Pergamon

0031-9422(94)00962-7

Phytochemistry, Vol. 39, No. 4, pp. 737–749, 1995

0031-9422/95 $29.50 + 0.00

REVIEW ARTICLE NUMBER 104

MOLECULAR ORGANIZATION OF THE SHIKIMATE PATHWAY IN HIGHER PLANTS

JÜRG SCHMID and NIKOLAUS AMRHEIN

Institute of Plant Sciences, Swiss Federal Institute of Technology, Universitätstrasse 2, CH-8092 Zürich, Switzerland

(*Received* 14 *November* 1994)

Key Word Index—Biosynthesis of the aromatic amino acids; shikimate pathway; phenylalanine; tyrosine; tryptophan; genetic organization; higher plants.

Abstract—The shikimate pathway produces the three proteinogenic aromatic amino acids, phenylalanine, tyrosine and tryptophan, which are, in addition to several intermediates of the shikimate pathway, intermediates in the biosynthesis of numerous aromatic natural products in higher plants. While there is only little difference in the sequence of the chemical reactions of the pathway in bacteria, fungi and plants, considerable differences exist in the organization and regulation of the shikimate pathway in plants, fungi and bacteria. The recent isolation and characterization of cDNAs and genes coding for enzymes of the shikimate pathway in higher plants have confirmed that plastids are the major, if not only site of aromatic amino acid biosynthesis in plants. Furthermore, the observed differential spatial and temporal expression of genes coding for isozymes of the pathway indicates a complex regulation that we are only beginning to understand.

INTRODUCTION

In nature, thousands of compounds are known which contain one or more aromatic rings. These compounds are predominantly synthesized via the shikimate pathway and its many branches, the most prominent and essential products being the three proteinogenic aromatic amino acids, i.e. phenylalanine, tyrosine and tryptophan. The occurrence of the pathway is restricted to plants, fungi and bacteria, therefore making aromatic amino acids essential in the diets of animals. As important as the aromatic amino acids are, they represent only a fraction of known products of the shikimate pathway and are themselves precursors to a myriad of other aromatic natural products (for recent reviews, see [1–5]).

Due to the diversity of aromatic metabolism in various classes of organisms, this review will be confined to the plant kingdom. Our main aim is to provide a summary of recent data on aromatic amino acid biosynthesis in higher plants, focusing on results obtained by applying techniques of molecular biology.

Aromatic amino acids as structural units of proteins and precursors of secondary metabolites

In plants, proteins are synthesized in three different compartments: in the cytoplasm, in the plastids, and in the mitochondria. Therefore, the aromatic amino acids must either be synthesized *in situ* in the respective protein synthesizing compartment or they are synthesized outside this compartment and have to be imported. In the case of mitochondria, there is no evidence to suggest that the aromatic amino acids are synthesized in these organelles; therefore, one can envisage carrier-mediated transport of the aromatic amino acids across the mitochondrial membranes. On the other hand, it is well established by the work of Schultz and associates, that all the three aromatic amino acids can be synthesized in plastids [6, 7]. It is a matter of debate, however, whether there is, in addition, a complete pathway in the cytoplasm, or whether the aromatic amino acids are synthesized exclusively in the plastids [5, 8–11].

All three aromatic amino acids, besides being structural units of proteins, serve as precursors of secondary plant products and, in this respect, represent pathway intermediates. Some of the shikimate pathway intermediates serve also as direct precursors of secondary plant products. Some of these are widely represented across the plant kingdom whereas others may be specific for only certain plant species.

All three aromatic amino acids, as well as the tryptophan precursor anthranilate are precursors in the biosynthesis of alkaloids [12, 13]. Furthermore, phenolic compounds encompass a considerable range of substan-

ces such as simple phenols, phenolic acids, acetophenols, phenylacetic and hydroxycinnamic acids, phenylpropanes, (iso)coumarins, chromones, xanthones, stilbenes, flavonoids, lignans, neolignans, lignins, catechol melanins and others. All these classes of compounds are derived either from phenylalanine and, to a limited extent, from tyrosine, or directly from shikimate pathway intermediates [14, 15].

The chemical diversity of these compounds is also reflected in the diversity of their function. In woody plants, up to one third of photosynthetically fixed carbon can be incorporated into lignin via phenylalanine and hydroxycinnamyl alcohols [16]. Many plant pigments and UV protectants [17] are derived from aromatic amino acids, as well as some membrane constituents and electron carriers [18], plant hormones [19], and phytoalexins [20, 21].

Molecular biological techniques

Molecular biology provides techniques which can be used to study problems of a biochemical or physiological nature. These techniques thus complement more classical methods and together, will ultimately allow a more comprehensive understanding of metabolism in the intact plant. One advantage of molecular biological tools is the fact that, as a rule, the interpretation of the results is straightforward and unambiguous. Statistical evaluation of the results is not usually required. The strategy used in molecular biology follows a general path. The first step is the isolation and characterization of the gene(s) and/or cDNA(s) of interest, which is a prerequisite for all other analyses which can then be conducted. Several approaches are available to isolate cDNA clones encoding an enzyme from higher plants. For shikimate pathway enzymes, antibodies have been raised against purified enzymes and used to screen cDNA expression libraries, or enzymes were purified, their N-terminal or tryptic peptide sequences determined and oligonucleotides corresponding to the amino acid sequences were used to screen cDNA libraries. Probes generated by approaches based on the polymerase chain reaction (PCR), as well as fragments of yeast genes have also been used. Complementation of appropriate mutants of yeast and *E. coli*, respectively, with plant cDNA libraries has been a successful strategy to isolate certain genes.

Several methods have been established to analyse the regulatory mechanisms of gene expression, such as Northern blot analysis or promoter studies with promoter–reporter gene fusions in transgenic plants. To analyse regulatory mechanisms at the post-translational level, various methods have been perfected, such as the expression of antisense RNA in transgenic plants or the overexpression of a given gene or cDNA. Expressing a cDNA of interest in a heterologous system, e.g. yeast, *E. coli*, or insect cells, may presently be the method of choice to obtain large quantities of a protein compared to tedious purification from plant tissue, which frequently does not even provide a homogeneous enzyme preparation.

BIOSYNTHESIS OF THE AROMATIC AMINO ACIDS

Overview

As pointed out above, plants, fungi and bacteria are able to synthesize the three aromatic amino acids via the shikimate pathway. Most of the biochemical reactions in the synthesis of phenylalanine, tyrosine and tryptophan are identical in all three classes of organisms indicating an ancient origin of the pathway. The shikimate pathway can be divided into three parts. The pathway from phospho*enol*pyruvate and erythrose 4-phosphate to chorismate is common for the synthesis of all three aromatic amino acids, and hence is often called the prechorismate pathway. Beyond chorismate, the shikimate pathway branches into a pathway leading to phenylalanine and tyrosine and into another one leading to tryptophan. The only pronounced difference between plants, fungi and bacteria can be seen in the branch leading to phenylalanine and tyrosine (Fig. 2). In plants, the biosynthetic sequence proceeds from chorismate via prephenate to arogenate and then to either phenylalanine or tyrosine. In *Neurospora crassa* and *Saccharomyces cerevisiae*, the pathway diverges already after prephenate, i.e. via phenylpyruvate to phenylalanine, and via 4-hydroxyphenylpyruvate to tyrosine. In many bacteria either of the pathways or even both are present [10].

Other substantial differences between classes of organisms able to synthesize the three aromatic amino acids relate to the organization of the participating enzymes. Whereas all enzymatic activities are separable from each other in some bacteria [22], in some fungi the activities of five consecutive steps of the prechorismate pathway reside on a single polypeptide, called *arom* complex [23, 24]. In plants, only the 3-dehydroquinate dehydratase and the shikimate: NADP oxidoreductase activities are found as a bifunctional enzyme [25] (for EC numbers see the respective subtitles). An *arom* complex has also been described in *Euglena gracilis* [26]. The organization of the enzymes of the pathway from chorismate to tryptophan is rather complex for the three classes of organisms. In plants, all steps are catalysed by separable enzymes [27, 28], whereas different multifunctional enzyme complexes exist in bacteria and fungi [29]. In *Euglena gracilis*, the organization of these enzymes seems again to be different. The anthranilate phosphoribosyl transferase, phosphoribosyl anthranilate isomerase, indole-3-glycerol phosphate synthase and tryptophan synthase activities could not be separated from each other, which led to the conclusion that they are arranged in a polyfunctional enzyme complex [30].

Compartmentation of the shikimate pathway in plants

The shikimate pathway appears to be the only amino acid biosynthetic pathway for which a controversy exists concerning its subcellular localization [5, 8–11]. As mentioned above, it is still a matter of debate whether or not the shikimate pathway actually is a dual pathway, i.e. whether two full sets of the biosynthetic pathway operate

in two different subcellular compartments. The presence of a plastidic pathway is well documented and generally accepted, but a cytosolic pathway is, at least by some groups working in the field, disputed. The presence or absence of a cytosolic pathway has, of course, many important physiological implications. If only a plastidic pathway exists, aromatic amino acids, or possibly their precursors, have to be transported out of the plastids into the cytosol from where they can be allocated to other organelles for utilization or storage. If an additional cytosolic pathway exists, the aromatic amino acids produced in plastids are either exclusively utilized within these organelles (which would imply that plastids are autonomous with respect to the aromatic amino acids), or their carrier-mediated exchange with the cytosol can be envisaged. Such carriers must be postulated if only a plastidic pathway exists.

