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Review

Evolution and current status of research in phenolic compounds

Alain-Michel Boudet *

UMR CNRS-UPS 5546 IFR40, Pôle de Biotechnologie Végétale, 24, chemin de Borderouge, B.P. 42617, Auzeville 31326, Castanet Tolosan, France

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Abstract

Phenolic compounds are ubiquitous in plants which collectively synthesize several thousand different chemical structures characterized by hydroxylated aromatic ring(s).

These compounds play several important functions in plants. They represent a striking example of metabolic plasticity enabling plants to adapt to changing biotic and abiotic environments and provide to plant products colour, taste, technological properties and putative health promoting benefits.

Phenolic compounds represent the most studied phytochemicals and have been widely exploited as model systems in different areas of plant research. Initial studies in the field concerned the analytical characterization of a wide range of structures and of relevant enzymes with PAL being one of the most studied plant enzymes. This research is still active due to the complexity of the structures and the biosynthetic pathways As an example, the nature and functions of enzymes involved in lignin synthesis have been revisited several times, even in recent years.

More recently, molecular biology and genomics have provided additional understanding of the mechanisms underlying the synthesis of these compounds with special emphasis on the regulation of gene expression by environmental factors. The extensive characterization of genes encoding the different enzymatic steps of flavonoid synthesis and cytochrome P450 genes have been among the most recent advances in this area.

Metabolic engineering of lignins and flavonoids has been deeply investigated. Significant positive results have been obtained in both areas but the negative European opinion towards genetically modified organisms has considerably hampered potential applications. From a more basic point of view, global approaches (such as transcript and metabolite profiling) have investigated the repercussions of these engineered modulations of specific phenolics synthesis on other branches of plant metabolism. These studies have revealed a substantial and sometimes unexpected network of regulatory interactions.

In the present time, the societal demand and an increasing interest for practical applications has stimulated a wide range of biological and epidemiological studies aiming at characterizing the health promoting properties of specific phenolic compounds with antioxidant activities towards cancer, cardiovascular and neurodegenerative diseases or for use in antiaging or cosmetic products.

Increased emphasis on sustainable development should stimulate innovative investigations on phenolic synthesis for improving plant biomass and for a better control of plant and animal health.

Keywords: Phenolic compounds; Phenylpropanoids; Lignins; Flavonoids; Biosynthetic pathways; Multigene families; Transcription factors; Cytochrome P450 genes; Metabolic engineering; Antioxidant activities; Health promoting properties

Abbreviations: AFLP, Amplified fragment length polymorphism; cDNA, complementary desoxyribonucleic acid; CYP, cytochrome P450; 4CL, 4-hydroxycinnamoyl-coA ligase; C3H, *p*-coumarate 3-hydroxylase; C4H, cinnamate 4-hydroxylase; CAD, cinnamyl-alcohol dehydrogenase; CCoAOMT, caffeoyl-CoA *O*-methyltransferase; CCR, cinnamoyl-CoA reductase, COMT I, caffeic/5-hydroxyferulic acid *O*-methyltransferase; F5H, ferulate 5-hydroxylase; HCT, hydroxycinnamoyltransferase; PAL, Phe ammonia-lyase; SAD, sinapyl-alcohol dehydrogenase.

* Tel.: +33 0 5 62 19 35 21/35 42; fax: +33 0 5 62 19 35 02.
E-mail address: amboudet@scsv.ups-tlse.fr
URL: http://ifr40.scsv.ups-tlse.fr

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Contents

1. Introduction

How could we imagine plant species without polyphenols? They have been a feature of plants since early colonisation of the land. These so called secondary metabolites are indeed crucial for many important functional aspects of plant life. These include structural roles in different supporting or protective tissues, involvement in defence strategies, and signalling properties particularly in the interactions between plants and their environment.

Collectively, higher plants synthesize several thousand known different phenolic compounds and the number of these fully characterized is continually increasing. Leaves of vascular plants contain esters, amides and glycosides of hydroxycinnamic acids, glycosylated flavonoids especially flavonols and proanthocyanidins and their relatives. Lignin, suberin and pollen sporopollenin are examples of phenolic containing polymers. Some soluble phenolics are widely distributed e.g. chlorogenic acid but the distribution of many other structures is restricted to specific genera or families making them convenient biomarkers for taxonomic studies. Their common feature is the presence of a hydroxy-substituted benzene ring within their structure.

Different physicochemical methods have, since the beginning of the 20th century, permitted the characterization of a plethora of complex structures and at the present time the technical advances both in chromatographic techniques and in identification tools particularly the diverse forms of mass-spectrometry allow us to meet the challenges of separation and characterization of compounds of increasing complexity, of high molecular weight, low stability and low abundance (Whiting, 2001).

The biosynthetic routes of phenolic metabolism derive from primary metabolism and there is increasing evidence that duplications of essential genes of primary metabolism have been an important basis for gene recruitment in secondary metabolism. In the course of evolution, these duplicated genes have acquired new functions and have been optimized and diversified for their roles in new pathways (Dixon and Steele, 1999; Lehfeldt et al., 2000). The phenylpropanoid pathway leading to lignins involves a common set of biochemical reactions in vascular plants already present 400 million of years ago with the emergence of erect vascular land plants. The biosynthesis of other phenolic compounds including flavonoids for use as sunscreens was also a likely early event in land plant evolution. From an evolutionary point of view these metabolic backbones have been progressively enriched to provide specific adaptations to different plant families and the remarkable biochemical diversity we can observe.

It is particularly important to note that phenolics have wide impacts on human activities in the agroindustrial and food sectors exerting either positive or negative influences on the processes or on the quality of the products and these characteristics have motivated specific research programmes.

Beyond a continuous flow of information related to the characterization of new structures several phases may be schematically delineated in phenolic research during the last 50 years:

1 The characterization of the enzymatic steps leading to the common precursors of the phenylpropanoid pathway and to specific branches of phenolic metabolism.
2 The use of molecular biology to probe the changes in gene expression associated with the plasticity of phenolic metabolism.
3 The emergence of functional genomics, providing a more accurate picture of the diversity of the genes/enzymes involved in phenolic metabolism.
4 The exploitation of genetic engineering for optimizing the phenolic profiles of plants (particularly lignin and flavonoid patterns).
5 The explosion of epidemiological studies supporting the protective role of food polyphenols in human health.

It is not possible due to space limitations to cover all these different topics in depth, which have been in some cases treated in recent reviews, including in this journal (see for example the issue related to Tannins and related

Polyphenols, vol. 66, No. 17, September 2005). This paper will highlight some of the major advances in these areas and discuss the most exciting future perspectives open to phenolic research, with special reference to the recent literature.

