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Review

Metabolome diversity: too few genes, too many metabolites?☆

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Abstract

The multitude of metabolites found in living organisms and the calculated, unexpected small number of genes identified during genome sequencing projects discomfit biologists. Several processes on the transcription and translation level lead to the formation of isoenzymes and can therefore explain at least parts of this surprising result. However, poor enzyme specificity may also contribute to metabolome diversity. In former studies, when enzymes were isolated from natural sources, impure protein preparations were hold responsible for broad enzyme specificity. Nowadays, highly purified enzymes are available by molecular biological methods such as heterologous expression in host organisms and they can be thoroughly analyzed. During biochemical analysis of heterologously expressed enzymes poor specificity was observed for enzymes involved in fruit ripening, e.g. in flavour and color formation. Surprisingly broad specificity was shown for the reactants in the case of alcohol acyl-CoA transferase, *O*-methyltransferase, glucosyltransferase, P450 monooxygenases as well as polyketide synthases and for the product in the case of monoterpene synthases. Literature data confirm the assumption of limited specificity for enzymes involved in metabolism and bioformation of secondary metabolites. It is concluded that metabolome diversity is caused by low enzyme specificity but availability of suitable substrates due to compartmentation has also taken into account.

Keywords: Plant secondary metabolites; Enzyme specificity; Substrate availability; Diversity

Contents

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1. Introduction

Plants are the source of an almost uncountable number of metabolites whose structure, function and usability have been explored only partially. More than 100,000 plant secondary metabolites have already been identified, which probably represent only 10% of the actual total in nature (Wink, 1988). A large fraction of this diversity is derived from differential modification of common backbone structures, which requires the evolution of enzymes with the respective product specificity, catalyzing the different chemical reactions. Out of a total of 5,000 different flavonoids, 300 different glycosides of a single flavonol, quercetin, have already been identified (Harborne and Baxter, 1999) and individual plant tissues, such as berries of grapevine (*Vitis vinifera*) accumulate more than 200 different aglycones conjugated to glucose (Sefton et al., 1993, 1994). Consequently, the complement of all glycosylating activities in any given individual plant must therefore be regarded as broad.

On the other side genomes such as that of *Arabidopsis* carry not more than 25,500 genes (Bevan et al., 2001) and the screening of the consensus sequence of glycosyltransferases in the *Arabidopsis* genome yielded 107 sequences (Ross et al., 2001). It is feasible that a proportion of these consensus sequences are not expressed or involved in primary metabolism rather than secondary metabolism. Although the direct comparison of the cited numbers is not legitimate it becomes obvious that the number of metabolites found in one species exceeds the number of genes involved in their biosynthesis. The concept of one gene—one mRNA—one protein—one product is collapsing. It turns out that there are many more proteins than genes in cells because of post-transcriptional modification. Therefore, many proteins cannot be anticipated from single knowledge of an organism's genome. This hypothesis is also supported by the fact that increasing metabolome diversity does not simply correlate with increasing gene number (Copley et al., 2001; Mayer and Mewes, 2001). There are several mechanisms providing multiple mRNAs from one gene, multiple proteins from one mRNA and multiple products from one protein.

Consequently, diversity can occur on the DNA, mRNA, and protein level (Table 1). On the mRNA level alternative pre-mRNA splicing can lead to multiple proteins being made from one single DNA coding sequence. Depending on the remaining exons different specificities arise (Reddy, 2001). On the gene level alternative reading frames provide multiple mRNAs. A recent publication demonstrated that two transcripts are synthesized from different promotors and have different first exons but share exon 2 and 3. The transcripts are encoded in two distinct reading frames, in a process that yields two entirely different protein products (Ruas and Peters, 1998). The proteins are clearly the products of the same genomic locus but they are unusual and entirely distinct splice variants. The difference to the first example consists in the two promotors using the same DNA coding sequence but encoding two different reading frames. Gene fusion represents another example of gene diversification. A single gene isolated from *Mucor circinelloides* (*carRP*), coding for a protein with two different enzymatic activities, lycopene cyclase and phytoene synthase was functionally expressed in *E. coli* (Velayos et al., 2000). The gene product contains two domains: the R domain is located at the N-terminus and determines lycopene cyclase activity; the P domain is located at the C-terminus and displays phytoene synthase activity. A similar gene homologous to *carRP* has been reported: the *crtYB* gene from the basidiomycetous yeast *Xanthophyllomyces dendrorhous* (Verdoes et al., 1999). On the protein level post-translational modifications and heterodimer formation can lead to multiple

Table 1
Mechanisms explaining the diversity of metabolites

Level	Mechanism	Reference
DNA	Alternative reading frame	Ruas and Peters, 1998
	Gene fusion	Velayos et al., 2000
mRNA	Alternative splicing	Reddy, 2001
Protein	Post-translational modification	Bachmair et al., 2001
	Heterodimer formation	Frick and Kutchan, 1999; Frick et al., 2001
	Specificity	this publication

enzymes (Bachmair et al., 2001; Frick and Kutchan, 1999). Recently, it was shown that heterodimers consisting of two isoenzyme monomers exhibit different specificities in comparison with their parent enzymes (Frick et al., 2001). It will be shown in the present paper that although enzymes are considered highly regioselective they often accept more than one substrate. Thus, depending on substrate availabililty multiple products emerge explaining a lot of metabolome diversity.

2. Enzyme specificity

Generally, enzymes show three levels of specificity: for the substrate, for the reaction and for the product. In reality also enzymes involved in primary metabolism are not absolutely specific. Hexokinase, for example accepts D-glucose but also D-fructose as substrate and forms the corresponding 6-phosphates. Some glycolytic enzymes may be multifunctional proteins involved in processes other than carbohydrate metabolism (Plaxton, 1996). Yeast hexokinase exhibits protein kinase activity. Enolase is a structural protein of the eye lens. In yeast enolase is a heat shock protein that may confer thermotolerance (Fothergill-Gilmore and Michels, 1993). Concerning the reaction specificity, triose phosphate isomerase, the enzyme catalyzing the reversible transformation of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate forms also methylglyoxal by an elimination reaction beside the isomerisation reaction (Richard, 1991). Finally, there is also a number of multiple product enzymes such as the (+)-bornyldiphosphate synthase (Wise et al., 1998). This enzyme forms at least seven products. Modeling studies indicated greater access of water to the active site of this enzyme compared to olefin synthases, thus enabling the formation of additional products (Schwab et al., 2001). Although (+)-bornyldiphosphate is the major product, (+)-bornyl diphosphate synthase cannot really considered selective. Many more present-day proteins are known to have the capability of accepting more than one substrate, catalyzing more than one reaction and forming more than one product. They are called multifunctional enzymes.