Those authors in favour of a dual pathway have speculated that the aromatic amino acids synthesized in plastids are primarily utilized in protein synthesis (i.e. both in the plastids and in the cytosol!), whereas those synthesized in the cytosol are mainly utilized as precursors in the synthesis of secondary plant products [5]. Considering that in this scenario the aromatic amino acids need to be exported from the plastid for cytosolic protein synthesis, little thought has been given to the question of how separate pools of aromatic amino acids are maintained in the cytosol. To add to the complexity, a model of a dual pathway within plastids has been proposed. The aromatic amino acids produced by one pathway would be used for protein biosynthesis and those produced by the other for the biosynthesis of secondary plant products [31]. However, this would imply the unlikely situation that the two pathways are either sequestered in different compartments within the plastid or within different plastids. But none of these speculations is based on convincing experimental evidence. The proportion of aromatic amino acids which is incorporated into proteins as compared to that which is utilized for the synthesis of secondary plant products has never been determined by any tissue by a metabolic flux analysis.

The identification of cytosolic isozymes of the shikimate pathway has mainly been based on biochemical fractionation studies. But none of these activities has ever been purified to homogeneity or their respective cDNAs or genes cloned. Since fractionation methods invariably produce results with a certain degree of inaccuracy, this technique will not unambiguously solve the problem of the existence of a cytosolic shikimate pathway. The purification of the enzymatic activities, and the isolation of the corresponding genes will be required to provide meaningful answers to these questions.

The enzymes of the shikimate pathway

The prechorismate pathway

*3-Deoxy-*D-arabino-*heptulosonate 7-phosphate synthase* (EC 4.1.2.15). 3-Deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) synthase is the first enzyme of the prechorismate pathway and catalyses the condensation of phospho*enol*pyruvate and erythrose 4-phosphate to yield DAHP. DAHP synthase activity has been demonstrated in extracts from tissues of several plant species, often resulting in the demonstration of the presence of two different isozymes. One of these is stimulated exclus-

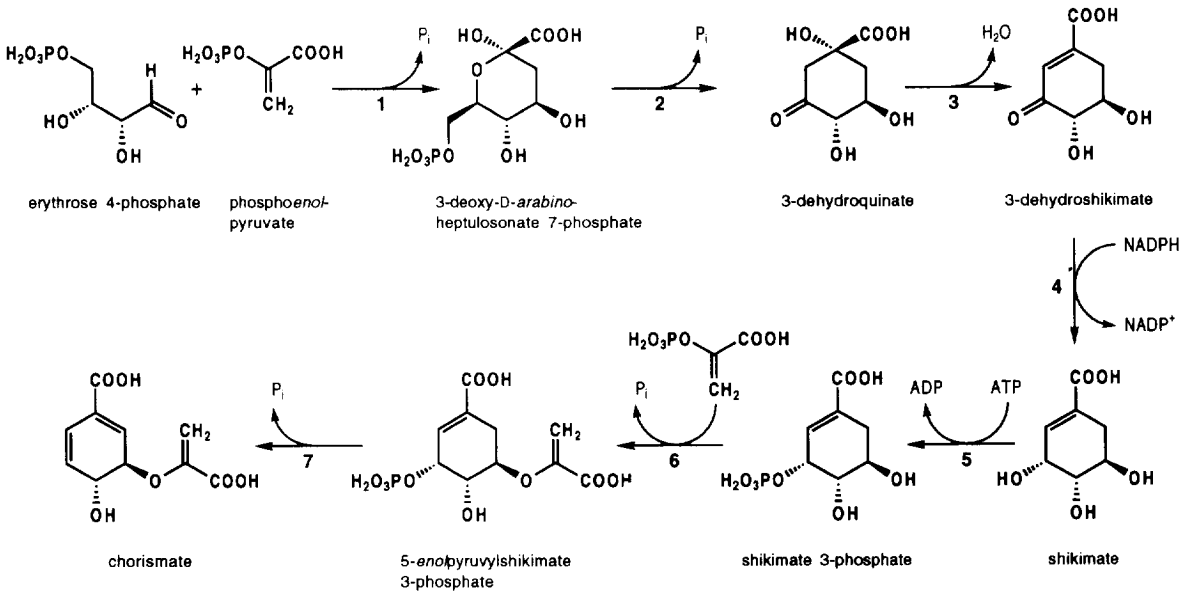

Fig. 1. The pathway from erythrose 4-phosphate to chorismate. The enzymes are: **1**, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate synthase; **2**, 3-dehydroquinate synthase; **3**, 3-dehydroquinate dehydratase; **4**, shikimate: NADP oxidoreductase; **5**, shikimate kinase; **6**, 5-*enol*pyruvylshikimate 3-phosphate synthase; **7**, chorismate synthase.

ively by Mn^{2+}, whereas the other requires either Co^{2+}, Mg^{2+}, or Mn^{2+} for activity. For *Nicotiana silvestris*, spinach and parsley, the Mn^{2+} stimulated DAHP synthase isozyme has been assigned to the chloroplast [32, 33], whereas the Co^{2+} requiring isozyme appears to be located in the cytoplasm. Extractable DAHP synthase activity can be induced by wounding [34, 35], elicitor treatment [33, 36], and light [36], correlating with the accumulation of secondary metabolites derived from the shikimate pathway. Elicitor treatment of suspension cultured parsley cells, or wounding of potato tubers induces only the plastidic DAHP synthase isozyme but not the cytosolic form. As both treatments lead to enhanced secondary product synthesis, it follows from this observation that the plastidic isozyme must be involved in secondary product synthesis, contrary to the postulate of the dual pathway hypothesis. The induction by elicitor, wounding, or light appears to be due to an induced expression of the corresponding gene, since the induction by the elicitor can be inhibited by both cycloheximide and actinomycin D, and also because the abundance of DAHP synthase-specific mRNA increases after wounding of potato and tomato tissues [33, 34]. cDNA clones encoding DAHP synthases have been isolated from several plant species, which based on comparisons of the deduced amino acid sequences, seem to be plastid-specific (Table 1). It is interesting to note that the similarity between the amino acid sequences of the plant and microbial DAHP synthases is rather low when compared with other enzymes of the pathway (see below). The identity between the DAHP synthase sequences of tomato, potato, tobacco, and *Arabidopsis* is in the range of 74–98% [37], while the identity between the plant and microbial enzymes is only in the range of 11–16% [38–40]. A comparison of DAHP synthase sequences within the Solanaceae revealed that they fall into two

Table 1. Enzymes of the shikimate pathway in higher plants of which cDNAs and genes have been isolated

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate synthase:		
Isolated cDNAs:	two from *Solanum tuberosum*	[38, 123]
	one from *Nicotiana tabacum*	[40]
	two from *Arabidopsis thaliana*	[39]
	two from *Lycopersicon esculentum*	[37]
Isolated genes:	two from *Arabidopsis thaliana*	[39]
3-Dehydroquinate dehyratase-shikimate: NADP oxidoreductase:		
Isolated cDNAs:	one from *Pisum sativum*	[47]
	one from *Nicotiana tabacum*	[47a]
Shikimate kinase:		
Isolated cDNAs:	one from *Lycopersicon esculentum*	[50]
Isolated genes:	one from *Lycopersicon esculentum*	J.S. and N.A., unpublished results
5-*Enol*pyruvylshikimate 3-phosphate synthase:		
Isolated cDNAs:	one from *Petunia hybrida*	[54]
	one from *Lycopersicon esculentum*	[55]
Isolated genes:	one from *Arabidopsis thaliana*	[56]
	one from *Petunia hybrida*	[55]
	one from *Brassica napus*	[56a]
Chorismate synthase:		
Isolated cDNAs:	one from *Corydalis sempervierens*	[65]
	two from *Lycopersicon esculentum*	[66]
Isolated genes:	two from *Lycopersicon esculentum*	[67]
Chorismate mutase:		
Isolated cDNAs:	one from *Arabidopsis thaliana*	[79]
Anthranilate synthase α-subunit:		
Isolated cDNAs:	two from *Arabidopsis thaliana*	[31]
Isolated genes:	two from *Arabidopsis thaliana*	[31]
Anthranilate synthase β-subunit:		
Isolated cDNAs:	two from *Arabidopsis thaliana*	[97]
Isolated genes:	three from *Arabidopsis thaliana*	[97]
Anthranilate phosphoribosyl transferase:		
Isolated cDNAs:	one from *Arabidopsis thaliana*	[99]
Isolated genes:	one from *Arabidopsis thaliana*	[99]
Tryptophan synthase β-subunit:		
Isolated cDNAs:	two from *Zea mays*	[102]
Isolated genes:	two from *Arabidopsis thaliana*	[28, 103]

