to (+)-secoisolariciresinol. Consequent oxidation of the dibenzylbutane by secoisolariciresinol dehydrogenase (SDH) results in the production of matairesinol [56].

2.2 Biosynthesis of Flavonone, Isoflavonoids, and Anthocyanins

Flavonoids are a major group of plant secondary metabolites emerging out of the phenylpropanoid pathway. They are basically represented by the pigments that color most of the flowers, leaves, and fruits. Extensive research has been carried out on them, and this has resulted in the identification of more than 6000 flavonoids in a wide variety of plants [57]. They are broadly classified into seven major groups including flavones, flavonols, chalcones, flavondiols and anthocyanins, pro-anthocyanins, and aurones [57–59]. Legumes are characterized by the presence of specific flavonoid structures called as isoflavonoids [60, 61], and grapes, peanuts, and few unrelated species synthesize a chalcone derivative called as stilbenes [62], while few other species produce phlobaphenes [59]. A high diversity in their structure also accounts for myriads of biological functions including protection from phytopathogens and other physiological stresses, protection from photo-oxidative damages, nutrient retrieval, coloration of flowers, pollination, male sterility, nodular signaling, and hormonal transport [59, 63, 64]. 4-Coumaroyl CoA is transformed into various kinds of flavonoids by a series of downstream enzymatic activities [59] (Fig. 2). The first enzyme in the process is chalcone synthase (CHS), a type III polyketide synthase which catalyzes the claisen ester condensation concomitant with CO_2 liberation from p-coumaroyl-CoA to produce chalcone scaffolds from which all flavonoids are derived [65]. This is followed by subsequent isomerization and hydroxylation by chalcone isomerase (CHI) and flavonone 3-hydroxylase

(F3H), respectively, to produce dihydrokaempferol [66]. Dihydrokaempferol is further hydroxylated with F3H to synthesize dyhydroquercetin. Subsequently, flavonone synthase (FLS) catalyzes an oxidoreductase reaction by removing the dihydroxyl group from dihydrokaempferol and dihydroquercetin to synthesize kaempferol and quercetin, respectively [67]. Dihydrofavonols are consecutively catalyzed by dihydrofavonol reductase (DFR) and anthocyanidin synthase (ANS) to produce the anthocynidins. Subsequent glycosylation and acylation reactions convert the anthocyanidins into anthocyanin molecules [68, 69]. A multiple enzyme-based branching reaction emerges out from the central isoflavanone intermediate naringenin in the leguminous plants for the biosynthesis of isoflavonoids [60]. This first step in this process involves the migration of an aryl group from the two to three carbon position in the B ring of naringenin resulting in the formation of 2-hydroxyisoflavanone [70, 71]. This reaction is catalyzed by a CYP450 enzyme isoflavonone synthase (IFS). The 2-hydroxyisoflavanone is subsequently dehyadrated by 2-hydroxyisoflavanone dehydratase (HID) to form corresponding isoflavonoids [72].

Although the fundamental pathway for flavonoid biosynthesis is conserved, specific alteration in the basic skeleton of flavonoid metabolism could be seen amid plant species due to multiple steps driven by a wide range of enzymes such as isomerases, reductases, and dioxygenases [73]. Further, most of the flavonoid biosynthetic enzymes are present as enzyme complexes bound to the endoplasmic reticulum, while the synthesized pigments are located in the vacuoles or cell wall. However, recent reports also suggest the localization of some flavonoid biosynthetic enzymes in the nucleus as well as the tonoplast [74, 75].

3 Metabolic Engineering of Phenylpropanoids

Plant secondary metabolites constitute several groups and have multiple vital functions in plant life cycle. Besides, they are characterized by high medicinal and economic significance apart from natural in-plant functions [76]. In the attempt of producing high-yielding plants, often the productions of some specialized secondary metabolites are compromised [77]. Also, many plants producing important secondary metabolites grow slowly and are almost impossible for the in vitro cultures [2]. Therefore, it is equally important to maintain and/or improve the production of important secondary metabolites at the time of producing an elite cultivar. Metabolic engineering is a promising tool for the enhancement of production and quality of plant secondary metabolites in a highly selective and cost-effective approach (Fig. 3). Additionally, the engineering of metabolic pathways can lead to metabolite structural modifications, improvements, or even synthesis of new products [78]. Several plant secondary metabolites and their associated metabolic pathways have been improved by using metabolic engineering tools [79]. In some cases of pathway manipulation, the change of biosynthetic site also helps in improved yield of the particular secondary metabolite by overriding the plant regulatory mechanisms [80, 81]. Pathway engineering for enhanced production of secondary metabolites can