2. New insights on PAL a key enzyme of phenolic metabolism

Most plant phenolic natural compounds are derived from trans-cinnamic acid, formed by deamination of L-Phenylalanine (L-Phe) by L-Phenylalanine ammonia-lyase (PAL; EC 4. 3. 1. 5). This enzyme, first described by Koukol and Conn (1961), has been the focus of a wide range of investigations and for a long time has represented one of the most extensively studied plant enzymes.

At the gateway from primary metabolism, PAL plays a pivotal role in phenolic synthesis and thousands of reports emphasize the correlation between increases in the corresponding PAL gene/protein expression/activity and increases in phenolic compounds in response to different stimuli. Pioneering work in this area was performed in Hahlbrock's lab (see for example Hahlbrock and Scheel, 1989). In addition to this regulation via *de novo* synthesis, other regulatory mechanisms involve feedback effects (Blount et al., 2000) or post-translational modifications (Allwood et al., 1999; Cheng et al., 2001). PAL has also been involved in the formation of enzymatic complexes responsible for specific metabolic channelling. It has thus been suggested for a long time (Stafford, 1974) that consecutive reactions of phenolic biosynthesis may be organized as complexes, known as metabolic compartments or metabolons, through which pathway intermediates can be channelled without equilibration with free cytoplasmic pools. A large body of experimental evidence obtained from cell suspension cultures or *in vitro* isolated microsomes supports the channelling of cinnamic acid between PAL and cinnamate hydroxylase (C4H) *in vivo* (Rasmussen and Dixon, 1999), to produce *p*-coumaric acid, a central hydroxycinnamic acid phenylpropanoid intermediate. The situation seems to be indeed more complex than expected. Recent experiments on transgenic tobacco plants independently expressing epitope tagged versions of two PAL isoforms (PAL1 and PAL2) and Cinnamate 4-hydroxylase suggest that the subcellular location of two PAL isoforms is different and dependent on the amount of C4H able to organize a complex with PAL1 (Achnine et al., 2004). PAL1 associated with the endoplasmic reticulum is thought responsible for channelling cinnamic acid. PAL2 would then produce cinnamic acid able to reach C4H by diffusion through the cytosol. Differential subcellular distributions of cinnamic acid arising from the activities of differentially localized PAL isoforms could help partitioning of phenylpropanoid biosynthesis into different branch pathways including those which bypass the C4H reaction (e.g. 2-hydroxylation of cinnamic acid). Interestingly this work suggests that changes in the subcellular location of enzyme isoforms might provide an extra and unsuspected level of metabolic regulation. Other data from co-expression of PAL and C4H in yeast as a heterologous system, however, show that physical interaction between PAL and C4H proteins is not required for efficient channeling of carbon from phenylalanine into *p*-coumaric acid (Ro and Douglas, 2004). In this case, the enzymatic properties of C4H, a P450 enzyme with high substrate affinity catalyzing a thermodynamically nearly irreversible reaction, as discussed below, appear to be sufficient, when paired with PAL activity, to channel metabolism into the biosynthesis of phenylpropanoids.

Recent studies have elucidated the crystal structure of the PAL enzyme at high resolution and suggested a reaction pathway for the catalysis (Ritter and Schulz, 2004). The enzyme resembles histidine ammonia-lyase but contains 207 additional residues, mainly in an N-terminal extension rigidifying a domain interface and in an inserted α-helical domain restricting the access to the active center. The unusual non-oxidative deamination reaction of PAL requires an electrophilic group in the enzyme, which is not available among the 20 standard amino acid residues. These studies suggest that it contains the unusual electrophilic 4-methylidene-imidazole-5-one group, which is derived post-translationally from a tripeptide segment in two autocatalytic dehydration reactions. The green plant and fungal PALs are presumably derived from histidine ammonia lyase, an enzyme of central metabolism in early evolution when fungi and vascular plants established their secondary phenylpropanoid metabolism based on trans-cinnamic acid.

However in terms of evolution a few procaryotic PALs have been identified and they are associated with the biosynthesis of specific microbial secondary metabolites. A recent report describes in detail PALs from two cyanobacteria (Moffit et al., 2007). In comparison to eukaryotic homologues, the cyanobacterial PALs are 20% smaller in size but share similar substrate specificities and kinetic activities toward L-phenylalanine over L-tyrosine. The two cyanobacterial PALs are similar in tertiary and quaternary structure to plant and yeast PALs.

In the future, PALs may have applications in enzyme substitution therapy for reducing toxic levels of L-phenylalanine in the blood of patients suffering from phenylketonuria by converting it to cinnamate and ammonia. These prokaryotic PALs with convenient structural, biochemical and immunogenic properties represent alternatives to plant and yeast PALs for such treatments (Moffit et al., 2007).

PAL inhibitors have been for a long time investigated and a wide range of active molecules are available for fundamental studies or for applied purposes. Recently Miziak et al. (2007) identified a synthetic compound 2-amino-4-bromoindane-2-phosphoric acid as a potent inhibitor of PAL activity *in vitro* and of anthocyanin biosynthesis *in vivo*. Cinnamaldehyde was also shown to inhibit PAL (Fujita et al., 2006) and interestingly this compound suppresses

the browning of cut lettuce when they are immersed in a solution of the inhibitor.

3. Cytochrome P450 monoxygenases: newly recognized important players in phenolic metabolism

Most phenolic compounds are derived from phenylalanine via the core phenylpropanoid pathway leading to 4-coumaroyl-CoA. From there, branches dispatch precursors to the biosynthesis of the diverse phenylpropanoid classes, including monolignols, flavonoids, coumarins, stilbenes, xanthones, phenolic esters, benzoic derivatives, etc.

More than sixteen cytochrome P450 monooxygenases have been recently shown to be involved in all these branch pathways. Given their high exothermy reactions catalyzed by P450s constitute ''points of no return'' in metabolic networks and thus channel flow irreversibly into specific branches (Ehlting et al., 2006). We will present here some of the members of this family with an emphasis on the new vision they have provided in specific sections of phenolic metabolism.