However, multifunctionality of proteins is not restricted to catalytic activities. Beside enzymes, proteins act as inhibitors, transporters, hormones, storage proteins, defense agents, etc. Moreover, the same protein can be involved in several processes, i.e., reveal multifunctional properties (Mosolov et al., 2001). An unusual protein capable of inhibiting both trypsin and alpha-amylase was first isolated from ragi seeds (*Eleusine coracana* Gaertn.) and in the meanwhile additional bifunctional inhibitors have been published. Examples of enzymatic activity of proteinase inhibitors have also been reported. Trypsin inhibitors display dehydroascorbate reductase activity and a storage protein from yam (*Dioscorea batatas* Decne) tubers, was found to both inhibit trypsin and catalyze the carbonic anhydrase reaction, the latter playing an important role in photosynthetic CO_2 fixation (Mosolov et al., 2001). The R domain of the *carRP*-protein has also two functions. It exhibits lycopene cyclase activity and contains a transmembrane domain serving as membrane anchor for phytoene synthase (Velayos et al., 2000).

Diverse functional roles of multifunctional proteins arise from either their independent functional domains or dual activities mediated through a single active site (Kumar and Deobagkar, 1996). The presence of multifunctional proteins significantly enhances the metabolic efficiency of a cell. The concept of one enzyme-one activity is rapidly vanishing as more and more multifunctional proteins are being identified (Kirschner and Bisswanger, 1976; Ramasarma, 1994, Eckermann et al. 1998; Frick and Kutchan, 1999; Chiron et al., 2000; Ross et al., 2001; Hefner et al., 2002). The following examples will support the hypothesis that poor enzyme specificity contributes to metabolome diversity.

3. Alcohol acyl-CoA transferase

Recently, the first alcohol acyl-CoA transferase gene was isolated from a plant source, cloned and functionally expressed in *E. coli* (Aharoni et al., 2000). The encoded protein catalyzes the formation of volatile esters in strawberry fruits. Ester formation is the result of transacylation from acyl-CoA to an alcohol. Comparison of the kinetic data obtained for the partially purified enzyme isolated from strawberry fruits and the recombinant protein showed that the preferred substrates of the enzyme are acetyl-CoA or pentyl-CoA and medium chain alcohols e.g. octanol (Aharoni et al., 2000; Olias et al., 1995). However, octyl pentanoate has never been identified as constituent of strawberry fruits (Fig. 1). The observed selectivity of the enzyme has not been sufficient to explain the specificity of the ester profile obtained. Thus, ester formation in strawberry is governed more by the supply of specific suitably functionalized substrates and less by enzyme specificity. A similar observation was obtained for the formation of volatile branched chain esters in bananas (*Musa sapientum* L.) (Wyllie and Fellman, 2000). It was suggested that substrate supply is a major determinant of the quantitative and qualitative composition of the aroma profile of banana fruit.

4. *O*-Methyltransferase

Work on *O*-methyltransferases indicated that this class of enzymes has varying methylating capabilities