groups. In tomato and potato, a higher divergence is found between the respective gene pairs within the same organism as compared to the corresponding genes in different plant species [37]. The two *Arabidopsis* DAHP synthase genes (DHS1 and DHS2), which both encode plastidic enzymes, respond differentially to wounding and pathogenic attack [39]. The level of only the DHS1-specific RNA increased in leaves which had either been physically wounded or infiltrated with pathogens. The two tomato DAHP synthase genes (LeDHS1 and LeDHS2) which both code for putative plastidic DAHP synthase isozymes, differ completely in their organ-specific expression [37, 41]. Southern blot analyses strongly suggest that no related genes exist other than those of which the corresponding cDNAs have been isolated [37]. In tomato, the patterns of the organ-specific expression of the two DAHP synthase genes differ strongly from each other, and both are again different from those of other genes of prechorismate pathway enzymes (i.e. shikimate kinase, 5-*enol*pyruvylshikimate 3-phosphate synthase and chorismate synthase) [41]. There is at most a two-fold difference of LeDHS1-specific transcripts between their lowest abundance in stems and their highest abundance in flowers. The differences in the abundance of the LeDHS2-specific transcripts are far more pronounced. There is a 16-fold difference between the mRNA levels in leaves as compared to roots [41]. In roots, in particular, its abundance is extremely high compared to that in the other organs. The differential expression patterns of the two DAHP synthase genes indicate their differential regulation which sets them clearly apart from each other as well as from the other analysed genes of the prechorismate pathway enzymes from tomato [41]. Analysis of the effect of different elicitors on the two DAHP synthase genes in suspension cultured tomato cells revealed that only one of them (LeDHS2) was induced. The abundance of LeDHS2-specific transcripts reached a peak after four to five hours which was about 15 times higher than that before addition of the elicitor [Görlach *et al.*, unpublished results].

The relationship between the two groups of plastidic DAHP synthase isozymes analysed at the DNA level on the one hand, and separable cytosolic and plastidic DAHP synthase activities analysed biochemically on the other hand, is presently not clear at all. The cytosolic and the plastidic proteins must be structurally very different. The cytosolic enzyme from potato, for example, is not recognized by antibodies raised against the plastidic potato DAHP synthase [35]. Furthermore, the sequences of the genes encoding the cytosolic and the plastidic forms are expected to be different to such an extent that they do not cross-hybridize and are undetectable in Southern blots. Lastly, they have not been detected in PCR experiments. Alternatively, the cytosolic enzyme may not even be a specific DAHP synthase after all, because it readily uses aldehydes other than erythrose 4-phosphate as substrates and, in fact, exhibits the highest catalytic turnover with glycolaldehyde [42]. Consequently, a more appropriate name for this enzyme may be 4,5-dihydroxy-2-oxovalerate synthase and its involvement in DAHP synthesis *in planta* appears questionable.

3-Dehydroquinate synthase (EC 4.6.1.3). 3-Dehydroquinate (DHQ) synthase catalyses the conversion of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate to 3-dehydroquinate, i.e. the cyclization step in the shikimate pathway. DHQ synthase requires Co^{2+} and bound NAD^+ as cofactor. So far neither a cDNA clone nor a gene coding for DHQ synthase have been isolated.

3-Dehydroquinate dehydratase (EC 4.2.1.10)–*shikimate: NADP oxidoreductase* (EC 1.1.1.25). In higher plants, the next two steps of the prechorismate pathway are catalysed by the bifunctional enzyme 3-dehydroquinate dehydratase (DHQase)–shikimate: NADP oxidoreductase (SORase), catalysing the two sequential reactions from 3-dehydroquinate to shikimate via 3-dehydroshikimate. The pea enzyme has been purified to homogeneity and an apparent molecular mass of $59 \times 10^3 M_r$ was determined. By chromatofocusing techniques, three isoforms were separated, two of which appear to be of plastidic origin [43]. The extractable activities of the pea isozymes are found in a tissue- and a developmental-specific manner [44]. Wounding of roots of sweet potato leads to an increase in SORase activity [45]. An ageing-specific expression of SORase activity, which was not affected by ethylene, was reported in swede root disks [46]. The pea enzyme was purified to near homogeneity and the amino acid sequence of tryptic peptides used to generate oligonucleotides which allowed the isolation of DHQase–SORase specific PCR fragments [47]. A near full length cDNA clone encoding the bifunctional enzyme has been isolated from tobacco and was shown to complement both the *aroD* and *aroE* mutants of *E. coli* [47a].

Shikimate kinase (EC 2.7.1.71). Shikimate kinase (SK) phosphorylates shikimate to yield shikimate 3-phosphate. Schmidt and Schultz [48] described a stimulation of the spinach SK activity by thioredoxin but more recent data have not confirmed this observation [49; A. Schaller and N.A., unpublished results]. A cDNA clone encoding SK has been isolated from tomato [50]. The N-terminal section of the deduced amino acid sequence resembles known chloroplast transit peptides, and the existence of such a signal peptide was proven by the uptake and processing of the *in vitro* synthesized peptide by isolated chloroplasts. The precursor peptide is enzymatically active, as it catalyses the phosphorylation of shikimate in the presence of ATP in a time-dependent manner [50]. The isolation of the SK gene and Southern blot analysis are consistent with the existence of only one SK gene per haploid genome in tomato [J. S. and N. A., unpublished results]. This gene is expressed in an organ-specific manner similar to the expression of the 5-*enol*pyruvylshikimate 3-phosphate synthase gene and the two chorismate synthase genes of tomato. The abundance of SK-specific transcripts was highest in flowers, lower in roots and again lower in stems, cotyledons and leaves [41]. The gene is also induced by elicitors. The abundance of transcripts in suspension cultured tomato cells reached its maximum already two hours after addi-

tion of the elicitor and the magnitude of induction was about 17 times the control level (Görlach *et al.*, unpublished results).

5-Enol*pyruvylshikimate 3-phosphate synthase* (EC 2.5.1.19). 5-*Enol*pyruvylshikimate 3-phosphate (EPSP) synthase catalyses the addition of the *enol*pyruvyl moiety of phospho*enol*pyruvate to the 5-hydroxyl group of shikimate 3-phosphate. This enzyme is the best analysed of the shikimate pathway because of the interest in it as the target of the broad-spectrum herbicide glyphosate [51]. In pea, most of the enzyme activity was localized in chloroplasts and only a minor fraction could be detected in the cytosolic fraction [9]. Three different isoforms have been characterized in *Sorghum bicolor*, but their subcellular localization has not been determined [52]. The three-dimensional structure of the *Escherichia coli* enzyme has been solved by crystallographic techniques [53]. Several EPSP synthase genes and cDNA clones have been isolated from higher plants: one cDNA clone and the corresponding gene from *Petunia hybrida* [54, 55], one cDNA clone from tomato [55] and one gene each from *Arabidopsis* [56] and *Brassica napus* [56a]. Two EPSP synthase genes per haploid genome seem to be present in tomato and *Arabidopsis* [55, 56]. All EPSP synthase-specific genes and cDNAs isolated so far encode plastidic enzymes. While in tomato one gene has been analysed in detail, the second gene was detected only by genomic Southern blot analysis. It was postulated that it also encodes a chloroplastic isozyme since the restriction fragment can also be detected with a transit peptide-specific probe (G.M. Kishore, personal communication). Both the petunia and the *Arabidopsis* EPSP synthase genes have seven introns at precisely the same positions. The *Arabidopsis* gene is significantly smaller than the petunia gene which is exclusively due to smaller intron sizes [56]. The identity between the protein sequences of the mature enzymes is 84% between petunia and *Arabidopsis*, and 93% between petunia and tomato. The sequences of the transit peptides of the three EPSP synthases are far more divergent than those of the mature enzymes. The respective homologies between the petunia and *Arabidopsis* sequences on the one hand, and between petunia and tomato sequences on the other hand are 23 and 58%, respectively [55, 56].

The EPSP synthase genes are differentially expressed in organs of petunia. The highest steady-state level of EPSP synthase mRNA was found in petals, less in anthers and barely detectable levels were found in leaves and pistils of mature plants. In seedlings, mRNA levels are high in roots and stems and much lower in leaves [55]. In tomato, in contrast, the levels of EPSP synthase mRNA vary only slightly between pistils, anthers, petals and leaves [55]. Compared with other tomato genes encoding enzymes of the prechorismate pathway, the organ-specific expression pattern of the EPSP synthase gene is similar to that of the shikimate kinase gene and the two chorismate synthase genes [41]. The expression of the tomato EPSP synthase gene can also be induced by different elicitors [Görlach *et al.*, unpublished results]. The tissue-specific expression has been analysed in transgenic petunia and tobacco plants harbouring a reporter gene (chloramphenicol acetyltransferase or β-glucuronidase) under the control of an EPSP synthase promoter fragment [57, 58]. In petunia seedlings, the expression was strongest in root cortex cells and in trichomes [58]. The results of these experiments also confirmed the observation that the EPSP synthase genes are highly expressed in petunia petals whereas in petals of transgenic tobacco plants no expression above background could be detected. Histochemical analysis showed that the expression was restricted to the upper and lower epidermis of leaves, the vascular tissue, and the mesophyll of mature petunia petals. In the reproductive parts of the flower, the expression was restricted to immature ovules and pollen. In transgenic tobacco plants, expression was found in pollen [57]. A detailed analysis of a petunia EPSP synthase promoter in transgenic petunia provided evidence that the minimal sequence required for high level expression in petals is larger than 500 base pairs (bp). Two different promoter fragments, each about 500 bp long, confer tissue-specific expression of a reporter gene which indicates a functional redundance for the transcriptional regulation of this EPSP synthase gene [58].