Cinnamate hydroxylase (C4H) was one of the first P450s to be characterized in plants (Russel and Conn, 1967). In 1993, cDNAs encoding C4H were isolated from different plants and called CYP73, based on standard P450 enzyme nomenclature (http://drnelson.utmem.edu/CytochromeP450.html) homologous CYP73s were identified in numerous species by means of library screening and they form a cluster of closely related sequences highly similar to each other. These sequences contain a classical N-terminal targeting signal and it has been shown that C4H is indeed targeted to the endoplasmic reticulum (Ro and Douglas, 2004). This class of P450 enzymes was shown to be highly specific for the conversion of cinnamate, and promoter regions of C4H genes do contain putative *cis*-acting elements known from other phenylpropanoid genes further supporting a coordinated transcriptional regulation of the genes of the phenylpropanoid pathway. Down-regulation of C4H in transgenic plants (Reddy et al., 2005) and C4H mutation (Ruegger and Chapple, 2001) induce pleiotropic effects on phenolic patterns including reduction in chlorogenic acid, flavonoids and lignins. All these data converge to support the view of Anterola et al. (2002) that C4H constitutes a rate-limiting step for channeling carbon flux into the phenylpropanoid pathway. This is further supported by the fact that C4H drives the efficient channeling of carbon from phenylalanine into *p*-coumaric acid biosynthesis in the yeast heterologous system (Ro and Douglas, 2004).

The enzyme responsible for the 3-hydroxylation of phenolic intermediates (formation of caffeic acid from *p*-coumaric acid) long remained uncharacterized. In the last 5 years three groups, using complementary approaches, independently identified the CYP98 family of cytochrome P450 enzymes as the major 3-hydroxylase in the phenylpropanoid pathway (Schoch et al., 2001; Nair et al., 2002; Franke et al., 2002). Using recombinant protein expressed in yeast, these groups determined the substrate specificity of CYP98A3 and demonstrated that the shikimate and quinate esters of 4-coumaric acid are very actively hydroxylated in the meta-position on the phenolic ring, with shikimate esters being the preferred substrate (Fig. 1).

CYP98A3 was thus confirmed as a *meta*-hydroxylase of the phenolic ring functioning as a 5-*O*-(4-coumaroyl) shikimate/quinate-3′hydroxylase (C3′H). This enzyme is in particular responsible for the final step of the biosynthesis of chlorogenic acid, a widespread phenolic in plants. Two CYP98 genes were recently characterized from the coffee tree, a chlorogenic acid accumulator, one of them CYP98A35 being able to hydroxylate the chlorogenic acid precursor *p*-coumaroyl quinate (Mahesh et al., 2007). In addition to these shikimate and quinate esters other 4-coumaroyl esters (with phenyllactic acid for example) or amides can be *meta*-hydroxylated by CYP98s in species which accumulate specific compounds such as rosmarinic acid or tyramine derivatives (Gang et al., 2002; Morant et al., 2007).

A related important enzyme/gene is an acyltransferase named hydroxycinnamoyl-CoA: shikimate/quinate hydroxycinnamoyltransferase. The enzyme catalyzes the synthesis of shikimate and quinate coumaryl esters, the substrates of CYP98s and also reversibly the formation of caffeoyl-CoA from 5-*O*-caffeoyl shikimate ester (Fig. 1). It corresponds to a step in the synthesis of lignins (Hoffmann et al., 2003) and its downregulation impacts, as expected, the lignin content and composition (Hoffmann et al., 2004). Another specific transferase seems to contribute to formation of chlorogenic acid in accumulating species (Niggeweg et al., 2004). Together these data suggest that CYP98 is a major meta-hydroxylase in phenylpropanoid metabolism. However, recent results (Abdulrazzak et al., 2006) indicate that an alternative hydroxylation pathway may exist at least in Arabidopsis. Indeed, Arabidopsis CYP98A3 T-DNA mutants produce detectable levels of sinapyl derivatives including S units of lignins, mainly in the roots. In the past other enzymes such as phenolase have been shown to possess *p*-coumaroyl hydroxylase activity (Vaughan and Butt, 1970).

The 5-hydroxylase of phenylpropanoids was initially considered to be a ferulate 5-hydroxylase. First characterized biochemically (Grand, 1984) and later genetically (Chapple et al., 1992; Meyer et al., 1996) it corresponds to a cytochrome P450 from the CYP 84 family. However further results demonstrated that the 5-hydroxylation occurs predominantly, if not exclusively, on coniferaldehyde and coniferyl alcohol and not, as previously assumed, on the free acid (Humphreys et al., 1999; Osakabe et al., 1999). The corresponding enzyme should be preferably named coniferylaldehyde 5-hydroxylase (CA5H) but the previous name F5H still continues to be used (Fig. 1).

A large amount of data resulting from genetic and transgenic studies show the central role of this enzyme in regulating flux into the syringyl fraction of lignin (Reddy et al.,