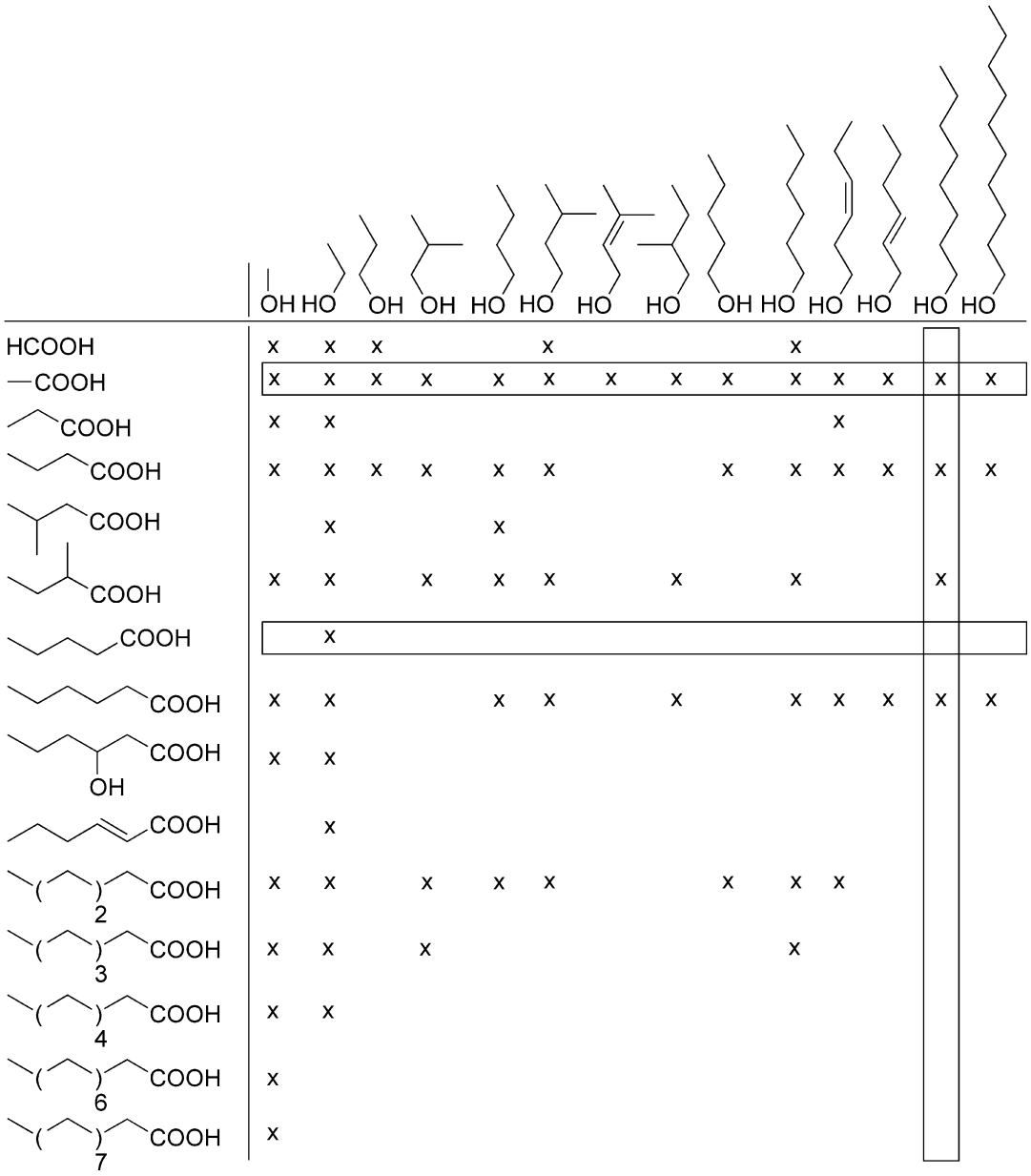

Fig. 1. Volatile esters identified in strawberry fruits. Alcohol moieties are shown on the top and acid moieties are depicted on the left. Bars illustrate the products formed by the preferred substrates of the strawberry alcohol acyl-CoA transferase (data are taken from Honkanen and Hirvi, 1990 and Aharoni et al., 2000).

accepting e.g. hydroxylated cinnamic acids, alkaloids, and flavonoid aglycons (Ibrahim et al., 1998; Joshi and Chiang, 1998). One single *O*-methyltransferase has been shown to act on more different natural products like simple catechols, phenylpropanoids and structurally complex isoquinoline alkaloids (Frick and Kutchan, 1999). Two caffeic acid *O*-methyltransferases (COMT I and II) have been isolated from alfalfa internodes (Inoue et al. 2000). COMT II exhibited an unusually low K_m value for caffeic acid but could also methylate flavonoids and simple phenols. In contrast, only caffeic acid and 5-hydroxyferulic acid were accepted by COMT I.

Plant *O*-methyltransferases use *S*-adenosyl-L-methionine (SAM) as methyl source, yielding S-adenosyl-L-homocysteine and the methyl ether derivative as product. This family of enzymes shows conserved SAM binding while affording sufficient degree of active site diversity to bind and correctly position a variety of disparate small molecules. Only recently, the first crystal structures of two plant *O*-methyltransferases involved in (−)-medicarpin biosynthesis in *Medicago sativa* (alfalfa) have been published (Zubieta et al., 2001). This work provides a structural basis for understanding the substrate specificity of the divers family of plant *O*-methyltransferase but still it is impossible to predict the correct

substrates. Surprisingly, the isoflavone-*O*-methyltransferase, which is induced along with other enzymes involved in medicarpin biosynthesis in alfalfa, methylates the A-ring 7-hydroxyl group of daidzein in vitro, a reaction that probably does not occur in vivo. The enzyme also accepted substrates not present in alfalfa (He et al., 1998). Nevertheless, this *O*-methyltransferase was proposed as the enzyme that catalyzes the formation of formononetin in vivo. It was assumed that the methylation reaction may occur in a metabolic channel in which a microenvironment is created that favors the methyl transfer to a yet unknown unstable intermediate. While this enzyme will methylate daidzein, this compound is not the in vivo substrate. The *O*-methyltransferase almost certainly never encounters daidzein and thus produces no isoformononetin in vivo. In other words, the access to the substrate (substrate channeling) defines the product pattern.

Also tobacco possess an array of *O*-methyltransferase isoforms with variable efficiency toward the diverse plant *ortho*-diphenolic substrates (Maury et al., 1999). In angiosperms, two types of structurally and functionally distinct lignin pathway *O*-methyltransferases, caffeic acid 3-*O*-methyltransferases (COMT) and caffeoyl CoA 3-*O*-methyltransferases (CCoAOMT) have been reported and extensively studied. In gymnosperms an *O*-methyltransferase exhibiting COMT and CCoAOMT activities and thus the potential to mediate a dual methylation pathway in lignin biosynthesis was demonstrated (Li et al., 1997). Molecular cloning and functional expression of a stress-induced multifunctional *O*-methyltransferase with pinosylvin methyltransferase activity from Scots pine (*Pinus sylvestris* L.) showed that this new enzyme catalyzes the methylation of diverse phenolic substrates (Chiron et al., 2000). The very broad substrate specificity found for the expressed protein was unexpected because substrates of several branches of the phenylpropanoid metabolism, such as stilbenes, flavonols and hydroxycinnamic acids, were methylated.

An *O*-methyltransferase, catalyzing the transfer of a methyl group from *S*-adenosyl-L-methionine to an alcohol forming a methyl ether was recently detected in red ripe strawberry fruits but not in immature fruits. Surprisingly, the enzyme accepts substrates such as caffeic acid and catechol but also the key strawberry aroma compound 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone (Fig. 2). The ratios of the enzyme activity towards the three different substrates were constant in three different strawberry cultivars. This implies that only one enzyme is present or three different enzymes are formed in a defined ratio. An *O*-methyltransferase gene was isolated from strawberry fruits and functionally expressed in *E. coli* (Wein et al., 2002). The recombinant protein showed the same substrate preference as the native enzyme. Thus, the existence of different enzymes can be excluded. The 2,5-dimethyl-4-hydroxy-3(2*H*)-furanone occurs as a mixture of four isomers due to keto-enol tautomerisation (Fig. 2). We assume that only one isomer, the dienol, resembling the *ortho*-diphenyl structure of the *O*-methyltransferase substrates is the actual substrate of the enzyme. Kinetic data showed that 3,4-dihydroxybenzaldehyde is the preferred substrate. However, the product vanillin has only been identified as a trace compound in strawberry but 2,5-dimethyl-4-methoxy-3(2*H*)-furanone and its precursor are major components of the volatile extracts obtained from