Chorismate synthase (EC 4.6.1.4). The last common step in the synthesis of all three aromatic amino acids, catalysed by chorismate synthase (CS), is the formation of chorismate by a 1,4-*trans* elimination of phosphoric acid from 5-*enol*pyruvylshikimate 3-phosphate [59]. CS is one of a few known enzymes which require flavin and yet catalyse a reaction that does not appear to involve a change in the redox state of the substrate(s) (for a review, see [60]). In the case of CS the flavin has recently been shown to be actively involved in the catalytic cycle and a flavin adduct has been proposed as a reaction intermediate [61]. CS was the last enzyme of the shikimate pathway, the activity of which was discovered in an extract from a higher plant. It was first described from *Pisum sativum* seedlings, and at least part of the activity was found in chloroplasts [62]. The CS activity has been purified to homogeneity from a *Corydalis sempervirens* cell culture [63] and 1200-fold from *Euglena gracilis* [64]. A comparative study of CS from *E. gracilis*, a higher plant (*C. sempervirens*), a bacterium (*Escherichia coli*) and a fungus (*Neurospora crassa*) revealed that these enzymes are very similar in terms of cofactor specificities, kinetic properties, isoelectric points and pH optima. All four enzymes react with polyclonal antisera directed against CS from either *C. sempervirens* or *E. coli*. The closely associated flavin mononucleotide reductase activity present in CS preparations from *E. gracilis* and *N. crassa*, in contrast to the monofunctional CS enzymes from *C. sempervirens* and *E. coli*, is the main distinguishing feature amongst CS from these sources [64].

A CS-specific cDNA clone from the higher plant *C. sempervirens* was first isolated by screening a cDNA expression library with a monospecific antiserum [65]. Recently also tomato CS cDNA clones were obtained [66]. The deduced amino acid sequence of the *C. sempervirens* CS is 48% identical to the *E. coli* sequence which is

substantially higher than, for example, the homology of plant DAHP synthases to their bacterial counterparts, but within the range of EPSP synthase sequence homologies between higher plants and *E. coli*. The amino terminal domains of the plant sequences do not correspond to the *E. coli* sequence, but show striking similarities to known plastid-specific transit peptides. Northern and Southern blot analyses are consistent with the presence of only one CS gene per haploid genome of *C. sempervirens* [65], but there are at least two CS genes in tomato [66]. Analysis of the 3'-ends of different CS cDNA clones from *C. sempervirens* revealed a heterogeneity of the poly(A) addition sites. Out of seven analysed cDNAs only two clones contained the same 3'-end [65]. Similar observations were made when DAHP synthase, shikimate kinase and CS specific cDNA clones from tomato were analysed [37, 50, 66]. There is no evidence, however, that these different polyadenylation sites have any physiological or regulatory significance for CS expression.

Southern blot analyses strongly suggest that no related genes exist in tomato other than those of which the corresponding cDNAs have been isolated (LeCS1 and LeCS2; [66]). The organ-specific expression appears to be identical for the two genes, with the only difference that the abundance of transcripts of one gene (LeCS1) is consistently higher than that of the other one (LeCS2; [41, 66]). The pattern of high steady-state levels of LeCS1- and LeCS2-specific transcripts in flowers and roots as compared to leaves, cotyledons and stems, is comparable to the expression pattern of the shikimate kinase gene and the EPSP synthase gene in tomato [41]. Only one of the two CS genes (LeCS1) was induced by elicitors in suspension cultured tomato cells (Görlach *et al.*, unpublished results). In tomato, additional CS isozymes may be generated by a differential processing of primary transcripts. The LeCS2-specific primary transcript is alternatively spliced which may lead to the synthesis of a third type of CS [67].

The pathway from chorismate to phenylalanine and tyrosine

Chorismate mutase (EC 5.4.99.5). Chorismate mutase (CM) is the first enzyme of the branch of the pathway leading to phenylalanine and tyrosine and catalyses the intramolecular rearrangement of the *enol*pyruvyl side chain of chorismate to form prephenate. The activity has been found in a variety of higher plants, often existing as two (CM-1 and CM-2) or even three separable isozymes [68–74]. The enzymatic properties of different CM isozymes have been studied in detail in mung bean [75], *Nicotiana silvestris* [72], *Sorghum bicolor* [73, 74] and *Solanum tuberosum* [76, 77]. One CM isozyme (designated CM-1) is activated by tryptophan and feedback inhibited by phenylalanine and tyrosine, whereas a second isozyme (CM-2) is insensitive to the aromatic amino acids. In *N. silvestris*, the CM-1 activity was reported to be localized in plastids, while the CM-2 activity was recovered from the cytoplasmic fraction [33, 78]. The ratio of the activities of CM-1 to CM-2 in aged potato tuber disks is 9: 1 whereas it is 1:4 in green leaves. This observation suggests an organ-specific regulation of the expression of these isozymes [77]. Wounding of potato tubers produces a 4.5-fold increase in CM activity within 48 hr (The two forms were not differentiated.). This in-

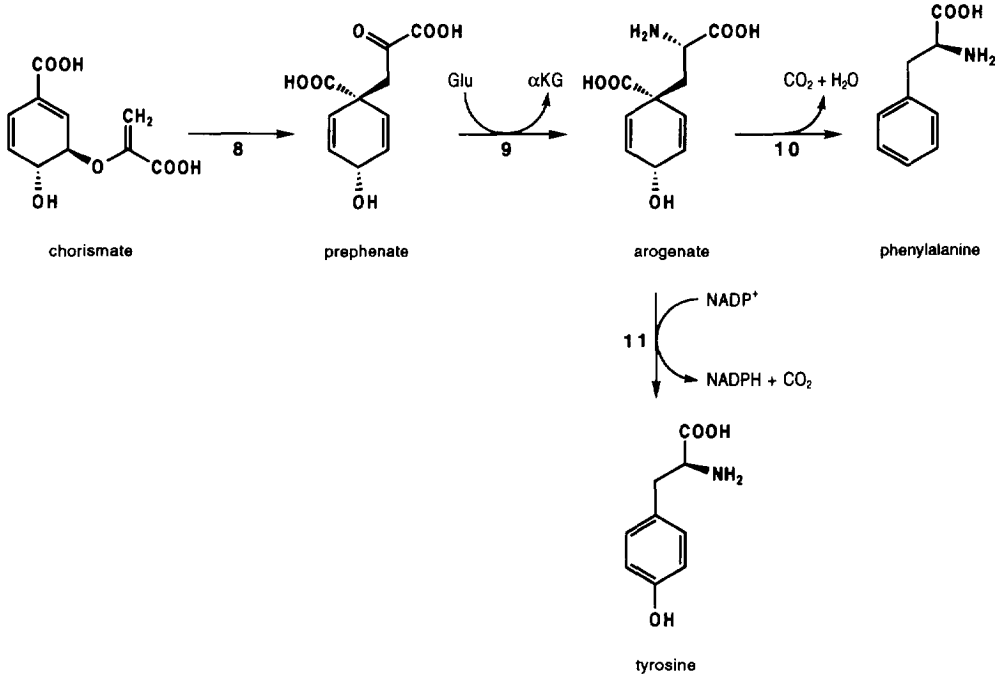

Fig. 2. The pathway from chorismate to phenylalanine and tyrosine. The enzymes are: **8**, chorismate mutase; **9**, prephenate aminotransferase; **10**, arogenate dehydratase; **11**, arogenate dehydrogenase.

crease is completely inhibited by cycloheximide or actinomycin D, suggesting *de novo* synthesis of CM and the corresponding mRNA under these conditions [76].

Complementation of a CM$^-$ yeast mutant with a complete *Arabidopsis thaliana* cDNA library led to the identification and characterization of a cDNA coding for CM [79]. The deduced amino acid sequence exhibited a sequence identity of 41% with the CM sequence of *Saccharomyces cerevisiae*. Hardly any similarity was found in known sequences of bacterial chorismate mutases. The N-terminal region of the deduced *Arabidopsis* sequence did not correspond to the *S. cerevisiae* sequence, but rather resembled known plastidic transit peptides [80]. The *Arabidopsis* enzyme expressed in yeast is regulated by the three aromatic amino acids as has been reported for plant chorismate mutases of the CM-1 type [79].

Prephenate aminotransferase (EC 2.6.1.). The last common step in the biosynthesis of phenylalanine and tyrosine, the transamination of prephenate to arogenate, is catalysed by the prephenate aminotransferase (PAT). PAT was isolated from different tissues of *Sorghum bicolor* leaves and is predominantly located in plastids [81]. The activity was purified to homogeneity from cultured *Anchusa officinalis* cells. The enzyme had an apparent molecular mass of $220 \times 10^3 M_r$ and the subunits had apparent molecular masses of 44 and $57 \times 10^3 M_r$, respectively, indicating a possible $\alpha_2\beta_2$ subunit structure [82]. So far neither a cDNA clone nor a gene coding for this enzyme have been isolated.