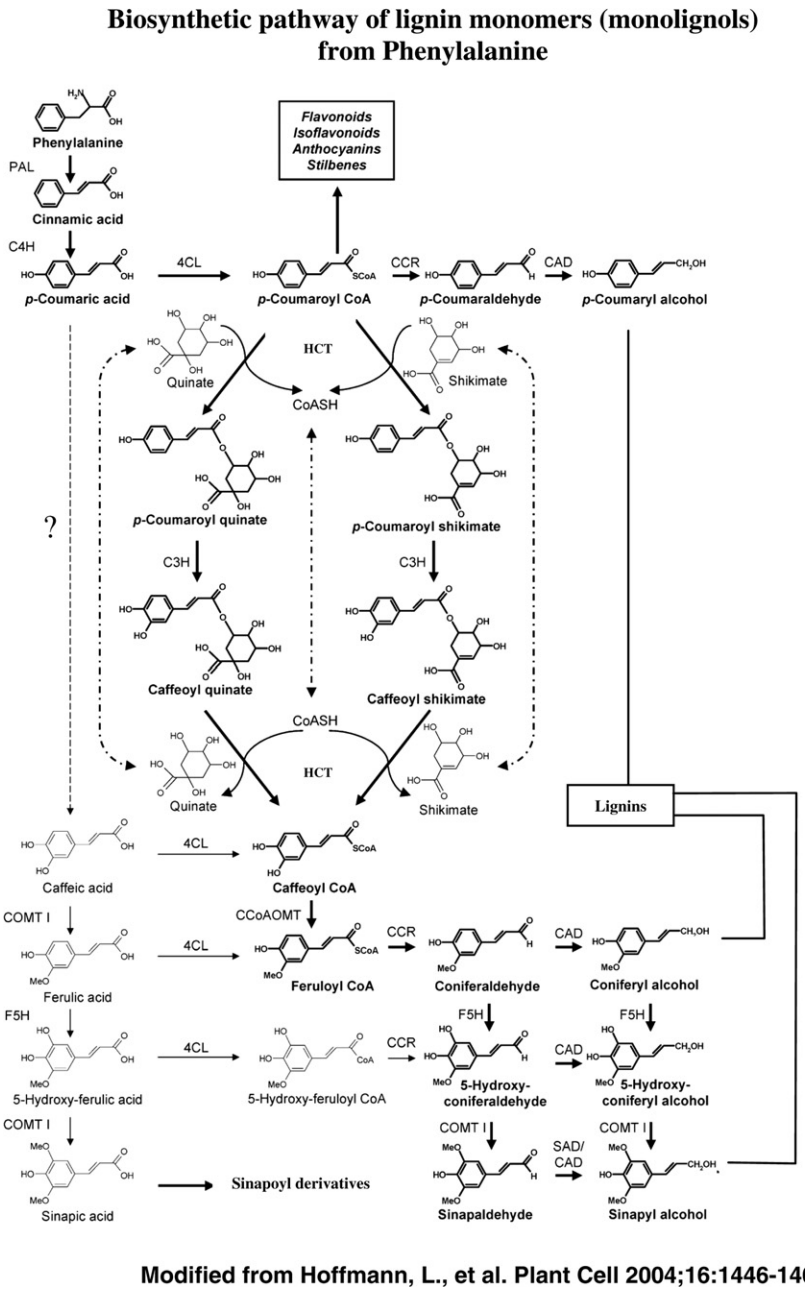

Fig. 1. Biosynthetic pathway of lignin monomers (monolignols) from phenylalanine.

2005). In addition to these three major hydroxylation steps of the main phenylpropanoid pathway common to all vascular plants, cytochromes P450 are also involved in a wide range of other reactions (Ehlting et al., 2006). Some of them are of widespread occurrence such as in the reactions controlling the biosynthesis of salicylic acid and benzoate derivatives or the hydroxylation of the B-ring of flavonoids. The latter activity is determined by two members of the cytochrome P450 family, the flavonoid 3′-hydroxylase (F3′H) and the flavonoid 3′–5′ hydroxylase (F3′5′H) (Seitz et al., 2006). Phylogenetic analysis of known sequences of F3′H and F3′5′H have indicated that F3′5′H was recruited from an F3′H enzyme before the divergence of angiosperms and gymnosperms. Cytochrome P450 are also involved in the synthesis of compounds of more limited occurrence, such as lignans (podophyllotoxin and 6-methoxypodophyllotoxin) and xanthones.

The diversity of cytochromes P450 characterized to date in the context of phenolic synthesis is remarkable and suggests a more complex array of reactions in this metabolism than initially supposed. Cytochromes P450 are largely responsible for the diversity and flexibility of phenolic metabolism and are important in controlling flux into different phenolic branch pathways. Future investigations will likely contribute to the characterization of a large number of newly identified cytochrome P450 enzymes and of their

specific functions. Beyond an increased basic knowledge of these pathways new cytochrome P450s may provide attractive targets for breeding and engineering crops with novel biochemical profiles for specific purposes.

4. Multigene families: increased complexity for fine tuning or unuseful redundancy?

Genomic studies on phenolic metabolism in diverse plant taxa have demonstrated that most genes, at least those involved in the core phenylpropanoid pathway and in monolignol biosynthesis, exist in small gene families. In some plants the cloned genes encode identical or nearly identical proteins whereas in other plants structurally divergent forms have been isolated. I will discuss here some examples related to phenylalanine ammonia lyase, 4-coumarate CoA ligase and cinnamyl alcohol dehydrogenase.

PAL is encoded by multigene families in all plant species investigated. Two genes have been characterized in tobacco and the Arabidopsis genome harbors four PAL genes that fall into two phylogenetic groups consisting of PAL1 and PAL2 on the one hand and PAL3 and PAL4 on the other. Cochrane et al. (2004) have cloned the four putative Arabidopsis pal genes and purified the corresponding recombinant proteins to characterize their kinetic and physical parameters. At-PAL1, 2 and 4 were able to preferentially deaminate L-phenylalanine with similar K_m values, whereas At-PAL3 showed very low activity. All four PAL displayed similar pH optima but not temperature optima. Activities with L-tyrosine were very low or undetectable. In order to understand the individual functions of PAL1, PAL2 in-depth studies of PAL1, PAL2 T-DNA insertion mutants in the first intron of both genes which totally disrupted the expression of these genes and of PAL1 PAL2 double mutant have been undertaken (Rohde et al., 2004). No clear visible phenotypic alterations were seen in the Arabidopsis pal1 and pal2 single mutants suggesting some functional redundancy. Only small visible alterations were observed in the double mutant. However, molecular phenotyping (transcriptome and metabolome) of pal1 and pal2 mutants revealed distinct roles for pal1 and pal2 in phenylpropanoid production. Indeed, the differential expression of several genes from diverse metabolic pathways correlated with only one of the two mutations (e.g. PAL3, CH3 1 and CCoAOMT 7 with pal2 and CCR2 with pal1). On the other hand, pal1 mutants are more altered in phenylpropanoid related transcripts and contain a reduced amount of total extractable phenolics. When considered together, mutation of PAL1 has more severe consequences for the phenylpropanoid pathway than a PAL2 mutation. Whereas both enzymes contribute to the production of lignin precursors. PAL1 is of greater importance for the generation of flavonoids in the inflorescence stem. More generally the disruption of PAL led to an altered transcriptome profile, suggesting adaptation of components of the carbohydrate and amino acid metabolism at the level of gene expression to reduced flux into phenylpropanoid metabolism. All together these data revealed redundant and specific functions for PAL1 and PAL2, and complex interactions at the level of gene expression between the different pathways of primary and secondary metabolism.

The functional differences between the members of the 4-coumarate CoA ligase (4CL) gene family are more obvious. In aspen, (Populus tremuloides) Pt4Cl1 has been associated with lignin biosynthesis since its has activity towards substituted hydroxycinnamic acids and the corresponding gene is expressed in lignifying xylem. Aspen Pt4Cl2 is considered to be involved in the biosynthesis of other phenolics such as flavonoids since it has higher activity towards coumarate and the corresponding gene is preferentially expressed in the epidermis of leaves and stems (Hu et al., 1998). In *Arabidopsis thaliana* the 4CL gene family include four members At4CL1, At4CL2, At4CL3, and At4CL4 which encode enzymes with distinct substrate preferences and specificities (Ehlting et al., 1999; Hamberger and Hahlbrock, 2004). Phylogenetic comparisons indicate that, in angiosperms, 4CL can be classified in two major clusters, class I including At4CL1, At4CL2, and At4CL4 and class II, including At4CL3. Class I 4CLs from all angiosperm species examined are more similar to each other than class II 4CLs from the same plant, indicating an evolutionarily ancient divergence of these 4CL sub-families. Based on enzymatic properties and expression characteristics At4CL3 is likely to participate in the biosynthesis of flavonoids, while At4CL1 and At4CL2 are probably involved in lignin formation. This distinction is also evident in other plants for example in aspen, in which Pt4CL2 is a class II 4CL. The *in vivo* function of At4CL4, which has the unusual property of high activity towards sinapic acid, is not clear. However, a sinapate-utilizing 4CL isoform have also been identified in soybean (Lindermayr et al., 2002), and the role of these unusual enzymes in monolignol and other phenylpropanoid branch pathways remains to be investigated.