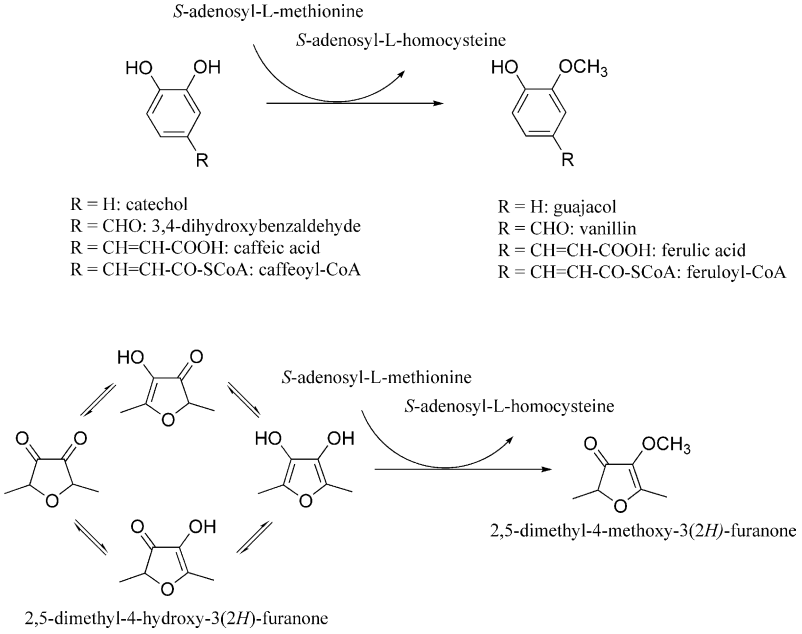

Fig. 2. Substrates and products of the strawberry *O*-methyltransferase.

strawberry fruits. It is concluded that in the case of the strawberry *O*-methyltransferase, substrate availability governs the formation of the methylated products.

5. Terpene synthases

A cDNA from peppermint encoding (*E*)-β-farnescene synthase was cloned by random sequencing of an oil gland library and was expressed in *E. coli* (Crock et al., 1997). The recombinant, partially purified (*E*)-β-farnescene synthase showed a K_m value of 150 μM for Mg^{2+} and a K_m value of 7.0 μM for Mn^{2+}. The sesquiterpenoids produced by the recombinant enzyme are (*E*)-β-farnescene (85%), (*Z*)-β-farnescene (8%), and Δ-cadinene (5%) with farnesyl diphosphate and Mg^{2+} as cofactor, and (*E*)-β-farnescene (98%) and (*Z*)-β-farnescene (2%) with Mn^{2+} as cofactor. No Δ-cadinene or other sesquiterpenoid was synthesized in the latter instance, indicating that a structural alteration in the binding of Mn^{2+} to the substrate and/or enzyme improves the fidelity of the reaction and reduces the diversity of the products (Crock et al., 1997). With the C_{10} analog, geranyl diphosphate, as substrate, the monoterpenes limonene (48%), terpinolene (15%), and myrcene (15%) are produced. Although the relative velocity at saturated levels of geranyl diphosphate was only 3% of the velocity with farnesyl diphosphate for the synthase, the calculated K_m value for geranyldiphosphate was only 3-fold higher than that for farnesyl diphosphate, suggesting that the binding of the C_{10} analog was reasonably efficient. Thus, depending on the available substrate monoterpenes or sesquiterpenes are formed and the type of metal cofactor takes on the fine tuning of the sesquiterpene pattern.

Citrus limon possesses a high amount and large variety of monoterpenoids, especially in the glands of the fruit flavedo. By applying a random sequencing approach to a cDNA library from mRNA isolated from the peel of developing fruit, four monoterpene synthase cDNAs were isolated and functionally expressed in *E. coli* after removal of their plastid targeting signals (Lücker et al., 2002). The four enzymes account for the production of 10 out of the 17 monoterpene skeletons commonly observed in lemon peel oil, corresponding to more than 90% of the main components present (Table 2).

As already mentioned (+)-bornyl diphosphate synthase is an example for poor product specificity. In contrast, (−)-limonene and (−)-pinene synthase are more selective as these monoterpene cyclases produce only (−)-limonene or a mixture of only (−)-α- and (−)-β-pinene, respectively (Bohlmann et al., 1997). However, beside the natural substrate geranyl diphosphate all three monoterpene synthases also accept the artificial substrates 6,7-dihydrogeranyl diphosphate and 2,3-cyclopropylgeranyl diphosphate converting them into new products, never been detected in nature before (Fig. 3, Schwab et al., 2001). If by chance a non-selective reductase evolves, generating 6,7-dihydrogeranyl diphosphate from geranyl diphosphate, this new product can be further metabolized by present-day monoterpene synthases leading to a series of new secondary metabolites.