Arogenate dehydratase (EC 4.2.1.). The final step in the biosynthesis of phenylalanine is catalysed by the arogenate dehydratase which converts arogenate to phenylalanine thereby forming the aromatic ring. The enzyme activity is competitively inhibited by phenylalanine and is stimulated by tyrosine [83]. In a *Nicotiana silvestris* suspension culture, the arogenate dehydratase activity changes with growth conditions [84]. So far neither a cDNA clone nor a gene coding for this enzyme have been isolated.

Arogenate dehydrogenase (EC 1.3.1.43). Arogenate dehydrogenase catalyses the last step in the biosynthesis of tyrosine. Tyrosine, but neither phenylalanine, prephenate, tryptophan, or the tryptophan analogue 6-fluoro-DL-tryptophan, is a very effective inhibitor of arogenate dehydrogenase [85–87]. So far neither a cDNA clone nor a gene coding for this enzyme have been isolated.

The pathway from chorismate to tryptophan

Anthranilate synthase (EC 4.1.3.27). Anthranilate synthase (AS), the first enzyme of the tryptophan-specific branch of the shikimate pathway, catalyses the formation of anthranilate by elimination of the *enol*pyruvyl side chain of chorismate accompanied by a glutamine donated amino transfer. In microorganisms, AS is composed of two non-identical subunits: an α-subunit which binds chorismate and carries out its aromatization, and a β-subunit, which transfers the amino group from glutamine (for reviews, see [88, 89]). The α-subunit contains the tryptophan binding site, responsible for feedback inhibition of the enzyme by the end product of this branch [90, 91].

AS activities have been reported from several plant species. As in microorganisms, AS activity is inhibited by tryptophan but neither by phenylalanine or tyrosine

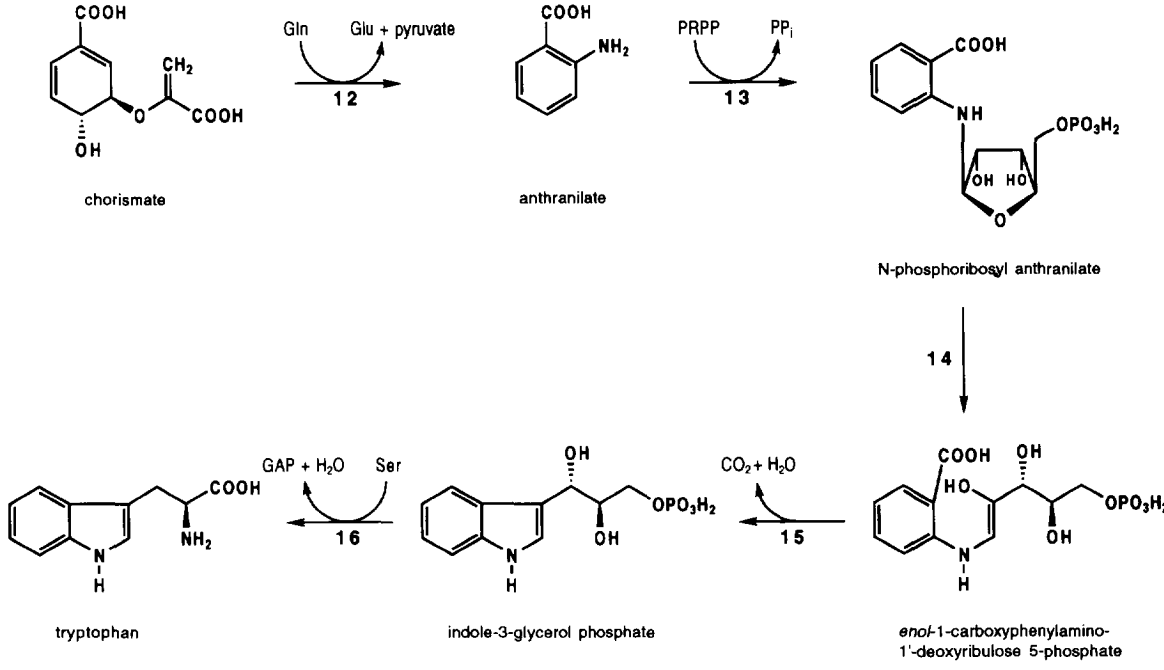

Fig. 3. The pathway from chorismate to tryptophan. The enzymes are: **12**, anthranilate synthase; **13**, anthranilate phosphoribosyl transferase; **14**, phosphoribosyl anthranilate isomerase; **15**, indole-3-glycerol phosphate synthase; **16**, tryptophan synthase.

[92], but feedback resistant forms of AS have been reported for potato [93], *Nicotiana otophora* and *Nicotiana tabacum* [94]. The activity is localized in etioplasts of dark grown pea seedlings [95] and in leucoplasts of *Juglans regia* seeds [96]. In tobacco plants and cultured cells, the two isozymes were localized in different compartments: the tryptophan sensitive form was recovered from the particulate fraction, and the tryptophan resistant form was assigned to the cytoplasm [94]. The apparent molecular masses of the two isozymes were determined to be $150 \times 10^3 M_r$ and $200 \times 10^3 M_r$ for the sensitive and the resistant forms, respectively [94].

From *Arabidopsis thaliana*, two genes coding for AS α-subunits (ASA1 and ASA2) and three genes coding for AS β-subunits (ASB1, ASB2 and ASB3) were cloned by screening a library with a yeast probe and by complementing an *E. coli* mutant, respectively [31, 97]. The identity of the two deduced amino acid sequences of ASA1 and ASA2 is 67%. The identity to microbial sequences is in the range of 30–36%. The peptide domain which has been shown to be involved in feedback inhibition by tryptophan in bacteria, is conserved in both *Arabidopsis* AS α-subunits. The N-terminal regions of the plant sequences are characteristic of plastid-specific transit peptides and are absent from the corresponding microbial proteins [31, 97]. The steady-state levels of ASA1- and ASA2-specific mRNAs vary in different *Arabidopsis* tissues. The ASA1 and mRNA is about 10-fold more abundant than the ASA2 mRNA, and the ASB1 gene is the predominantly expressed ASB gene. The abundance of ASA1-specific transcripts was found to be highest in roots and levels, lower in stems, and undetectable in flowers, developing siliques, and mature, dry seeds. ASA2-specific transcripts were, although at a reduced level with respect to ASA1, detectable in roots, stems, leaves and developing siliques. In leaves, the expression of the ASA1-specific mRNA was induced by wounding or by infiltration with pathogenic *Pseudomonas* strains, whereas the level of ASA2-specific transcripts gradually declined after either treatment [31]. The expression of at least one of the ASB genes was also induced by pathogenic *Pseudomonas* strains [97].

Anthranilate phosphoribosyl transferase (EC 2.4.2.18). The conversion of anthranilate to *N*-phosphoribosyl anthranilate is catalysed by the anthranilate phosphoribosyl transferase (APRT) which involves the addition of the phosphoribosyl moiety of 5-phosphoribosyl 1-pyrophosphate to the amino function of anthranilate. APRT activity was found in plastids isolated from peas [95] and *Juglans regia* [96]. Screening for *Arabidopsis* tryptophan auxotrophs was based on selection for growth on media containing a mixture of 5-methylanthranilate and tryptophan. In contrast to previous experiments which led only to the isolation of amino acid auxotrophic plant cells in culture, this strategy resulted in the identification of the first amino acid auxotroph at the whole plant level. The mutant *Arabidopsis* (*trpl*) lacks the APRT activity [98]. An APRT-specific cDNA clone from *Arabidopsis* has been isolated by complementation of an *E. coli* strain (*trpD*) which is defective for the APRT activity. The N-terminal region of the deduced amino acid sequence was not found in microbial APRT sequences and resembles plastid-specific transit peptides. The corresponding gene from *Arabidopsis* was cloned and RFLP mapping showed that it is the one which is defective in the mutant *trpl* [99].

Phosphoribosyl anthranilate isomerase (EC 5.3.1.). The Amadori rearrangement of *N*-phosphoribosyl anthranilate to *enol*-1-carboxyphenylamino-1′-deoxyribulose 5-phosphate is catalysed by the phosphoribosyl anthranilate isomerase (PRAI). PRAI activity has been localized in pea and *Juglans regia* plastids [95, 96]. So far neither a cDNA clone nor a gene coding for this enzyme have been isolated.

Indole-3-glycerol phosphate synthase (EC 4.1.1.48). The next step in the biosynthesis of tryptophan is catalysed by indole-3-glycerol phosphate synthase (InGPS) which converts *enol*-1-carboxyphenylamino-1′-deoxyribulose 5-phosphate to indole-3-glycerol phosphate by decarboxylation, followed by ring closure of the side chain. InGPS activity has been localized in plastids of pea *Juglans regia* [95, 96]. So far neither a cDNA clone nor a gene coding for InGPS have been isolated.