The previous data on 4CL genes clearly demonstrate the interest of gene families for a tight control of specific branches of phenolic synthesis in a highly compartmentalized metabolism. The situation is not so clear for other multigene families such as those characterized for cinnamyl alcohol dehydrogenase.

4.1. Two unrelated enzyme families corresponding to gene analogues can catalyze the conversion of coniferaldehyde into coniferyl alcohol

Using the nomenclature first proposed by Goffner et al. (1998) two CADS: CAD 1 and CAD2 exhibit common enzymatic properties (reduction of coumaryl and coniferaldehydes to the corresponding alcohols) but do not display any structural homologies. They belong to two different families of alcohol dehydrogenases: short-chain dehydrogenases and zinc-binding dehydrogenases, respectively. The active form of CAD2 is a dimer while CAD1 is active

as a monomer and is related to cinnamoyl CoA reductase and dehydroflavonol reductase sequences (70% and 62% similarity respectively).

CAD1 is well conserved in angiosperms and gymnosperms and homologous sequences are also present in non-vascular land plants (*Physcomitrella patens*) suggesting that the gene could represent an ancestor of cinnamyl-alcohol-producing proteins.

Functional studies performed in our laboratory (Damiani et al., 2005) on transgenic tobacco plants reveal a complex involvement of CAD1 both in lignan (oligomers of monolignols) and in lignin synthesis. These observations raise the general problem of the specific control of the synthesis of these two end products of monolignol polymerisation. Moreover, the occurrence of two structurally unrelated classes of enzymes: CAD1 and CAD2 involved in the reduction of coniferaldehyde into coniferyl alcohol represents an interesting example of analogous (as opposed to homologous) genes/enzymes, having evolved by the recruitment of existing genes.

4.2. Classical CAD and CAD like genes

Twelve CAD and CAD-like (CADL) genes have been identified in rice although their individual roles in lignin metabolism are poorly known. In Arabidopsis, the CAD gene family involves nine potential candidates (Sibout et al., 2003) that have been placed into four different classes based on the amino acid similarities of the corresponding proteins. A first class involves CAD-C and CAD-D which are highly similar to other well-characterized angiosperm CAD proteins. A second class contains CAD-A, CAD-B1 and CAD-B2, which are closely related to the poplar sinapyl alcohol dehydrogenase (SAD) identified by Li et al. (2001). The third class is composed of CAD1,CAD-E and CAD-F three genes closely related to the alfafa CAD2 (Brill et al., 1999). The last and fourth class contains CAD-G without significant homologies with other CAD-like genes. Exploiting a set of Arabidopsis single and double mutants, Sibout et al. (2005) clearly demonstrated that CAD-C and CAD-D act as the primary genes involved in lignin biosynthesis and the corresponding gene products display a synergistic role in reducing coniferaldehyde and sinapaldehyde to the corresponding alcohols. The functions of the other genes are still unknown. This assignment is supported by functional testing of *in vitro* activities of CAD and CADL enzymes (Kim et al., 2004) and by microarray expression profiling (Ehlting et al., 2005).

In poplar, annotation of the poplar CAD and CAD-like gene repertoire revealed a total of 17 CAD and CADL Genes (Douglas, personal communication). Phylogenetic studies have shown that the major CAD and CADL clades have genes from all three species with fully sequenced genomes (Arabidopsis, rice, poplar), suggesting that CAD/CADL functions are ancient and conserved in angiosperms. The situation is different and more complex in two large clades containing multifunctional CAD-related genes (Clades B2 and C) where lineage-specific events have led to the specific expansion of certain gene families in rice and poplar (Hamberger and Douglas submitted).

In poplar, a CAD-like enzyme specific for sinapylaldehyde, SAD, has been described and is hypothesized to be required for S lignin biosynthesis (Li et al., 2001). However poplar seems to contain a single bona fide CAD gene involved in monolignol biosynthesis (Douglas, personal communication) and the *in vivo* function of poplar SAD remains to be clarified.

Beyond description of this molecular diversity, further functional studies are needed to better appreciate the respective involvement of these different genes in the synthesis of specific lignins and/or of related products. Accurate metabolic surveys and further *in vitro* activity studies of the corresponding recombinant proteins will be necessary to detect such specific functions, or alternatively to demonstrate functional redundancy, which may be required to maintain the enzymatic capacity in the event of impairment of any member of the gene of the family.

5. A growing family of transcription factors that regulate phenolic metabolism

The synthesis of major classes of phenolic compounds such as flavonoids or lignins corresponds to strictly regulated processes both in space and time during plant development. The last decade has seen the characterization of a wide range of transcription factors controlling, in a coordinated way, different sets of structural genes in these specific pathways. These transcription factors are regulated by internal or external signals leading to controlled responses (Vom Endt et al., 2002).

These studies have been particularly significant in the context of the synthesis of anthocyanin pigments, which represent convenient visual markers in genetic and biochemical investigations. Most of the structural genes that encode the 15 enzymes required for anthocyanin synthesis and modification have been isolated (Winkel-Shirley, 2001) and several regulatory genes have been characterized. These transcriptional regulators include proteins containing R2R3-MYB domains. MYB proteins are a superfamily of transcription factors and 126 R2R3-MYB genes have been recently identified in the Arabidopsis genome (Yanhui et al., 2006), where they are responsive to one or multiple types of hormone and stress treatments. In addition, basic helix-loop-helix, (bHLH) transcription factors and proteins with conserved WD 40 repeats are also involved as transcriptional regulators of flavonoid biosynthesis. Many of these have been identified by genetic studies in Arabidopsis, maize, petunia, Antirrhinum and other plants.