Table 2
Products formed by four *C. limon* monoterpene synthases (B93, D85, C62, M34) expressed in *E. coli* (data are taken from Lücker et al., 2002)

	B93 (%)	D85 (%)	C62 (%)	M34 (%)
α-Thujene	2.5			
α-Pinene	5.6	4.1	Trace	Trace
Sabinene	0.4	11.0		
β-Pinene	4.7	81.4		
β-Myrcene	0.9		0.8	0.8
α-Terpinene	1.7			
para-Cymene	Trace			
Limonene	9.1	3.5	99.2	99.2
γ-Terpinene	71.4			
Terpinolene	3.7			

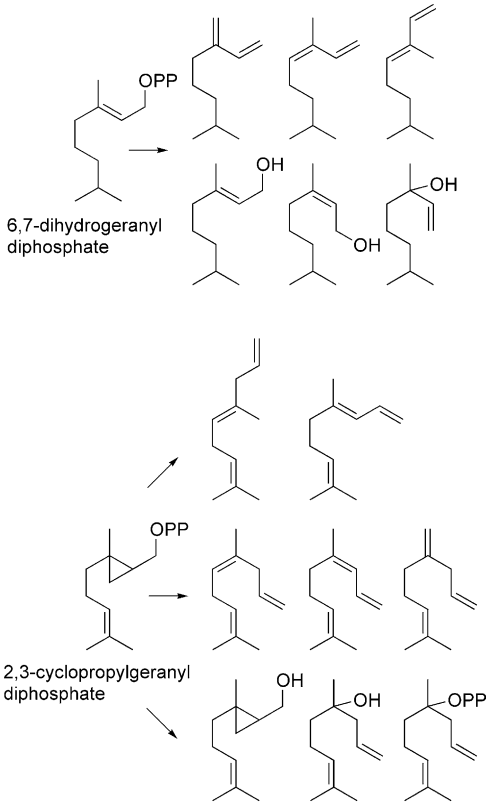

Fig. 3. Alternative, artifical substrates for monoterpene synthases: 6,7-dihydrogeranyl diphosphate and 2,3-cyclopropylgeranyl diphosphate and corresponding products (Schwab et al., 2001).

6. Glycosyltransferase

Poor enzyme specificity offers the opportunity to transform new products, which have been introduced into plant metabolism such as pesticides or by molecular biological methods. Recently, transgenic *Petunia* plants carrying the *S*-linalool synthase gene from *Clarkia breweri* were generated. First analyses showed that although the gene was expressed *S*-linalool was not detected. However, further analysis uncovered that the formed *S*-linalool was immediately metabolised to its glycoside as shown by LC–MS analysis (Lücker et al., 2001). Although *S*-linalyl glucoside is not present in *Petunia* plants there is already a non-specific glycosyltransferase present able to form the tertiary glucoside.

The cDNAs encoding a 3-*O*-glucosyltransferase and 5-*O*-glucosyltransferase were isolated from *Petunia hybrida* (Yamazaki et al., 2002) and the recombinant proteins were produced in yeast. The 3-GT converts not only anthocyanidins but also flavonols into the corresponding 3-*O*-glucosides and is most likely responsible for glucosylation of both compounds in vivo. In contrast, the recombinant 5-GT exhibits strict substrate specificity towards anthocyanidin 3-acylrutinoside.

Heterologous expression of two cDNAs encoding glucosyltransferases isolated from tobacco (*NtGTs*) in *E. coli* showed that the recombinant enzymes have glucosylation activity against both flavonoids and coumarins (Taguchi et al., 2001). Both enzymes also strongly react with 2-naphthol as a substrate and can utilize UDP-glucose as the sugar donor but also UDP-xylose as a weak donor. The results suggests that *NtGTs* encode the enzymes that can react with many kinds of naphthalene-like skeleton in tobacco cells.

Plant glucosyltransferases play a crucial role in natural product biosynthesis and metabolization of xenobiotics as shown by the heterologous expression, characterization, and molecular analysis of an arbutin synthase from *Rauvolfia serpentina*. Out of 74 natural and synthetic phenols and two cinnamyl alcohols, this enzyme accepts 45 substances as substrates, covering a broad range of structural features (Hefner et al., 2002).

Recently, the consensus sequences for glycosyltransferases were used to screen the genome data base of *Arabidopsis* to identify the number of putative enzymes of this class in a single plant species (Li et al., 2001). A large multigene family composed of 107 sequences was discovered. Ninety of these sequences have now been expressed as recombinant proteins in *E. coli*, and their in vitro catalytic activities have been analyzed. Using benzoates in a preliminary screen, 14 genes encoding enzymes with catalytic activity toward one or more of these substrates were identified (Lim et al., 2002).

The final step in the biosynthesis of the cyanogenic glucoside dhurrin is the transformation of the labile cyanohydrin into a stable storage form by *O*-glucosylation of (*S*)-*p*-hydroxymandelonitrile at the cyanohydrin function (Jones et al., 1999). The substrate specificity of the enzyme was established using isolated recombinant protein. In addition to mandelonitrile and *para*-hydroxymandelonitrile, benzyl alcohol, benzoic acid, and the monoterpenoid geraniol were also utilized as acceptor substrates by the *O*-glucosyltransferase. Geraniol was glucosylated at a rate of 11% compared with *para*-hydroxymandelonitrile. Tertiary structural modeling indicates that this unexpected and broad specificity may be explained by the similarity of geraniol to benzyl alcohol in particular configurations (Jones et al., 1999). The picture that is emerging regarding plant glucosyltransferase substrate specificity is one of limited by extended plasticity towards metabolites of related structure. This in turn ensures that a relatively high, but finite, number of glucosyltransferases can give rise to the large number of glucosides found in plants (Jones et al., 1999).

7. Miscellaneous

A new member of the chalcone synthase-like gene family that uses acetyl-CoA as primer in the synthesis of pyrones was recently discovered (Eckermann et al., 1998). The enzyme can also utilize benzoyl-CoA as primer to synthesize the backbone of phenylpyrones, synthetic derivatives. This work demonstrates that chalcone synthase-related enzymes are involved in the synthesis of a much larger range of plant natural products than at first expected. It is now apparent that chalcone synthase is a member of a family of closely related polyketide synthases that can utilize different starter molecules, and perform different numbers of condensation reactions, to yield a variety of natural products, such as benzalacetones, acridones, styrylpyrones, benzophenones leading to xanthones, and bibenzyls, as well as chalcones and stilbenes with different substitution patterns. This implies that one strategy adopted by nature for increasing the functional diversity of biosynthetic enzymes involves modifying polyketide assembly by altering the preference for the starter molecules. In vitro chalcone synthase accepts disparate CoA starter molecules, including aromatic and aliphatic CoA-thioesters of different length but it does not use *N*-methylanthraniloyl-CoA as a substrate (Jez et al., 2002). Site-directed mutagenesis yielded a mutant that preferentially accepts this CoA-thioester substrate to generate a novel alkaloid. A point mutation shifted the molecular selectivity of this enzyme but still a variety of substrates was accepted.