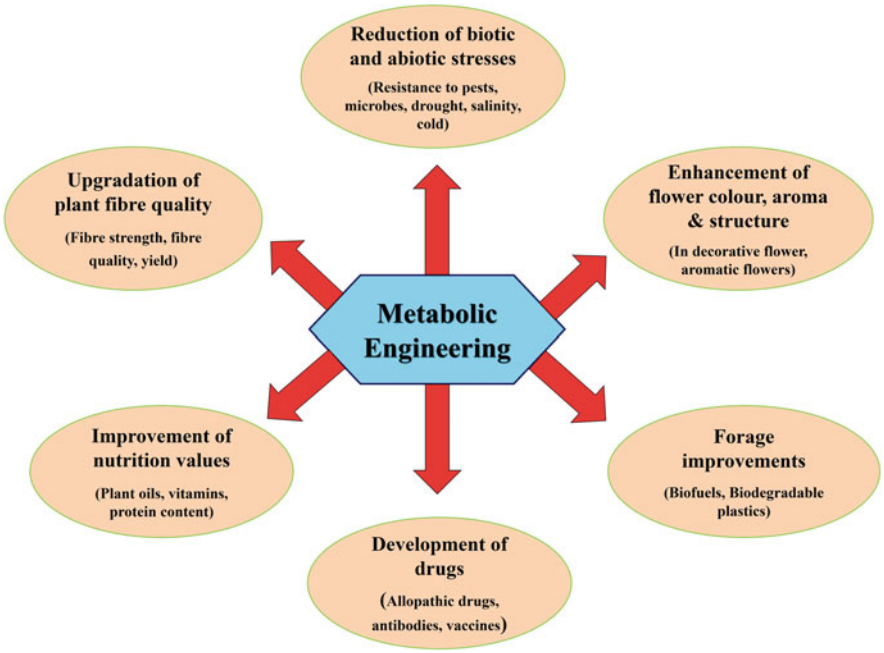

Fig. 3 Key applications of metabolic engineering in plants and microorganisms

be carried out in the native producer by the regulation of transcription factors and overexpression of biosynthetic genes [82]. Alternatively, the transfer of the metabolic pathways into heterologous hosts is a more acceptable strategy toward a successful metabolic engineering effort. Several heterologous hosts including *Streptomyces*, *Escherichia coli*, *Saccharomyces cerevisiae*, and *Nicotiana benthamiana* have been used to assemble large blocks of DNA for reconstruction of complex metabolic pathways for enhanced production of secondary metabolites [2].

Metabolic engineering of secondary metabolites can be achieved by bringing up alteration at many tiers [1]. The structural challenges in metabolic engineering should be addressed by the introduction of single or multiple rate-limiting enzymes encoding genes from the central metabolic pathway [83]. The overexpression of the rate-limiting enzymes in plants or their transformation into suitable microbes may lead to increased accumulation of the specific metabolite. Further, the biosynthesis of plant secondary metabolites is often governed at both transcriptional as well as translational stages. Therefore, the regulatory challenges have to be prevailed over by manipulation of transcriptional and translational apparatus [2]. Additionally, the generation of a particular metabolite is greatly affected by the metabolic pathways in-flux regulations. In such cases, the whole genomic or targeted transcriptomic and metabolomics analysis of a specific plant could be performed to overcome the flux-associated challenges. Lastly, the metabolic engineering should also result in high accumulation of the desired secondary metabolite, without any toxicity to the cells.