The evidence currently available demonstrates that these transcription factors are involved in a complex cascade of interactions. This is illustrated for example by anthocyanin 1 (AN1) of petunia, a transcription factor of the bHLH family (Spelt et al., 2002). It activates the transcription of

the structural anthocyanin gene dihydroflavanol reductase (DFR) and of a putative regulatory gene (MYB 27) whose function is unknown. The expression of AN1 is regulated by AN2 and AN4 two MYB domain transcription factors whose activity appears to be regulated post-transcriptionally by a cytosolic WD 40 repeat protein encoded by AN11 in petunia. In addition to the regulation of anthocyanin gene transcription, AN1 controls other aspects of cell differentiation including the acidification of vacuoles in petal cells. The situation can be still more complex when distinct combinatorial interactions of *cis*-acting elements recognized by different transcriptional activators determine specific responses to various stimuli (Hartmann et al., 2005). For example in *Arabidopsis thaliana* four genes required for the synthesis of flavonols are coordinately expressed in response to light and are spatially coexpressed in siliques, flowers and leaves. A bHLH and a R2R3-MYB factor cooperate in directing tissue-specific production of flavonoids, while an ACE-binding factor, potentially a bZIP, and a R2R3-MYB factor work together in conferring light responsiveness (Hartmann et al., 2005).

Another bHLH transcriptional regulator closely related to the petunia AN1 gene, the bHLH2 gene has been characterized in *Ipomea purpurea* mutants (Park et al., 2007). In these mutants a partial reduction in the expression of all structural genes encoding enzymes for anthocyanin biosynthesis was observed in the young flower buds and the production and accumulation of protoanthocyanidin pigments in the seed coats were drastically reduced.

MYB factors are also involved in the control of anthocyanin concentration in crop plants. For example in apple fruit MdMYB1 a gene encoding a R2R3 MYB transcription factor (Takos et al., 2006) and MdMYB10 a MYB transcription factor, that is similar in sequence to known anthocyanin regulators in other species (Espley et al., 2007), can induce anthocyanin accumulation.

In addition to the regulation of anthocyanin synthesis, MYB factors of the small subfamily of R2R3-MYB transcription factors exert regulatory effects on different branches of the phenylpropanoid pathway. In grapevine, a member of this family, VvMYB5a, affects the expression of structural genes controlling the synthesis of phenylpropanoids and impacts on the metabolism of anthocyanins, flavonols, lignins and tannins (Deluc et al., 2006). More specifically, some members of this family seem particularly associated with the control of monolignol biosynthesis. EgMYB2 was cloned from Eucalyptus and the recombinant protein has been shown to specifically bind the *cis*-regulatory regions of the promoters of two lignin biosynthetic genes, namely cinnamoyl-coenzyme A reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD), which contain MYB consensus binding sites (Goicoechea et al., 2005). In transgenic tobacco plants over-expressing EgMYBb2, transcript abundance of genes encoding enzymes specific to lignin biosynthesis was increased whereas core phenylpropanoid genes were not significantly affected. A dramatic increase in secondary cell wall thickness and an alteration in lignin profiles were also observed supporting a targeted impact of EgMYB2 on lignification.

In the same way PtMYB1 and PtMYB4 from pine and AtMYB61 a member of the Arabidopsis R2R3-MYB family are closely associated to lignification (Newman et al., 2004; Patzlaff et al., 2003a). PtMYB1 was shown to bind AC elements (DNA motifs rich in adenosine and cytosine that have been implicated in the tissue-specific regulation of genes encoding phenylpropanoid and lignin biosynthetic enzymes) and may regulate transcription from *cis*-acting AC elements in pine xylem (Patzlaff et al., 2003b).

In addition to their roles in regulating developmental expression, these MYB factors are also regulators of the activation of phenolic metabolism, observed in plant responses to different stimuli. For example methyl jasmonate (MJ) induced in tobacco BY-2 cells the accumulation of specific phenylpropanoid conjugates in correlation with the induction of a R2R3MYB-type transcription factor (NtMYBJS1) that was co-expressed in a close temporal pattern with the core phenylpropanoid genes phenylalanine ammonia-lyase (PAL) and coumarate: CoA ligase (4 CL). It is clear that NtMYBJS1 functions with MJ in signal transduction pathways inducing phenylpropanoid genes and phenylpropanoid polyamine conjugates during stress (Galis et al., 2006).

These transcription factors have been exploited in metabolic engineering strategies for crop improvement. For example high-flavonol tomatoes resulted from the simultaneous expression of the heterologous maize transcription factor genes LC (MYC Type) and C1 (MYB type) (Bovy et al., 2002). These flavonols, which are potent antioxidants, are present in significant amounts in fruit flesh, a tissue that normally does not produce any flavonoids. Since the presence of proanthocyanidins (PAS) in forage crops is an important agronomic trait, preventing pasture bloat in ruminant animals a controlled increase in their synthesis has been investigated. The combined expression of the PAP1 MYB transcription factor and of the anthocyanidin reductase in tobacco leaves has led to the accumulation of epicatechin and gallocatechin monomers and of a series of dimers and oligomers consisting primarily of epicatechin units (Xie et al., 2006). These levels of PAS reached values in forage species that would confer bloat reduction.

6. Engineering the phenolic content of plant biomass towards its improved utilization

The strategies and limits of engineering biosynthetic pathways leading to plant natural have been the subject of a recent excellent review by Dixon (Dixon, 2005). The lack of clear understanding of the biosynthesis of the targeted products, the complex interplay between different branches of metabolism and the need for coordinate regulation of multiple gene activities are among the main limiting factors.

The exploitation of plant biomass for non-food uses is a growing concern in the general context of sustainable development. Indeed plant biomass is a reservoir of renewable carbon, which could be more extensively used for the production of fuels and "green" chemicals. The design of biomass for these specific purposes is of interest in combination with other technical and agronomical strategies. Lignin is a key component of biomass with both negative and positive effects on biomass processing and utilization. Until now, many studies have targeted lignin biosynthetic genes for transgenic downregulation to improve paper pulping or forage digestibility, and in some cases have generated plant varieties with a reduced lignin content or altered lignin composition. One of the most significant examples which could be of immediate utility corresponds to the production of poplar trees down-regulated for cinnamyl alcohol dehydrogenase which exhibit lignins with an altered structure more easily extractable during the pulping process with resulting benefits on pulp yield and consumption of energy and chemical. The agronomic performance of these transgenic trees in field trials did not reveal any disadvantages that could counterbalance the positive characteristics (Pilate et al., 2002). Only the reluctance to deploy genetically modified organisms at the European level and the lack of regulatory clarity in transgenic tree release in other jurisdictions are precluding exploitation of these transgenics.

Beyond the modified expression of one gene, more sophisticated strategies have been envisaged such as the simultaneous introduction by cotransformation, of a sense and an antisense construct to simultaneously upregulate one enzyme and downregulate another. For example aspen trees (*Populus tremuloides*) expressing both antisense 4-coumarate-CoA ligase and sense coniferaldehyde 5-hydroxylase had a reduced lignin content (52% less) and a 64% higher S/G ratio (Li et al., 2003).