NAD(P)H:quinone oxidoreductases are another group of multifunctional enzymes recently expressed in host organisms. The recombinant protein encoded by a

cDNA isolated from *Triphysaria versicolor* reduces a variety of quinones and naphtoquinones, including several of allelopathic significance, using either NADH or NADPH as electron donors (Wrobel et al., 2002). The ζ-crystallin gene *P1* of *Arabidopsis thaliana*, known to confer tolerance toward the oxidizing drug 1,1′-azo-bis(*N*,*N*-dimethylformamide) (diamide) to yeast was expressed in *E. coli* to characterize the biochemical properties of the protein. The recombinant protein showed NADPH:quinone oxidoreductase activity with specificity to several quinones and it also catalyzed the divalent reduction of diamide to 1,2-bis(*N*,*N*-dimethylcarbamoyl)hydrazine, with a k_{cat} comparable with that for quinones (Mano et al., 2000).

Cytochrome P450 enzymes are heme-containing proteins that catalyze many, but not all, of the hydroxylation reactions of plant natural product biosynthesis. Major advances in the characterization have come from the employment of PCR-based strategies for generation of candidate cytochrome P450 sequences, and the use of heterologous systems for functional expression. Many of these enzymes are multifunctional and therefore fewer enzymes than might be expected are required to synthesize the various hydroxylated structures. For example, it was shown that two P450s encode multifunctional enzymes catalyzing the three oxidation steps from *ent*-kaurene to *ent*-kaurenoic acid and the four oxidation steps from *ent*-kaurenoic acid to gibberellin 14 (Tudzynski et al., 2002). The first six reactions in the conversion of L-tyrosine to dhurrin are catalyzed by just two cytochrome P450s (Halkier and Du, 1997). These are only some examples of metabolic channeling in natural product biosynthesis.

8. Conclusion

8.1. Substrate specificity

The multiplicity of secondary plant products can be viewed as the current status of nature's activities in combinatorial biochemistry (Dixon, 1999). During years of natural selection pressure (e.g. resistance) evolutionary changes in gene structure, gene expression, protein structure, enzyme activity, substrate specificity and availabilty have generated the premises for the multitude of secondary plant metabolites. Recent studies have shown that a relatively small number of conserved enzymatic mechanisms can generate the biosynthetic diversity (Lazcano et al., 1995; Roy, 1999; Henrissat et al., 2001; Hrmova and Fincher, 2001; Nishida, 2001). Additionally, molecular cloning and functional expression of proteins will rapidly lead to the characterization of an increasing number of multifunctional enzymes. Most enzymes analysed until now, either in vitro or in vivo, have been shown to accept only a small range of substrates. Since the substrate specificity of enzymes at least of enzymes isolated from natural sources have not been thoroughly investigated, due to the limited amount of pure protein it is justifiable to assume that some of these proteins belong also to the category of multifunctional enzymes. Recent extensive studies using recombinant *O*-methyltransferases from *Thalictrum tuberosum* or recombinant *O*-glycosyltransferases from *A. thaliana* demonstrated their broad substrate specificities (Frick and Kutchan, 1999; Lim et al., 2002).

Amino acid sequences are responsible for the structures and properties of proteins. It is evident that despite the enormous variability of proteins, particular structural elements are rather conservative and these elements govern to a large extent the function of the protein molecule. This is most pronounced in the case of proteins possessing the catalytic functions. For example, only five types of sites forming the catalytic structure were found for hydrolases, which comprise about one-third of all known enzymes (Varfolomeev and Gurevich, 2001). It can be deduced that multifunctionality of enzymes, i.e. acceptance of different substrates is not really surprising.

Progress in defining the three-dimensional structures of plant enzymes has been generally slow, but in the last 5 years momentum has picked up considerably (Hrmova and Fincher, 2001). The emerging conclusion is that proteins are comprised of an unexpectedly small number of protein folds, which can be combined, adapted, and fine-tuned to achieve the diverse and sometimes quite specific functions mediated by the very large number of proteins that operate at the cellular level. A relatively small number of structural elements has been conserved, but these are used over and over again in the diversification of protein function during evolution (Hrmova and Fincher, 2001).

8.2. Substrate availability

On the basis of the presented examples it is proposed that the qualitative and quantitative composition of secondary metabolites appears to be determined by two factors. One is the selectivity of the enzymes involved in the transformation. The other important factor determining the composition is the supply of specific suitable functionalized substrates. The supply of suitable substrates, in turn, is affected by the temporal and spatial expression of their synthesizing enzymes, leading to compartmentation.

It has been shown that metabolic pathways compete for limited pools of substrates. For example, the overexpression of farnesyl diphosphate synthase (FPS) in transgenic *A. thaliana* led to a cell death/senescence-like phenotype correlating with the endogenous level of cytokinins (Masferrer et al., 2002). Increasing FPS activity reduces the pool of isopentenyl diphosphate and

dimethylallyl diphosphate available for cytokinin biosynthesis. Thus, metabolome diversity is governed by enzyme specificity and to a high extent by availability of suitable substrates as demonstrated for the alcohol acyl-CoA transferases, *O*-methyltransferase, terpene synthases, glucosyltransferase, polyketide synthases, and P450s. This list will rapidly be extented when more and more biochemical analysis of heterologously expressed enzymes are being performed. Our hypothesis is further supported by the metabolism of pesticides and other exogenously applied compounds by endogenous non-specific plant enzymes. Intensive studies on pesticides have shown the capability of plants to deal with unknown chemicals (Hoagland et al., 2001). Novel xenobiotic substances are glucosylated by many plant species (Pflugmacher and Sandermann, 1998). Thus, cellular compartmentalization has to be taken into account to understand the in vivo function of the multifunctional enzymes. Compartmentalization might be more important than in vitro enzyme activity (Chiron et al., 2000; Zubieta et al., 2001).