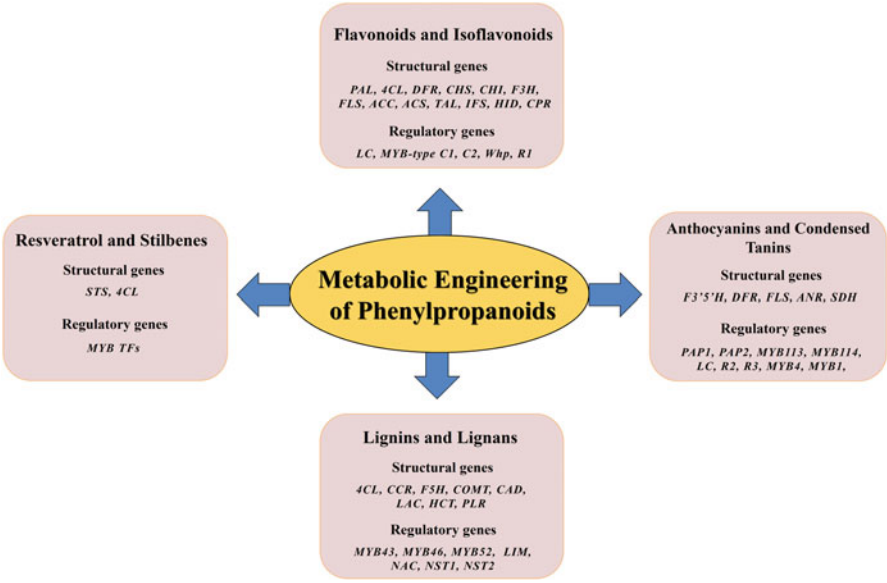

Fig. 4 Engineering of phenylpropanoid pathway through the manipulation of structural genes and regulatory factors. The names of the structural genes are mentioned in the legend of Fig. 2. The regulatory genes are as follows: *LC* leaf color, *MYB* myeloblastosis family of transcription factors, *NAC* NAM, ATAF and CUC family of transcription factors, *PAP* production of anthocyanin pigment

Therefore, specific transporters must be designated for specific secondary metabolites to ensure their movement to proper subcellular and/or extracellular location for sequestration, without resulting in any toxicity. Phenylpropanoid being an important group of secondary metabolites offers significant bioengineering potential for improvements [77]. Several key enzymes involved in both early and late stages of phenylpropanoid metabolism have been already cloned and characterized [84]. These enzymes and their sequence information serve as an asset for the metabolic engineering processes and improvement of the end products. A significant review of engineering strategies for the manipulation of structural and regulatory genes from the phenyl propanoid pathway is presented here (Fig. 4). We have discussed many specific examples on the engineering of different kinds of phenylpropanoids to point out crucial advancement in the field.

3.1 Engineering Major Flavonoid Metabolism

Flavanoids are the most significant group of commercial phenylpropanoids owing to multitude of functions in plant systems [85]. However, the flavonoid in plants is very low and largely depends upon developmental and environmental conditions. This limited availability is always a challenge for commercial production of flavonoids.

Besides, the alternative approaches for flavonoid production are highly challenging and cost expensive due to the long growing stages of the plants, poor culturability together with extreme reaction conditions, and toxicity of the chemical synthetic processes. Therefore, the engineering of phenylpropanoid pathway in plants or microbes could be a suitable alternative to produce novel flavonoid with important commercial and medicinal activities. Since the first report on heterologous expression of maize *dihydro quercetin 4-reductase* (*DFR*) gene resulting in orange *Petunia* flowers [86], the flavonoid biosynthetic pathways have been engineered in many horticultural plants for floral coloration [87] and disease resistance [88]. Tomato being an agriculturally important crop has been used as model plant for engineering of the flavonoid pathway for enhanced nutraceutical properties [89]. Both the structural and regulatory genes have been modified from the flavonoid pathway of tomato to increase the yield of the secondary metabolite. The overexpression of two flavonoid regulatory transcription factors, the maize *leaf color* (LC) and MYB type *C1* (*colorless*), resulted in enhanced accumulation of kaempferol in the flesh of the tomato fruits which normally doesn't produce flavonoids [90]. A previous heterologous expression of *LC* and *C1* genes in *Arabidopsis* also resulted in anthocyanin accumulation even in those tissues which do not show any pigmentation [91]. Interestingly, the tomato fruits did not accumulate any anthocyanin because of the low expression of the gene encoding flavanone-3,5-hydroxylase (F3,5-H) in tomato fruit together with strong preference of tomato dihydroflavonol reductase (DFR) enzyme to use F3,5-H-derived dihydromyricetin as substrate for the synthesis of anthocyanins [92]. Among the structural genes associated with flavonoid biosynthesis in tomato, *CHI* which converts naringenin chalcone to naringenin is the rate-limiting enzyme in the pathway leading to rutin. An ectopic overexpression of *CHI* gene of *Petunia* led to 70% enhancement in the accumulation of quercetin in the peels of tomato [24, 93]. Later, Colliver et al. [94] demonstrated that a concomitant expression of four *Petunia* genes *CHS*, *CHI*, *F3H*, and *FLS* enhanced the synthesis of quercetin and kaempferol in peel and flesh of tomato, respectively. In recent times, RNA interference has been widely used to downregulate the expression of specific flavonoid genes. RNAi inhibition of CHS resulted in drastic reduction of total flavonol together with significant phenotypic variations in tomato [95]. The majority of fruits were parthenocarpic in the RNAi lines suggesting that flavonoids have a significant role in fertilization and fruit and seed development. Engineering of flavonoid pathway has been reported in some plants for the regulation of pollen fertility. In maize, male sterile lines were produced through the mutation of *CHS* genes *C2* and *Whp* [87]. Antisense expressions of *CHS* and *STS* have also resulted in male sterility in *Petunia* and tobacco plants [96, 97].