One of the drawbacks of targeted induced modifications of key enzymes of phenolic metabolism aiming to increase or decrease a specific compound is the observation of unexpected effects due to the combined outcome of a complex interplay of various metabolic pathways. These interactions may be observed between different branches of phenolic biosynthesis as recently shown by Besseau et al. (2007). In Arabidopsis plants silenced for hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) expression lignin repression leads to the direction of the metabolic flux into flavonoids through chalcone synthase activity. Correlated with this, a strong reduction of plant growth is observed. Interestingly, the suppression of flavonoid accumulation by repression of chalcone synthase expression in HCT silenced plants restored wild-type plant growth. The data show that the reduced size phenotype was due to an indirect effect of ectopic flavonoid accumulation altering auxin transport.

A recent study was undertaken in Boerjan's group (Dauwe et al., in press) on the consequences of altering the expression of cinnamoyl CoA reductase (CCR) or cinnamyl alcohol dehydrogenase (CAD) or of both genes in tobacco on the transcriptome and metabolome. A cDNA-AFLP based survey of 11,000 transcript-derived fragments revealed that down-regulation of genes in monolignol biosynthesis may affect the expression of several genes in a number of metabolic pathways. These responses concern genes involved in secondary metabolism as well as in primary metabolism. Understanding these pleiotropic effects is important for several reasons. From a fundamental point of view, it provides a better knowledge of metabolic interplay between different pathways and may reveal new targets for pathway engineering. From an applied point of view it can help to predict any negative and unanticipated consequences of a mutation on plant health and quality. This knowledge opens possibilities to mitigate these effects through genetic engineering or breeding.

In addition to improvements in the context of the pulp industry that have motivated a large number of research programmes lignocellulosic materials can be used in different ways for the production of energy and "green materials". Investigations are emerging in this area in order to provide an optimized resource more adapted to downstream processes. Containing a higher proportion of carbon than polysaccharides, lignin has a higher calorific value and when wood is used directly for energy production, lignin-enriched plant species could be advantageous if this enhancement has no negative impacts on growth and development. Despite several attempts, efforts towards increasing lignin biosynthesis have been most of the time unsuccessful. Only in the case of the model plant, tobacco, the ectopic expression of R2R3MYB transcription factors from pine (Patzlaff et al., 2003b) or from Eucalyptus (Goicoechea et al., 2005) induced increased lignification.

The concept of the biorefinery envisages the conversion of biomass feedstocks, that largely consist of lignocelluloses, for producing biofuels, bioproducts and materials in integrated zero-waste systems. Plant cell walls contain lignins to resist breakdown and microbial attack and this resistance to breakdown is a massive bottleneck in the conversion processes. To replace the harsh chemical and physical treatments currently used in biorefineries, biological solutions should be more extensively used. They can proceed from a better adaptation of the plant resource and from the involvement of new microorganisms able to degrade cell wall components more efficiently (cellulases, hemicellulases, lignin-degrading oxidases). Metagenomics, a research field that analyses the genome of whole microorganism communities, should provide new enzymes more efficient in degrading the lignocellulosic material if special interest is focused on microbial communities that thrive on lignocellulosic substrates. In addition, progress has recently been made on the use of lignin as a feedstock for novel chemicals (reviewed in Boudet et al., 2003). Some bacteria can produce polyester and polyamide plastics from a wide variety of dimeric and monomeric lignin compounds. The production of lignin-derived end products is

still in its infancy at present but here again the progress of metagenomics and of engineering microorganisms might lead to new avenues for optimizing lignin use to produce novel chemicals.

7. An explosion of studies aiming to confirm the health benefits of phenolics

Considering that the role of dietary polyphenols in disease prevention has only attracted real scientific interest for about a decade, this field has seen a remarkable rate of progress.

Simple phenolics such as hydroxycinnamic acid conjugates and flavonoids are important constituents of fruits, vegetables and beverages. These compounds show a wide range of antioxidant activities *in vitro* (Rice-Evans et al., 1995) and are thought to exert protective effects against major diseases such as cancer and cardiovascular diseases. Oxidative stress imposed by reactive oxygen species (ROS) indeed plays a crucial role in the pathophysiology associated with neoplasia, atherosclerosis and neurodegenerative diseases. The ROS-induced development of cancer involves for example malignant transformation due to DNA mutations as well as modification of gene expression through epigenetic mechanisms (Lee and Lee, 2006). Proteggente et al., 2002 have evaluated, in a comparative way, the antioxidant activities of the major fruit and vegetables in the diet. Fruit and vegetables rich in anthocyanins (e.g. strawberry, raspberry and red plum) demonstrated the highest antioxidant activities, followed by those rich in flavonones (e.g. orange and grapefruit) and flavonols (e.g. onion, leek, spinach and green cabbage), while the hydroxycinnamate-rich fruits (e.g. apple, tomato, pear and peach) exhibited lower antioxidant activities.

A wide range of molecular, *in vitro* and epidemiological studies have been undertaken to confirm the postulated effects of these compounds. Epidemiological studies analyse the health implications of dietary phenolic intake on various pathological situations. These studies, in addition to their own complexity, must take into account the biochemical composition of the components of the diet and the bioavailability of the individual compounds under study. The absorption and bioavailability of phenolics in humans are still controversial as reviewed by Karakaya (2004). Increasing evidence shows that hydroxycinnamate derivatives and flavonoids can be absorbed into the human body in amounts that are, in principle, sufficient to exert antioxidant or other biological activities in vivo (Scalbert and Williamson, 2000; Olthof et al., 2001). However Rechner et al. (2002) have emphasized that intact conjugated polyphenols are detected at much lower levels than their cleavage products resulting from the action of colonic bacterial enzymes and subsequent metabolism in the liver. Consideration should also be given to these cleavage products.

A large number of epidemiological studies have revealed that a high consumption of antioxidant-rich fruits and vegetables is inversely correlated with the incidence of cancer (Levi, 1999; Knekt et al., 2002).

However recent studies reviewed by Lee and Lee (2006) show that the efficacies of antioxidant therapies based on antioxidative phenolics able to decrease ROS levels have been equivocal at best. Some antioxidants exhibit prooxidant activity under certain conditions and potential carcinogenicity under others and dietary supplementation with large amounts of a single antioxidant may be deleterious to human health. One of the most crucial conclusions at the present time is that a low risk of cancer is more closely related to a diet rich in multiple antioxidants than one supplemented with one individual antioxidant. The combination of antioxidative agents with different modes of action is suggested to increase efficacy and minimize toxicity (Lee and Lee, 2006) and additive and synergistic effects of dietary phytochemicals obtained from fruits and vegetables are likely responsible for the anticancer activities in a more efficient way than expensive dietary supplements (Liu, 2004).