Tryptophan synthase (EC 4.2.1.20). Tryptophan synthase (TS) catalyses the final step in the biosynthesis of tryptophan. The holoenzyme is a tetramer of two dissimilar subunits: one component (called either TS subunit-α, A component, of TRPA) catalyses the reaction from indole-3-glycerol phosphate to indole and glyceraldehyde 3-phosphate (G3P), and the second component (called either TS subunit-β, B component, or TRPB) catalyses the conversion of indole and serine to tryptophan. TS activity has been found in several plant species. A tryptophan-auxotrophic callus culture of *Hyoscyamus muticus* probably carrying a mutation in the α-subunit of TS has been described [100]. The maize mutant *orange pericarp* is caused by recessive mutations in the two unlinked genes *orp*1 ans *orp*2 [101]. This mutant accumulates both anthranilate and indole, which implies some defect in the biosynthesis of tryptophan. Further analysis revealed that the *orp* mutant is deficient in the activity of the TS β-subunit. Two different cDNA clones (corresponding to the genes TSB1 and TSB2) were isolated from maize which code for TS β-subunits. Their deduced amino acid sequences are 98% identical [102]. Restriction fragment length polymorphism mapping was used to establish the location of the two genes in the maize genome. TSB1 is located at position 76 on chromosome 4 and TSB2 at position 41 on chromosome 10, in good agreement with the genetic mapping data of *orp*1 and *orp*2, and thus strongly suggesting mutations in both TS β-subunit genes in the *orp* mutant [102]. A cDNA and the corresponding gene (TSB1), and a second gene (TSB2) encoding the TS β-subunit have been isolated from *Arabidopsis* [28, 103]. The N-terminal portion of the deduced amino acid sequences again have several features characteristic of chloroplast transit peptides. The identity of the two TS β-subunits is more than 95% (excluding the signal peptides), and the homology to TRPB sequences of several microorganisms is in the

range of 50–65% [28]. Southern blot analysis strongly supports the presence of only two TS β-subunit genes in *Arabidopsis*. Both genes have been mapped: TSB1 is localized on chromosome 5 and TSB2 on chromosome 4. Neither the TSB1 nor the TSB2 gene is fused to a gene coding for the α-subunit, suggesting that the α- and β-subunits of *Arabidopsis*, and most likely of all higher plants, are encoded by separate genes. A tryptophan-requiring *Arabidopsis* mutant (*trp*2-1) has been identified. This mutant has only about 10% of the wild-type TSβ activity. The mutation is correlated with a polymorphism in the TSB1 gene, strongly suggesting that *trp*2-1 is mutated in TSB1 [103].

The organ- and tissue-specific expression of the two *Arabidopsis* TSB genes was analysed in detail by determining the abundance of specific transcripts in different organs and by analysing the expression of promoter–reporter (β-glucuronidase) gene fusions in transgenic *Arabidopsis* plants [103, 104]. The expression of the TSB1 gene varied in an organ-specific manner in contrast to the expression of the TSB2 gene which did not show any variation between different organs. The abundance of TSB1 transcripts was highest in leaves, less in stems, roots and flower buds and even less in seed pods.

DISCUSSION

Does a dual shikimate pathway exist?

The available evidence for a dual shikimate pathway is solely based on isozyme analysis in subcellular fractions. Putative cytosolic isozymes have been described for DAHP synthase [32, 33], DHQase–SORase [43], EPSP synthase [9], CM [33, 78] and AS [94]. The substrate ambiguity of the cytosolic Co^{2+}-dependent DAHP synthase has been pointed out above. Another enzyme, which complicates the analysis of DAHP synthase activities, is the 3-deoxy-D-*manno*-octulosonate 8-phosphate synthase, which also possesses some DAHP synthase activity [105].

For DHQase–SORase, the results are again contradictory. By analysis of different plant species, several groups provided evidence for cytosolic isozymes [43, 106], while other groups have found only plastidic isozymes [107]. One must ask here to which extent a cytoplasmically synthesized precursor enzyme (destined for import into a plastid) could account for the respective enzymatic activity of 'cytosolic' isozymes [9], since the precursors of SK and EPSP synthase have been proven to be enzymatically active [50, 108]. No information is available, however, on the specific activities of the precursors and their steady-state levels in the cytosol, nor on the proportion of precursor to mature protein in a cell. But recent complementation experiments in our laboratory using a CS deficient *E. coli* strain indicated that the CS precursors of tomato are devoid of enzymatic activity, while the mature enzymes have full activity under these conditions [67]. This therefore suggests that chorismate may not be synthesized in the cytosol.

Without exception, cDNAs and genes encoding enzymes of the shikimate pathway from higher plants which have been cloned to date in our as well as in other laboratories encode proteins with an N-terminal, presumably plastid-specific transit peptide, which would lend no support to the existence of a complete cytosolic pathway. An explanation which cannot be ruled out as yet is the possible presence of cytosolic isozymes which are structurally grossly dissimilar to those already isolated. Again another possibility which could explain the occurrence of cytosolic isozymes, would be the presence of separate pathways which, at least in part, overlap the shikimate pathway. Quinic acid, for example, is catabolized by such a pathway in certain fungi which is encoded by the *qa* gene cluster in *Neurospora crassa* [109] and the *QUT* cluster in *Aspergillus nidulans* [110]. These fungi can utilize quinic acid as sole carbon source by first converting it to protocatechuic acid via 5-dehydroquinate and 5-dehydroshikimate, followed by ring fission. In *N. crassa*, the conversion of 5-dehydroquinate to 5-dehydroshikimate is catalysed by the *qa*-2 gene product which is a monofunctional isozyme of the dehydroquinate dehydratase domain of the *arom* complex [109].

An unexpected, and as yet not fully documented finding reported by Herrmann and coworkers [109a] further complicates the issue of the localization of the shikimate pathway enzymes in the plant cell. Using polyclonal monospecific antibodies against the purified potato tuber DAHP synthase in immunocytochemical experiments, these authors found the majority of the antigen localized to the secondary cell wall of the xylem, i.e. in the apoplast. On the other hand the *in vitro* synthesized DAHP synthase precursor was taken up and processed by isolated chloroplasts. This disturbing discrepancy needs to be resolved.

Implications of an exclusively plastidic pathway for the biosynthesis of the aromatic amino acids

If there exists only a plastidic shikimate pathway, all compartments of a cell must be provided with aromatic amino acids, or possibly intermediates of the pathway, by the plastids. Three compartments (plastids mitochondria and cytosol) utilize aromatic amino acids for the synthesis of proteins and other products. An example of a compound which is synthesized from both a shikimate pathway intermediate and a phenylalanine-derived metabolite is 5-*O*-(4-coumaroyl) shikimate [111]. Hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyl transferase catalyses the transesterification of shikimate (a pathway intermediate) and 4-coumaroyl-CoA (a phenylalanine derived metabolite) to 5-*O*-(4-coumaroyl) shikimate [112, 113]. The subcellular localization of this enzyme is as yet unknown. The next step in this pathway, the formation of *trans*-5-*O*-caffeoylshikimate, is catalysed by an enzyme in the microsomal fraction, presumably in the endoplasmic reticulum [114, 115].

The vacuole is an organelle in which aromatic amino acids and some of their precursors can be transiently

stored [116–118]. For example, suspension cultured buckweat cells, when treated with the EPSP synthase inhibitor glyphosate, accumulate shikimate in their vacuoles, and when the cells are treated with L-α-aminooxy-β-phenylpropionic acid, an inhibitor of phenylalanine ammonialyase, they accumulate phenylalanine in the organelle [117].

Therefore, since at least four pools of aromatic amino acids must be accounted for in a plant cell, the question arises as to the interconnection of these pools. The vacuolar and mitochondrial pools have to be fed from the cytosol by amino acids traversing the cytosol en route from the plastids. For this reason, one would expect to find carrier proteins in the membranes of these organelles which mediate the uptake and/or release of the aromatic amino acids. Since these carrier proteins have been poorly, if at all, characterized, it is not known whether and how they can regulate the flow of the aromatic amino acids from one compartment to another. These (putative) organellar amino acid carriers or their cDNAs, respectively, may not be as easily accessible as the cell membrane bound amino acid carriers recently cloned by Frommer and associates [119, 120] by complementation of appropriate yeast mutants, and other strategies for their identification will have to be employed.

Physiological significance of the shikimate pathway

An assessment of all results obtained so far on the organ- and tissue-specific as well as elicitor-induced expression of genes encoding enzymes of the shikimate pathway, leads to the conclusion that the traditional view of this pathway being of interest mainly because of the production of the aromatic amino acids is inaccurate. As Bentley [1] phrased it: "The shikimate pathway is a pathway with many branches". Several pathways branch off already from the prechorismate pathway. Quinic and gallic acid, for example, are compounds which may be synthesized via such branches. Another branch often previously neglected is the pathway from chorismate via isochorismate to naphtho- and anthraquinones [121, 122].

Since the organ-specific expression pattern of the phenylalanine ammonia-lyase genes in tomato plants is comparable to that observed for most genes encoding enzymes of the prechorismate pathway [41], one can conclude that the bulk of phenylalanine is mainly used as precursor for this synthesis of secondary metabolites rather than for the biosynthesis of proteins, and it may well be that the situation is comparable for the utilization to tyrosine and tryptophan.