E. coli has been the preferred ex-host system for metabolic engineering and heterologous production of flavonoids [98–100]. Hwang et al. [101] first reported the production of flavonoids in *E. coli* by engineering *PAL*, *CHS*, and *4CL* from *Streptomyces coelicolor*. However, the low levels of intracellular precursors greatly limited the flavonoid yield. Subsequently, Leonard et al. [102] introduced various strategies to reengineer the central metabolism in *E. coli* to enhance the production of intracellular precursor malonyl coenzyme A. Four acetyl CoA carboxylase (ACC)

subunits from *Photorhabdus lumenescens* were introduced into *E. coli* to realize a 5.8-fold increase in flavonoid production [102]. Zha et al. [103] performed additional engineering in the internal metabolic pathway by simultaneous overexpression of ACC and acetyl CoA synthase (ACS) leading to 15-fold increase in cellular malonyl coenzyme A. Fowler et al. [98] developed a highly innovative cipher of evolutionary design (CiED) to identify genetic perturbations that could improve the host genotypes for better production of the metabolites. Employing CiED, it was revealed that the improved *E. coli* genotypes channelled the flux toward malonyl coenzyme A (CoA) more effectively to increase the flavonoid biosynthesis. The targeted deletion of native genes from the TCA cycle of *E. coli* as predicted by CiED and overexpression of genes required for synthesis of the plant-derived flavanones and coenzyme A biosynthetic pathway enhanced the production of flavonoids. The elevated yields in the modified *E. coli* cells were over 660% for naringenin (15–100 mg/l/optical density unit [OD]) and by over 420% for eriodictyol (13–55 mg/l/OD) [98]. Likewise, Santos et al. [100] reported the production of naringenin directly from glucose, without the supplement of any precursor amino acid. A complete pathway consisting of four enzymes, i.e., *CHS* and codon-optimized *TAL*, *4CL*, and *CHI*, was transformed into *E. coli* and its heterologous expression produced 29 mg/l naringenin from glucose. Besides *E. coli*, many flavonoid metabolic engineering have been carried out in *Saccharomyces cerevisiae* cultures as it provide a number of specific advantages of overexpression in other systems. Ro and Douglas [104] introduced *PAL* and *C4H* into *S. cerevisiae* which could effectively deaminate phenylalanine into cinnamic acid consequently producing 4-coumaryl CoA. A subsequent experiment involving the introduction of PAL, 4CL, and CHS into yeast cells produced significant amount of naringenin from phenylalanine [105]. Besides, several other studies have demonstrated that metabolic engineering in yeast could result in enhance production of natural as well as novel flavonoids using chalcone substrates.