Nevertheless, the specific actions of individual molecules are supported by *in vitro* assays. Haddad et al. (2006) have, for example, evaluated the antiproliferative effects of different flavonoids on Pca cell lines corresponding to human prostate cancer. Five flavonoids induced cell cycle arrest in these Pca cell lines supporting the previous results of epidemiological studies having demonstrated an inverse association between flavonoid intake and prostate cancer. Indeed, some flavonoids are now in clinical trials in patients with hormone-refractory prostate cancer (Brown et al., 2005). In addition, and from a mechanistic point of view, a wide range of studies point out potential molecular targets of polyphenols. For example, the activity of protein kinase C, a major intracellular receptor for a mouse skin tumor promoter, can be modulated by plant phenolic acids, particularly tannic acid (Szaefer et al., 2006).

A more recent area is the emerging role of polyphenolic compounds in the treatment of neurodegenerative diseases, such as Alzheimer's and Parkinsons's diseases which are clearly associated to oxidative stress. Increasing numbers of studies demonstrate the efficacy of polyphenolic antioxidants from fruits and vegetables to reduce or to block neuron death. In addition to intrinsic antioxidant properties polyphenols seem to exert beneficial effects through different pathways such as signaling cascades, anti-apoptotic processes or the synthesis/degradation of the amyloid β peptide (Ramassamy, 2006). All together, plant-derived polyphenols appear to be a promising class of compounds for neuroprotection.

In other work, it has been shown, on the basis of epidemiological studies, that moderate intake of alcoholic beverages rich in polyphenols reduces the risk of cardiovascular, cerebrovascular and peripheral vascular diseases. Particularly, the phenolic compounds in red wine appear to interfere with the molecular processes underlying the initiation, progression and rupture of atherosclerotic plaques (Szmitko and Verma, 2005). However, large-scale randomized clinical trials of red wine intake are necessary before

final conclusions can be drawn, but focused studies support the involvement of polyphenols in the control of specific events related to atherosclerotic lesions (Oak et al., 2006). Recent excellent reviews cover the topic of polyphenols and prevention of cardiovascular diseases (Manach et al., 2005; Corder et al., 2006).

In-depth studies have dealt with phytoestrogen properties of different sources of polyphenols: isoflavonoids, stilbenes, coumestrans and lignans (reviewed by Cornwell et al., 2004). Preventing menopause symptoms through hormone replacement therapy may actually increase the risk of cancer but despite a hundred or so clinical trials, the understanding of the health effects of phytoestrogens is far from complete. The strongest body of evidence supports the view that a least soy isoflavonoids may be effective in preventing osteoporosis (Cornwell et al., 2004).

Independently, several reports suggest the involvement natural phenolics in the prevention of skin damage (Svobodova et al., 2003; Hsu, 2005).

Many interesting results indicate strongly positive correlations between the dietary intake of polyphenol-containing food and the prevention of major diseases. Future studies performed with standardized and structurally characterized mixtures of compounds or with isolated molecules will undoubtedly provide new opportunities to more clearly define the role of these food components, their health benefits and possible risks. Additive and synergistic effects of polyphenols with each other and with other protective compounds are likely to be the key of many of the observed effects. Results from these studies may catalyze increased research interest in plant phenolic metabolism, its diversity and its regulation in the future.

8. Concluding remarks

Plant biochemicals are gifts from nature. These visible or more discrete gifts are renewed each year through the energy from the sun. Phenolic compounds are typical representatives of these botanical gifts. They already have a long history of scientific investigation and represent the most abundant and the most widely represented class of plant natural products. The ability of plants to produce such an abundance of phenolic compounds is based upon the continual evolution of new genes brought about by gene duplication and mutation and subsequent recruitment and adaptation to specific functions. The inventory of these compounds is still uncomplete, every month provides new structures and the dissection of their metabolic pathways is far from being complete. For example recent reports underline that important questions still remain to be answered in the field of protoanthocyanidins and tannins (Xie and Dixon, 2005), and even the exact nature of the biosynthetic pathway(s) leading to lignin monomers is not fully clarified.

The factors motivating investigations into these chemical constituents were essentially the same as those driving similar work on other plant natural substances i.e. either scientific curiosity or the hope of finding some exploitable products of value to mankind. The field has successfully exploited a diversity of techniques and approaches, including the use of sophisticated analytical methods, isotopically labelled products, cell biology and microscopy techniques and more recently breakthroughs in molecular biology, genomics and genetic engineering.

This combination of different tools has revealed both the diversity and the high flexibility of this metabolism and its obvious involvement in various plant functions. Polyphenols are indeed key factors in defense and in morphological and biochemical adaptations to changing biotic and abiotic environments. The present phenolic profiles of higher plants are thus the result of long and complex processes of evolution and coevolution. It is clear that the regulation of their synthesis involves very sophisticated mechanisms driving the precise control of the production of specific molecules at the right place and the right time or in response to environmental cues. These integrated responses represent good examples of systems biology.

In addition to their biological functions in plants and in some cases to their aesthetic value, polyphenols are widely present in food products and agricultural/forestry raw materials. Research programmes driven by societal demand have increased significantly during the last decade. Two major objectives have been targeted: to rationalize the potential health benefits of polyphenols with antioxidant properties and to redesign plants better suited to specific agroindustrial processes (forage consumption, pulp industry, new uses of plant biomass). Many promising results have been obtained and the temptation to engineer or breed plants in relation to these different objectives is still great. However, metabolomics and microarray analysis of global expression patterns have revealed that playing with a piece of the jigsaw may induce unfavourable changes in the fragile equilibrium of the interconnected pathways. Accurate controls should be envisaged in future studies to check for these potential pitfalls.

The future of phenolic research will likely include surprising and unexpected advances in the characterization of new structures, new "in planta" functions and new exploitations in human activities.

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Alain Boudet has got a PhD from the University of Toulouse in Plant Biochemistry (metabolism of quinic and shikimic acid). He is professor of Plant Biochemistry and Plant Physiology at the University of Toulouse and at the Institut Universitaire de France. His scientific career benefited from several stays in excellent laboratories in England, USA, Germany and Belgium. He has published about 200 papers in refereed journals mainly on phenolic metabolism, lignin biosynthesis and lignin genetic engineering. He was for a long time director of the large Research Institute "Cell signaling and Plant Biotechnology" in Toulouse and he has coordinated several European programmes. He has been a co-editor or a member of the editorial board of several scientific journals including at the present time "Phytochemistry"and he was a recipient of the Tate and Lyle award of the Phytochemical society of Europe in 1987. Alain Boudet is also member of the French academies of Science and Technology.