8.3. In vivo situation

The extensive characterization of enzymes involved in the biosynthesis of secondary metabolites poses the question of whether the multitude of secondary plant metabolites results from the action of a relatively small number of highly promiscuous enzymes with broad substrate specificity or, at the other extreme, a large number of enzymes with a tight substrate specificity. The picture that is emerging, at least in vitro, is an intermediate situation.

For example, various cases of functional changes were observed in the NAD(P)-dependent malate and NAD-dependent lactate superfamily. The direction of evolution was found to always be polarized from enzymes with a high stringency of substrate recognition to enzymes with a broad substrate specificity (Madern, 2002). The in-depth characterization of the enzymes revealed that a finite number with some, but not very extended, plasticity towards structurally similar secondary metabolites exist. The effective substrate specificity can be further tightened in vivo, through the generation of only a specific set of substrates. Alternatively, if a multitude of secondary metabolites, all of which can act as substrates for a particular enzyme, are present simultaneously, then it is possible that enzyme promiscuity is exhibited in vivo. The consequences of modulating enzyme activity, in planta, then becomes an intriguing one, and it remains to be seen whether the metabolism of a single class of metabolites can be influenced in vivo through the modulation of a specific enzyme expression (Jones et al., 1999).

At the biochemical level, proteins rarely act alone; rather, they form dimers or polymers or interact with other proteins to perfrom particular cellular tasks (Alberts, 1998). These assemblies represent more than the sum of their parts by having a new function (Gavin et al., 2002). For example, the in vivo analysis of medicarpin biosynthesis in alfalfa suggests that isoflavone *O*-methyltransferase and isoflavone synthase form a complex upon induction of the defense response, which would provide for efficient channeling of the isoflavanone product of the isoflavone synthase to isoflavone *O*-methyltransferase. Thus, regardless of the substrate preference displayed in vitro, in vivo conditions most likely only allow for the presence of the isoflavanone substrate (Zubieta et al., 2001).

Also subtle modifications in the secondary and/or tertiary structures, for example in the microenvironments of tyrosin and trypthophan residues can change enzyme specificity (Pourplanche et al., 1994). The numerous variations observed in the behavior of lipoxygenase catalyzing either hydroperoxide synthesis or secondary reactions in modified micoenvironments can be attributed to various influences. In the presence of sorbitol, soybean lipoxygenase produces more 9-hydroperoxide and the conversion in oxodienic acids takes place faster than that of the 13-hydroperoxide. However, considerably more oxodienic acids are formed from 13-hydroperoxide under anaerobic conditions than under aerobic conditions. Activity of soybean lipoxygenase can be affected by a pH decrease, leading to the formation of 9-hydroperoxide at the expense of 13-hydroperoxide (Pourplanche et al., 1994).

8.4. Evolution

On the basis of the presented data it is possible to draw some conclusion for the evolution of secondary metabolites. It has been suggested that evolution seldom proceeds by the de novo synthesis of new structures (Wistow and Piatigorsky, 1988). A likely scenario of evolution of biosynthetic pathways is believed to have occurred by retro-evolution through recruitment of existing enzymes rather than generation of de novo classes (Roy, 1999). One prerequisite of such a scenario is the existence of broad-specificity enzymes which may be recruited for a new metabolic pathway, followed by further evolution towards more specific and efficient catalysts. Early in the development non-selective enzymes converted metabolites which appeared by chance due to new enzyme activities or loss of compartmentation. If the products were benefical for the plant, evolution adjusted the catalysts by increasing their specificities. It has been argued that primitive cells contained a relatively small number of enzymes, catalyzing a class of reaction with broad enzyme specificity (Jensen, 1976). Indeed, sequence homology analysis of proteins related to amino acid biosynthesis in the genome of *E. coli* demonstrated that some substrate-specific

enzymes evolved from an ancestor enzyme with broad substrate specificity (Nishida, 2001). Enzymes with broad substrate specificity still play an important role in the amino acid biosynthesis.

It is likely that enzymes present at the end of biosynthetic pathways have a broader substrate specificity than those preceding upstream, if there is to be any flexibility with respect to the evolution of novel secondary metabolite biosynthesis and xenobiotic catabolism. Recently, it was demonstrated that *O*-methyltransferase substrate preference is determined by a few amino acid residues and that new *O*-methyltransferases with different substrate specificity could begin to evolve from an existing *O*-methyltransferase by mutation of a few amino acids (Wang and Pichersky, 1999). Similar results were obtained for plant type III polyketide synthases (Jez et al., 2002).

Although, the step by step recruitment of proteins probably occurred frequently, an alternative proposal based on the multifunctional nature of many enzymes was suggested. This hypothesis, which is not necessarily mutually exclusive with that of the currently favored concept of enzyme recruitment, proposes the recruitment of a multifunctional enzyme capable of catalyzing several steps at a time, albeit inefficiently (Roy, 1999). In some cases one primordial multienzyme may have catalyzed the whole sequence of reaction of a biosynthetic pathway. Gene duplication and further evolution to more efficient enzymes led to extant pathways. Known homology between enzymes of the biosynthetic pathways and existence of bifunctional enzymes support this hypothesis of retro-evolution by jumps.

8.5. Multifunctional isoenzymes

Since many proteins involved in the biosynthesis of secondary plant metabolites are multifunctional, fewer enzymes are required to synthesize the various structures. However, several of the enzymes are encoded by multiple genes that are regulated differently in response to a range of environmental and developmental stimuli, adding genetic complexity. The isoenzymes encoded by a gene family show overlapping catalytic activity towards the different substrates as demonstrated for the glucosyltransferase genes in *Arabidopsis* (Lim et al., 2001) and *O*-methyltransferase genes in *Thalictrum tuberosum* (Frick and Kutchan, 1999) as well as overlapping expression pattern as shown for the alcohol dehydrogenase genes in *Vitis vinifera* (Tesniere and Verries, 2000). This implies that a deleterious mutation in one gene does not necessarily lead to the loss of secondary plant metabolites but results in a moderate shift of the product pattern. Secondary metabolism can be modulated by this concept. A loss of function has not really a significant effect. The collectivity of multifunctional isoenzymes resembles an A/D converter as a yes/no decision is translated to a gradual change in metabolite pattern (Fig. 4). A single amino