3.2 Engineering Isoflavonoid Biosynthesis

Isoflavonoids are important derivative of central phenylpropanoid pathway and have direct role in plant defense and nitrogen fixation in leguminous plants [106]. Metabolic engineering has opened up new opportunities to alter or enhance the accumulation of specific or new isoflavonoids in a variety of species. Genetic modification of key structural genes including *IFS* and *HID* is directly associated with enhance accumulation of isoflavonoids in many plants. Silencing of *IFS* gene through RNAi resulted in reduced production of isoflavonoids and less resistance to microbial infection soybean [107]. In contrast, a constitutive expression of *M. truncatula IFS* (*MtIFS1*) led to significant production of genistein glucoside besides accumulating several other isoflavonones including formononetin, medicarpin, and daidzein in alfalfa leaves [108]. Similarly, RNAi silencing of *CHS6* genes caused a drastic decrease in the accumulation of daidzein, coumestrol, and genistein in transgenic soybean roots [109]. Likewise, the overexpression of *HID* also leads to substantial

enhancement in the percentage of isoflavonoids daidzein and genistein in legumes as well as other plants [72, 110]. Additionally, the overexpression of regulatory transcription factor MYBs also plays an important role in the activation of isoflavonoid biosynthesis. Yu et al. [111] reported two to four folds increase in isoflavonoid levels of soybean seeds by ectopic expression of maize *C1* and *R1* TFs in conjunction with co-suppression of F3H. This has been replicated in various plant systems including maize, *Arabidopsis*, and tobacco [112, 113]. This suggests that activation/down-regulation of structural and regulatory genes is a viable strategy for the regulation of isoflavonoid biosynthesis in different plant species.

In addition to the native producers, heterologous hosts such as *E. coli* and *S. cereviceae* have also been used for the commercial production of isoflavonoids. However, the process is quite complex in bacteria owing to the absence of membrane-bound cytochrome P450 (CPR) enzymes and translational incompatibility due to the lack of endoplasmic reticulum. To overcome these problems, Leonard and Koffas [114] developed an artificial one component enzyme similar to bacterial P450BM3 that increases the turnover of bacterial P450 enzymes. These recombinant *E. coli* cells were found to have greater abilities for enhanced production of isoflavonoids genistein and daidzein. The heterologous expression of plant genes and the subsequent production of isoflavonoids in yeast are comparatively easy due to similarity in transcriptional and translational machinery. Co-expression of *CHI* and *IFS* in engineered yeast resulted in effective conversion of chalcone subtrates to flavones and subsequent isoflavones [115]. Recently, the co-expression of a three-enzyme system (IFS, HID, and CPRs) in *S. cereviceae* caused biotransformation of multiple natural and synthetic dihydroxy flavones into their corresponding isoflavones [116]. These results suggest that the identification of active enzymes and their engineering in heterologous hosts can be greatly used for mutasynthesis of isoflavonoids in commercial quantities.

3.3 Engineering the Production of Anthocyanin and Proanthocyanidins

Anthocyanins and proanthocyanidins (PA) (also known as condensed tannins) are major derivatives of phenylpropanoid pathway that occurs as focussed metabolites in various plants. Anthocyanins are glycosides of anthocyanidins, while proanthocyanidins are actually polymers of aglycones [117]. As they serve a variety of functions in plant systems, efforts have been taken to engineer their production for quality and quantity. Engineering of anthocyanin has been sought in many ornamental plants to generate novel floral colors. This includes the engineering of the anthocyanin and aurones biosynthetic pathway for the development of blue color in rose and *Petunia* or the introduction of red, orange, or yellow colors in other plants [118–120]. Engineering a functional *F3′5′H* gene to produce a delphidin-based anthocyanin together with the overexpression of a *Petunia DFR* gene led to blue coloration of the rose flower [118]. Likewise, the suppression of *F3′5′H* and overexpression of *Petunia DFR* genes in tobacco led to enhanced production of

pelargonidin-based anthocyanin resulting in orange-red flowers [119]. A previous study had shown that the anthocyanin production could be further induced by downregulation of *FLS* gene [121]. Many new genes related to anthocyanin biosynthesis have been identified through genomic and transcriptomic analysis whose engineering has resulted in delicate modifications of floral color [122, 123]. In the recent times, scientists are more comfortable in engineering the regulatory TFs owing to the complexity of the anthocyanin biosynthetic pathway and the associated structural genes. As many as four MYB TFs, *PAP1*, *PAP2*, *MYB113*, and *MYB114*, have been identified as major regulators of anthocyanin biosynthesis, and their overexpression results in high accumulation of anthocyanin in leaf, stem, flowers, and roots of various plants [124–126]. Recently, the co-expression of *PAP1* and *Lc* genes led to significant elevation of anthocyanin levels along with other phenylpropanoids in *Saussurea involucrate* [127]. Besides, the modification of anthocyanin regulatory factors also contributes to the marketability of the fruits and vegetables. For example, the mutation of a R2-R3 MYB gene in cauliflower conferred anthocyanin accumulation resulting in intense purple color in the curds [128].