Another important conclusion which can be drawn from these analyses concerns the synthesis of shikimate pathway metabolites in non-green tissues. If the steady-state levels of transcripts are assumed to be in proportion to the respective enzymatic activities, the rates of the production of certain shikimate pathway-derived compounds in photosynthetically inactive organs of tomato may well exceed those in photosynthetically active tissues [37, 41, 66]. Since erythrose 4-phosphate (an intermediate of the pentose phosphate pathway) and phospho*enol*pyruvate (an intermediate of glycolysis) are the initial substrates of the shikimate pathway, this pathway must be tightly linked to carbohydrate metabolism. In certain plant tissues the flow of carbon and investment of energy into this pathway are quite substantial. Presently we have no information which principles, at which level, regulate this flow and thus integrate the shikimate pathway into plant metabolism.

Acknowledgements—Support of the authors' research by the Swiss National Science Foundation and the Swiss Federal Institute of Technology is gratefully acknowledged.

REFERENCES

1. Bentley, R. (1990) *Crit. Rev. Biochem. Mol. Biol.* **25**, 307.
2. Braus, G. H. (1991) *Mirobiol. Rev.* **55**, 349.
3. Coruzzi, G. M. (1991) *Plant Science* **74**, 145.
4. Singh, B. K., Siehl, D. L. and Connelly, J. A. (1991) in *Oxford Surveys of Plant Molecular and Cellular Biology*, Vol. 7 (Miflin, B. J., ed.), p. 143. Oxford University Press, Oxford.
5. Hrazdina, G. and Jensen, R. A. (1992) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **43**, 241.
6. Bickel, H., Palme, L. and Schultz, G. (1978) *Phytochemistry* **17**, 119.
7. Schulze-Siebert, D., Heineke, D., Scharf, H. and Schultz, G. (1984) *Plant Physiol.* **76**, 465.
8. Gilchrist, D. G. and Kosuge, T. (1980) in *The Biochemistry of Plants*, Vol. 5 (Stumpf, P. K. and Conn, E. E., eds), p. 507. Academic Press, New York.
9. Mousdale, D. M. and Coggins, J. R. (1985) *Planta* **163**, 241.
10. Jensen, R. A. (1986) in *The Shikimic Acid Pathway* (Conn, E. E., ed.), p. 57. Plenum, New York.
11. Morris, P. M., Doong, R. -L. and Jensen, R. A. (1989) *Plant Physiol.* **89**, 10.
12. Fodor, G. B. (1980) in *Encyclopedia of Plant Physiology*, New Series, Vol. 8 (Bell, E. A. and Charlwood, B. V., eds), p. 92. Springer, Berlin.
13. Gröger, D. (1980) in *Encyclopedia of Plant Physiology*, New Series, Vol. 8 (Bell, E. A. and Charlwood, B. V., eds), p. 128. Springer, Berlin.
14. Harborne, J. B. (1980) in *Encyclopedia of Plant Physiology*, New Series, Vol. 8 (Bell, E. A. and Charlwood, B. V., eds), p. 329. Springer, Berlin.
15. Hahlbrock, K. and Scheel, D. (1989) *Ann. Rev. Plant Physiol. Plant Mol. Biol.* **40**, 347.
16. Higuchi, T. (1985) *Biosynthesis and Biodegradation of Wood Components*. Academic Press, New York.
17. Hahlbrock, K., Kreuzaler, F., Ragg, H., Fautz, E. and Kuhn, D. N. (1982) in *Biochemistry of Differentiation and Morphogenesis* (Jaenicke, L., ed.), p. 34. Springer, Berlin.
18. Fiedler, E., Soll, J. and Schultz, G. (1982) *Planta* **155**, 511.

19. Moore, T. C. (1989) *Biochemistry and Physiology of Plant Hormones.* Springer, Berlin.
20. Dixon, R. A., Dey, P. M. and Lamb, C. J. (1983) *Adv. Enzymol. Relat. Areas Mol. Biol.* **55**, 1.
21. Ebel, J. (1986) *Ann. Rev. Phytopathol.* **24**, 235.
22. Berlyn, M. B. and Giles, N. H. (1969) *J. Bacteriol.* **99**, 222.
23. Ahmed, S. I. and Giles, N. H. (1969) *J. Bacteriol.* **99**, 231.
24. Duncan, K., Edwards, R. M. and Coggins, J. R. (1987) *Biochem. J.* **246**, 375.
25. Berlyn, M. B., Ahmed, S. I. and Giles, N. H. (1970) *J. Bacteriol.* **104**, 768.
26. Patel, V. B. and Giles, N. H. (1979) *Biochim. Biophys. Acta* **567**, 24.
27. Delmer, D. P. and Mills, S. E. (1968) *Biochim. Biophys. Acta* **167**, 431.
28. Berlyn, M. B., Last, R. L. and Fink, G. R. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4604.
29. Hütter, R. and DeMoss, J. A. (1967) *J. Bacteriol.* **94**, 1896.
30. Cano Lara, J. and Mills, S. E. (1972) *J. Bacteriol.* **110**, 1100.
31. Niyogi, K. K. and Fink, G. R. (1992) *Plant Cell* **4**, 721.
32. Ganson, R. J., d'Amato, T. A. and Jensen, R. A. (1986) *Plant Physiol.* **82**, 203.
33. McCue, K. F. and Conn, E. E. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7374.
34. Dyer, W. E., Henstrand, J. M., Handa, A. K. and Herrmann, K. M. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 7370.
35. Muday, G. K. and Herrmann, K. M. (1992) *Plant Physiol.* **98**, 496.
36. Henstrand, J. M., McCue, K. F., Brink, K., Handa, A. K., Herrmann, K. M. and Conn, E. E. (1992) *Plant Physiol.* **98**, 761.
37. Görlach, J., Beck, A., Henstrand, J. M., Handa, A. K., Herrmann, K. M., Schmid, J. and Amrhein, N. (1993) *Plant Mol. Biol.* **23**, 697.
38. Dyer, W. E., Weaver, L. M., Zhao, J., Kuhn, D. N., Weller, S. C. and Herrmann, K. M. (1990) *J. Biol. Chem.* **265**, 1608.
39. Keith, B., Dong, X., Ausubel, F. M. and Fink, G. R. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 8821.
40. Wang, Y., Herrmann, K. M., Weller, S. C. and Goldsbrough, P. B. (1991) *Plant Physiol.* **97**, 847.
41. Görlach, J., Schmid, J. and Amrhein, N. (1994) *Planta* **193**, 216.
42. Doong, R. L., Gander, J. E., Ganson, R. J. and Jensen, R. A. (1992) *Physiol. Plant.* **84**, 351.
43. Mousdale, D. M., Campell, M. S. and Coggins, J. R. (1987) *Phytochemistry* **26**, 2665.
44. Feierabend, J. and Brassel, D. (1976) *Z. Pflanzenphysiol.* **82**, 334.
45. Minamikawa, T., Kojima, M. and Uritani, I. (1966) *Arch. Biochem. Biophys.* **117**, 194.
46. Rhodes, M. J. C., Hill, A. C. R. and Wooltorton, L. S. C. (1976) *Phytochemistry* **15**, 707.
47. Deka, R. K., Anton, I. A., Dunbar, B. and Coggins, J. R. (1994) *FEBS Letters* **349**, 397.
47a. Bonner, C. A. and Jensen, R. J. (1994) *Biochem. J.* **304**, 11.
48. Schmidt, C. L. and Schultz, G. (1987) *Physiol. Plant.* **70**, 65.
49. Schmidt, C. L., Danneel, H.-J., Schultz, G. and Buchanan, B. B. (1990) *Plant Physiol.* **93**, 758.
50. Schmid, J., Schaller, A., Leibinger, U., Boll, W. and Amrhein, N. (1992) *Plant J.* **2**, 375.
51. Steinrücken, H. C. and Amrhein, N. (1980) *Biochem. Biophys. Res. Commun.* **94**, 1207.
52. Ream, J. E., Steinrücken, H. C., Porter, C. A. and Sikorski, J. A. (1988) *Plant Physiol.* **87**, 232.
53. Stallings, W. C., Abdel-Meguid, S. S., Lim, L. W., Shieh, H.-S., Day-ringer, H. E., Leimgruber, N. K., Stegeman, R. A., Anderson, K. S., Sikorski, J. A., Padgette, S. R. and Kishore, G. M. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5046.
54. Shah, D. M., Horsch, R. B., Klee, H. J., Kishore, G. M., Winter, J. A., Tumer, N. E., Hironaka, C. M., Sanders, P. R., Gasser, C. S., Aykent, S., Siegel, N. R., Rogers, S. G. and Fraley, R. T. (1986) *Science* **133**, 478.
55. Gasser, C. S., Winter, J. A., Hironaka, C. M. and Shah, D. M. (1988) *J. Biol. Chem.* **263**, 4280.
56. Klee, H. J., Muskopf, Y. M. and Gasser, C. S. (1987) *Mol. Gen. Genet.* **210**, 437.
56a. Gasser, C. S. and Klee, H. J. (1990) *Nucl. Acids Res.* **18**, 2821.
57. Benfey, P. N. and Chua, N.-H. (1989) *Science* **244**, 174.
58. Benfey, P. N., Takatsuji, H., Ren, L., Shah, D. M. and Chua, N.-H. (1990) *Plant Cell* **2**, 849.
59. Onderka, D. K. and Floss, H. G. (1969) *J. Amer. Chem. Soc.* **91**, 5894.
60. Schloss, J. V., Ciskanik, L., Pai, E. F. and Thrope, C. (1990) in *Flavins and Flavoproteins* (Bray, R. C., Engel, P. C. and Mayhew, S. G., eds), p. 907. Walter de Gruyter & Co, Berlin.
61. Ramjee, M. N., Balasubramanian, S., Abell, C., Coggins, J. R., Davies, G. M., Hawkes, T. R., Lowe, D. J. and Thorneley, R. N. F. (1992) *J. Am. Chem. Soc.* **114**, 3151.
62. Mousdale, D. M. and Coggins, J. R. (1986) *FEBS Letters* **205**, 328.
63. Schaller, A., Windhofer, V. and Amrhein, N. (1990) *Arch. Biochem. Biophys.* **282**, 437.
64. Schaller, A., van Afferden, M., Windhofer, V., Bülow S., Abel, G., Schmid, J. and Amrhein, N. (1991) *Plant Physiol.* **97**, 1271.
65. Schaller, A., Schmid, J., Leibinger, U. and Amrhein, N. (1991) *J. Biol. Chem.* **266**, 21434.
66. Görlach, J., Schmid, J. and Amrhein, N. (1993) *Plant Mol. Biol.* **23**, 707.
67. Görlach, J., Raesecke, H.-R., Abel, G., Wehrli, R., Henstrand, J. M., Amrhein, N. and Schmid, J. (1995) submitted for publication.
68. Cotton, R. G. H. and Gibson, F. (1968) *Biochim. Biophys. Acta* **156**, 187.