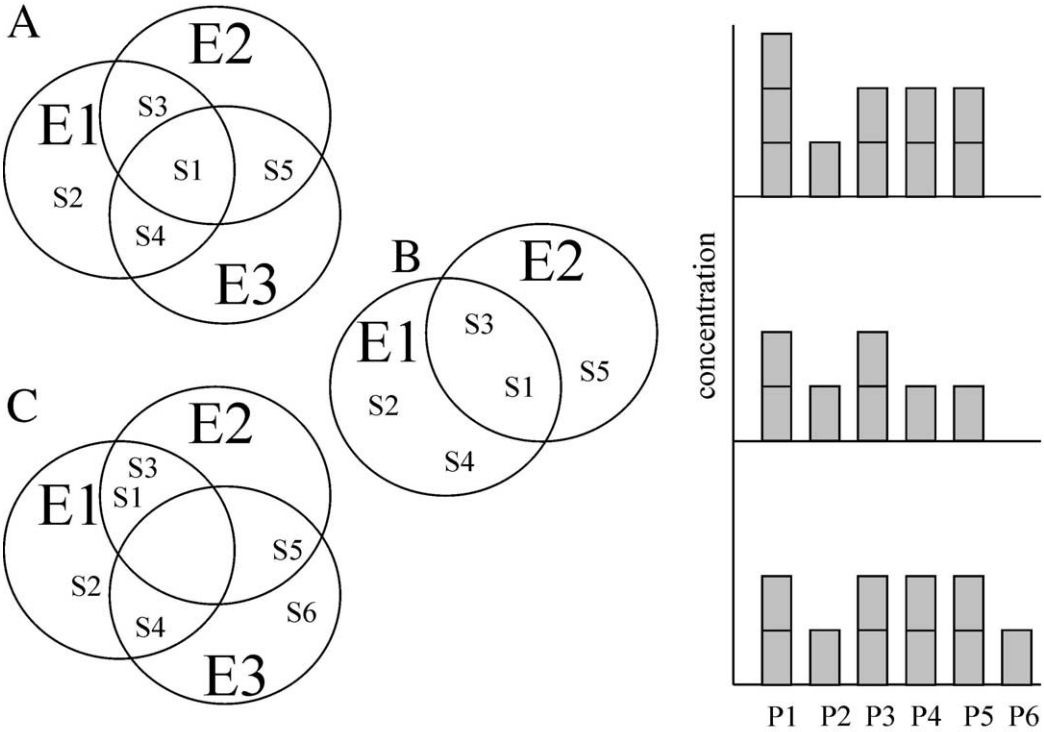

Fig. 4. Dependency of product pattern (P1 to P6) on the specificity of multifunctional enzymes (E1 to E3) and substrates (S1 to S6). Circles enclose the accepted substrates (S1 to S6) of the individual enzymes (E1 to E3). Substrates S1 to S6 form products P1 to P6, respectively. Enzymes have equal kinetic properties, for clarity. Starting condition (A), E3 is not present due to deleterious mutation or change in expression (B), and modified specificity of E3 (C). Substrate S1 is no longer accepted by enzyme E3 but S6 is a newly acquired substrate (C).

acid alteration in a protein can lead to a modified substrate preference and an increase of a small number of metabolites or even the formation of new compounds depending on substrate availabilty. If the compounds are profitable for the plant (e.g. resistence to pests, attractance of pollinators) the individuals carrying the mutation will rapidly propagate. A mutation in a multifunctional enzyme leads to a gradual shift in the composition of secondary plant metabolite rather than the appearance of a new metabolite at the expense of an existing compound. Consequently, we can see multifunctional isoenzymes as an ideal playground for evolution as natural selection can choose the appropriate combination leading to gradual changes in metabolite concentration. Plants appear to have adapted exisiting enzymes to their evolving needs, rather than inventing entirely novel mechanisms. Enzymes are becoming more and more specialized during evolution.

8.6. Gene duplication

Multifunctional enzymes have already been proposed as the ancestor of present-day enzymes (Jensen, 1976). These broad-specificity enzyme may be recruited for new metabolic pathways, followed by further evolution toward more specific and efficient catalysts. It follows that gene duplication (or even polyploidy) is an important factor in this concept as it provides the raw material for the acquisition of new biosynthetic pathways. For example, basils glands contain two *O*-methyltransferases exhibiting overlapping substrate specificities which means that they accept chavicol, eugenol, isoeugenol and others as substrates. (Gang et al., 2002). Molecular modeling suggested that a single amino acid difference was responsible for the difference in substrate discrimination between the two enzymes. This prediction was confirmed by site-directed mutagenesis and showed that a single amino acid change shifted the substrate preference and yielded a different product pattern. It is conceivable that both enzymes evolved from a multifunctional ancestor enzyme.

8.7. Further aspects

We can even assume that there is no evolution without multifunctional enzymes. Is it possible to develop new biosynthetic pathways with highly selective catalytic proteins? In the presence of enzymes exhibiting broad substrate specificity the appearance of a new chemical substance causes immediately the formation of a multitude of metabolites. Additionally, in the absence of a substance the enzymes in a following biosynthetic pathway are not necessarily useless as other metabolites rely on them.

These considerations provide new aspects for the endosymbiont theory. The appearance of endosymbionts leads to a gain of multifunctionality for the host and the guest. Totally new metabolic pathways are mutually offered, isoenzymes of an already existing pathway are delivered or an alterantive pathway, e.g. for terpenoid biosynthesis is obtained. Due to substrate exchange between the individual compartments new metabolites can emerge by catalysis of multifunctional enzymes.

In conclusion, it is suggested that further analysis of heterologously expressed proteins will confirm that multifunctional enzymes are ubiquitous in plant kingdom. Thus, formation of secondary plant metabolites is not only determined by enzyme specificity but also by substrate availability and compartmentation has to be taken into account.

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