PA production is significantly induced during herbivory and microbial attack. Therefore, engineering the PA biosynthesis has been attempted to regulate plant defense response against pests and pathogens. Overexpression of a catechin biosynthetic gene *PtrLAR3* in *Populus trichocarpa* showed PA accumulation and demonstrated enhance resistance to the fungus *Marsonina brunnea* [129]. Higher PA content contributes to the bitterness and astringency in fruits and vegetables. Metabolic engineering is also used to suppress the PA biosynthetic genes to make the fruits and vegetables edible without artificial treatments. Co-suppression of catechin biosynthetic gene *ANR*, *F3′5′H*, and *shikimate dehydrogenase* in a persimmon (*Diospyrus kaki*) mutant resulted in a no astringent phenotype with reduced accumulation of PA in the fruit [130]. A MYB TF *DkMYB4* was found to be synchronously downregulated similar to the structural genes in persimmon transgenic lines. A *DkMYB4* knockdown line of *Diospyrus kaki* also revealed similar pattern of gene expression and phenotype in accordance with the mutant line [130]. PA modification also contributes to the quality of forage plants for better ruminal digestibility. Ectopic expression of a strawberry transcription factor *FaMYB1* inhibits the biosynthesis of PA in the leaves of the forage plant *Lotus corniculatus* [131]. Altogether, this suggests that metabolic engineering of anthocyanin and PA is necessary for qualitative and quantitative improvement of plant varieties.

3.4 Metabolic Engineering of Resveratrol

Resveratrol are the non-flavonoid monomeric units of dihydroxy stilbene which are produced by a limited number of plants species especially from the family Vitaceae and Fabaceae [132]. They have phytoalexin activity and form a significant part of the general plant defense mechanism. The trans-resveratrol has myriads of medicinal benefits such as anticancerous, antitumor, and antiaging besides being used in the

treatment of neurological and cardiovascular disorders [62]. This has resulted in tremendous increase in the demand of trans-*R* and associated stilbenes in the recent times. As the native producers have a very low content of trans-*R*, metabolic engineering provides a suitable alternative for increasing the production of these natural phenolics compounds. Early application of trans-*R* engineering primarily focussed on increasing the production of phytoalexin for antimicrobial potential [133]. Since the first effort of transferring two grapevine *STS* genes *Vst1* and *Vst2* toward the development of tobacco resistance to *Botrytis cinerea* infection [134], the process has been reenacted in a variety of crop plants for protection against a wide range of phytopathogens (reviewed in Jeandet et al. [135]). However, the level of trans-*R* and its antimicrobial potential depended on the plant species, promoter used, and the ripening stage of the transgenic lines.

Current strategies for the bioproduction of trans-*R* largely involve the heterologous expression of either the entire phenylpropanoid pathway or selective genes in the baker yeast *S. cereviceae* [83, 99, 136]. Trantas et al. [99] introduced the entire pathway in *S. cereviceae* and produced 0.3 mg/l of trans-*R* from 10 mM phenylalanine as substrate. In a previous study, yeast strain transformed with *4CL* from tobacco and *STS* from grapevine resulted in higher quantity of trans-*R* only with p-coumaric acid as the substrate [137]. Further, Zhang et al. [138] constructed a 4CL::STS fusion to realize a tenfold increase in trans-*R* production. However, this yield is significantly lower as compared to the data obtained with bacterial host *E. coli* [139]. Recently, a new laboratory strain of yeast expressing *4CL1* from *A. thaliana* and *STS* from grapevine resulted in 262 mg/l of trans-*R* [136]. Additionally, the simultaneous expression of an *E. coli area E transporter* gene along with *4CL* and *STS* significantly increased the trans-R production in transformed yeast as compared to control cells [83]. These results suggest that a proper optimization of factors related to type of strains, precursors, and mode of transformation could result in higher level of trans-*R* production from microorganisms.