69. Gilchrist, D. G., Woodin, T. S., Johnson, M. L. and Kosuge, T. (1972) *Plant Physiol.* **49**, 52.
70. Woodin, T. S. and Nishioka, L. (1973) *Biochim. Biophys. Acta.* **309**, 211.
71. Woodin, T. S., Nishioka, L. and Hsu, A. (1978) *Plant Physiol.* **61**, 949.
72. Goers, S. K. and Jensen, R. A. (1984) *Planta* **162**, 117.
73. Singh, B. K., Connelly, J. A. and Conn, E. E. (1985) *Arch. Biochem. Biophys.* **243**, 374.
74. Singh, B. K., Lonergan, S. C. and Conn, E. E. (1986) *Plant Physiol.* **81**, 717.
75. Gilchrist, D. G. and Kosuge, T. (1974) *Arch. Biochem. Biophys.* **164**, 95.
76. Kuroki, G. W. and Conn, E. E. (1988) *Plant Physiol.* **86**, 895.
77. Kuroki, G. W. and Conn, E. E. (1989) *Plant Physiol.* **89**, 472.
78. d'Amato, T. A., Ganson, R. J., Gaines, C. G. and Jensen, R. A. (1984) *Planta* **162**, 104.
79. Eberhard, J., Raesecke, H.-R., Schmid, J. and Amrhein, N. (1993) *FEBS Letters* **334**, 233.
80. Gavel, Y. and von Heijne, G. (1990) *FEBS Letters* **261**, 455.
81. Siehl, D. L., Singh, B. K. and Conn, E. E. (1986) *Plant Physiol.* **81**, 711.
82. De-Eknamkul, W. and Ellis, B. E. (1988) *Arch. Biochem. Biophys.* **267**, 87.
83. Siehl, D. L. and Conn, E. E. (1988) *Arch. Biochem. Biophys.* **260**, 822.
84. Bonner, C. A. and Jensen, R. A. (1991) *Plant Science* **74**, 229.
85. Byng, G., Whitaker, R., Flick, C. and Jensen, R. A. (1981) *Phytochemistry* **20**, 1289.
86. Gaines, C. G., Byng, G. S., Whitaker, R. J. and Jensen, R. A. (1982) *Planta* **156**, 233.
87. Connelly, J. A. and Conn, E. E. (1986) *Z. Naturforsch.* **41c**, 69.
88. Hütter, R., Niederberger, P. and DeMoss, J. A. (1986) *Ann. Rev. Microbiol.* **40**, 55.
89. Crawford, I. P. (1989) *Ann. Rev. Microbiol.* **43**, 567.
90. Matsui, K., Miwa, K. and Sano, K. (1987) *J. Bacteriol.* **169**, 5330.
91. Caligiuri, M. G. and Bauerle, R. (1991) *J. Biol. Chem.* **266**, 8328.
92. Belser, W. L., Baron Murphy, J., Delmer, D. P. and Mills, S. E. (1971) *Biochim. Biophys. Acta* **237**, 1.
93. Carlson, J. E. and Widholm, J. M. (1978) *Physiol. Plant.* **44**, 251
94. Brotherton, J. E., Hauptmann, R. M. and Widholm, J. M. (1986) *Planta* **168**, 214.
95. Grosse, W. (1976) *Z. Pflanzenphysiol.* **80**, 463.
96. Grosse, W. (1977) *Z. Pflanzenphysiol.* **83**, 249.
97. Niyogi, K. K., Last, R. L., Fink, G. R. and Keith, B. (1993) *Plant Cell* **5**, 1011.
98. Last, R. L. and Fink, G. R. (1988) *Science* **240**, 305.
99. Rose, A. R., Casselman, A. L. and Last, R. L. (1992) *Plant Physiol.* **100**, 582.
100. Fankhauser, H., Pythoud, F. and King, P. J. (1990) *Planta* **180**, 297.
101. Wright, A. D. and Neuffer, M. G. (1989) *J. Heredity* **80**, 229.
102. Wright, A. D., Moehlenkamp, C. A., Perrot, G. H., Neuffer, M. G. and Cone, K. C. (1992) *Plant Cell* **4**, 711.
103. Last, R. L., Bissinger, P. H., Mahoney, D. J., Radwanski, E. R. and Fink, G. R. (1991) *Plant Cell* **3**, 345.
104. Pruitt, K. D. and Last, R. L. (1993) *Plant Physiol.* **102**, 1019.
105. Doong, R. L., Ahmad, S. and Jensen, R. A. (1991) *Plant Cell Environ.* **14**, 113.
106. Rothe, G. M., Hengst, G., Mildenberger, I., Scharer, H. and Utesch, D. (1983) *Planta* **157**, 358.
107. Fiedler, E. and Schultz, G. (1985) *Plant Physiol.* **79**, 212.
108. Della-Cioppa, G., Bauer, S. C., Klein, B. K., Shah, D. M., Fraley, R. T. and Kishore, G. M. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6873.
109. Giles, N. H., Case, M. E., Baum, J., Geever, R., Huiet, L., Patel, V. and Tyler, B. (1985) *Microbiol. Rev.* **49**, 338.
109a. Herrmann, K. M., Zhao, J., Pinto, J. E. B. P., Weaver, L. and Henstrand, J. M. (1992) in *Current Topics in Plant Physiology*, Vol. 7 (Singh, B. K., Flores, H. E. and Shannon, J. C., eds), p. 12. American Society of Plant Physiologists, Rockville.
110. Hawkins, A. R., Lamb, H. K., Smith, M., Keyte, J. W. and Roberts, C. F. (1988) *Mol. Gen. Genet.* **214**, 224.
111. Kühnl, T. and Wellmann, E. (1979) *Ber. Deutsch. Bot. Ges.* **92**, 741.
112. Ulbrich, B. and Zenk, M. H. (1980) *Phytochemistry* **19**, 1625.
113. Koch, U., Kühnl, T., Conradt, W. and Wellmann, E. (1990) *Plant Science* **70**, 167.
114. Heller, W. and Kühnl, T. (1985) *Arch. Biochem. Biophys.* **241**, 453.
115. Kühnl, T., Koch, U., Heller, W. and Wellmann, E. (1987) *Arch. Biochem. Biophys.* **258**, 226.
116. Wagner, J. G. (1979) *Plant Physiol.* **64**, 88.
117. Holländer-Czytko, H. and Amrhein, N. (1983) *Plant Sci. Letters* **29**, 89.
118. Homeyer, U., Litek, K., Huchzermeyer, B. and Schultz, G. (1989) *Plant Physiol.* **89**, 1388.
119. Frommer, W. B., Hummel, S. and Riesmeier, J. W. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 5944.
120. Kwart, M., Hirner, B., Hummel, S. and Frommer, W. B. (1993) *Plant J.* **4**, 993.
121. Ledüc, C., Ruhnau, P. and Leistner, E. (1991) *Plant Cell Reports* **10**, 334.
122. Poulsen, C., van der Heuden, R. and Verpoorte, R. (1991) *Phytochemistry* **30**, 2873.
123. Zhao, J. and Herrmann, K. M. (1992) *Plant Physiol.* **100**, 1075.