3.5 Metabolic Engineering of Lignans and Lignins

Lignin is a complex polymer generated by oxidative polymerization of cinnamyl alcohol derivatives called monolignols that are produced through the phenylpropanoid biosynthetic pathway [140]. They constitute a major part of the plant cell wall providing mechanical strength to plant stem and trunk. Lignin mostly occludes with plant cellulose preventing their enzymatic and microbial decomposition. This has tremendous impact on the usage of lignocellulosic biomass for their forage quality, pulping efficiency, and alternative source of fuels [141]. Metabolic engineering enables genetic alteration of the quality and quantity of lignin polymer in the lignocellulosic biomass. Downregulation of monolignol-specific pathway enzymes such as CCR, F5H, COMT, and CAD directly affects the lignin composition and increases the saccharification of the lignocellulosic biomass in many biomass crops (reviewed in Poovaiah et al. [142]). Besides, the modification of downstream laccases which helps in lignifications process also contributes to the

decrease in lignin quantity. Barthet et al. [143] reported that silencing of two *A. thaliana* laccase genes *LAC4* and *LAC17* demonstrated low lignin levels and increased saccharification in the transgenic lines as compared to wild-type plants. Additionally, the manipulation of transcription factor and other regulatory elements associated with lignin biosynthesis also have dramatic effect on lignin content and composition in forage plants [142]. The recent discoveries of the biosynthetic enzymes for the alternative lignin monomers also provide a viable option for engineering lignin composition by replacing the traditional monolignols with alternative monomers [142]. However, these are preliminary considerations and a complete metabolic engineering is yet to be attempted for introducing alternative monomers into the lignin biosynthetic pathway.

Lignans are oligomers of dibenzylbutane that are produced by dimerization of cinnamyl alcohol derivatives by the help of dirigent proteins. Lignans and their glucoside derivatives exhibit a wide variety of bioactivities in plants and animals, and this has led to the molecular characterization and engineering of lignan biosynthetic genes to enhance their production to commercial levels. Current metabolic engineering strategies for ligan production mostly involve cell and organ culture of lignan-rich plants such as *Linum* and *Forsythia* species [144]. *Forsythia* cell line transfected with a pinoresinol-lariciresinol reductase (PLR)-RNA interference (RNAi) (PLR-RNAi) construct showed complete loss of matairesinol and 20-fold accumulation of pinoresinol [145]. Additionally, the co-expression of *PLR-RNAi* and a sesem in producing gene *CYP81Q1* exhibited production of sesem in as well as pinoresinol glucoside in the transgenic cell line. Likewise, RNAi metabolic engineering has been carried out in many *Linum* species for the production of important lignans including podophyllotoxin, justicidin, and pinoresinol glucoside [144, 146]. Together, this suggests that *Forsythia* and *Linum* could be used as a suitable medium for engineering the production of endogenous as well as exogenous lignans.

4 Conclusions and Future Perspectives

Engineering of secondary metabolic pathways primarily aims at increasing or decreasing the quantity of certain metabolic compounds. With the advances in our knowledge of the enzymes from the phenylpropanoid pathway and associated biosynthetic branches together with efficient transformation protocols, it is now possible to engineer the composition and content of phenylpropanoids in plants. Besides, the functional reconstruction of biosynthetic pathways in highly characterized heterologous host such as *E. coli* and *S. cereviceae* provides greater opportunities for scaling the production of phenylpropanoids. However it is essential to understand and mitigate the structural, regulatory, and metabolic flux-associated challenges of the biosynthetic pathways to increase the efficiency of metabolic engineering in ex-host system. The advent of genome scale omics approaches such as whole genome/transcriptome sequencing, proteome analyses, molecular modelling, and metabolomics would be highly useful to iterate the complexities of

engineering targets and substrate usage. Additionally, these tools will not only provide information about the new hosts and reconstructed targets but also will explore their conditions for the maintenance of engineered pathways and accumulation of exogenous phenolic compounds. These are interesting times to initiate more in-depth studies to develop and optimize new platforms of metabolic engineering for viable and large-scale production of phenylpropanoids as per the human requirements.

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