

Stilbene and Chalcone Synthases: Related Enzymes with Key Functions in Plant-Specific Pathways

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Several years of extensive research using the new powerful techniques of molecular biology have enabled the direct comparison of functionally or evolutionarily related genes and their products at the nucleotide and amino acid sequence levels. Two types of synthase with similar functions are discussed as an interesting example. Stilbene synthases, *e.g.* resveratrol synthase, produce the stilbene backbone as a key reaction in the biosynthesis of stilbene-type phytoalexins. Chalcone synthase is a key enzyme in the biosynthesis of flavonoids, including certain phytoalexins derived from a 6'-deoxychalcone which is synthesized by cooperation of chalcone synthase with a reductase. Resveratrol and chalcone synthases utilize the same substrates (4-coumaroyl-CoA and 3 molecules of malonyl-CoA) and catalyze the same condensing type of enzyme reaction (resulting in sequential addition of acetate units *via* malonyl-CoA), but the products differ in the newly formed ring systems (resveratrol and naringenin chalcone). A comparative analysis of cloned DNA sequences and of the reaction mechanisms indicates that the two enzymes are closely related. It seems likely that the proteins possess a common scaffold for substrate recognition and for the condensing reaction, and that the different folding of an enzyme-bound intermediate prior to closure of the new aromatic ring is responsible for the formation of the different products. The same type of condensing reaction is utilized by the 2-ketoacyl-ACP synthases of fatty-acid biosynthesis. However, the available data indicate that these enzymes share little overall homology with either resveratrol or chalcone synthase. One exception may be a short amino acid sequence which corresponds to the active center of the condensing reaction in 2-ketoacyl-ACP synthases.

Introduction

The combination of molecular biological techniques and the more conventional biochemical methods provides a powerful approach to understanding the function and evolution of genes and proteins. This approach has been widely used with bacterial and animal systems, and it becomes evermore important with plants (see [1, 2] for some advanced examples). Many of these systems analyze the relation between genes and proteins for one specific function. Some of them deal with gene families coding for related proteins with different catalytic activities, *i.e.* the question of substrate/product specificity in closely related enzymes (see [3] as one example). This article focusses on recent

developments in such a system in plants: the relationship between two proteins which are closely related in amino acid sequence, use the same substrates and the same type of enzyme reaction, but nevertheless produce different products. Stilbene and chalcone synthases and their genes provide a unique system to investigate fundamental questions of specificity in enzyme catalysis.

Stilbenes and key enzymes of stilbene biosynthesis

A wide range of unrelated plants can synthesize stilbenes. Often they are found constitutively in the heartwood and/or in the sapwood of trees, or in roots and leaves. The induction of their synthesis is correlated with stress, which may be for example wounding or pathogen infection, or increased ozone concentrations [4]. Most stilbenes are toxic, and the toxicity usually correlates with the number of free phenolic hydroxyl groups which easily and non-enzymatically form covalent bonds with

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macromolecules. Stilbenes are phytoalexins [5–7], but as with most of these substances, it is difficult to determine precisely the extent to which they contribute to disease resistance, and each pathogen/host combination has to be tested separately (see [8] as example).

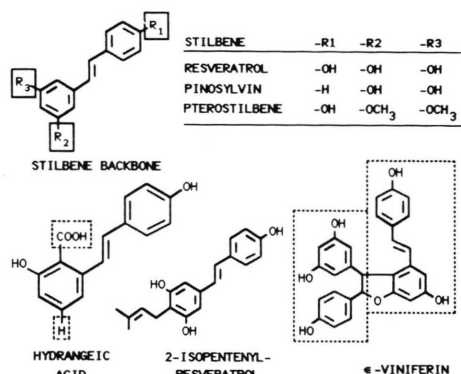


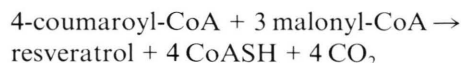
Fig. 1. The stilbene backbone and some representative stilbenes. The dotted lines in hydrangeic acid point out that this molecule, in contrast to typical stilbenes, possesses a carboxyl group and lacks one of the oxygen functions. The lines in ε-viniferin indicate that it is derived from two resveratrol molecules.

Stilbenes are a group of secondary plant products which share a diphenylethylene backbone, and Fig. 1 shows the structure of some examples. The basic molecule may be modified in a variety of ways such as (i) methylation of hydroxyl groups (e.g. pterostilbene), (ii) C-alkylation with isoprenyl groups (e.g. 2-isopentenyl-resveratrol), (iii) dimerization (e.g. ε-viniferin) or polymerization, and (iv) attachment of sugars to hydroxyl groups (glycosides). The existence of stilbene carboxylic acids (e.g. hydrangeic acid, Fig. 1) is of particular interest since it provided initial support for the theory that the stilbenes are products of the phenylpropanoid/polymalonate pathway. In addition, a large number of related substances may be derived from stilbene backbones by other or additional modifications (see [5, 9, 10] for reviews).

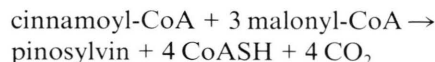
Like many other plant-specific compounds (see [11] for a recent review), stilbenes are phenylpropanoid derivatives, and the key elements of stilbene biosynthesis are the enzymes which synthesize the backbone from phenylpropanoid precursors. Two types of stilbene synthases have been characterized; they preferentially use either 4-coumaroyl-CoA or cinnamoyl-CoA as starter

molecules for addition of carbon units from malonyl-CoA (Fig. 2):

Resveratrol synthase



Pinosylvin synthase



Pinosylvin synthases have not yet been characterized in detail. The most thoroughly investigated enzyme is resveratrol synthase from peanut (*Arachis hypogaea*) [12], and cultured cells from this plant were the source for cDNA and genomic clones [13].

Until quite recently the interest in stilbenes has been mostly restricted to phytopathologists and biochemists investigating secondary plant products. With the advancement of molecular techniques in plant sciences this is changing rapidly. The gene for the key enzyme of stilbene biosynthesis has been cloned and analyzed for the first time, and the results open avenues for a number of

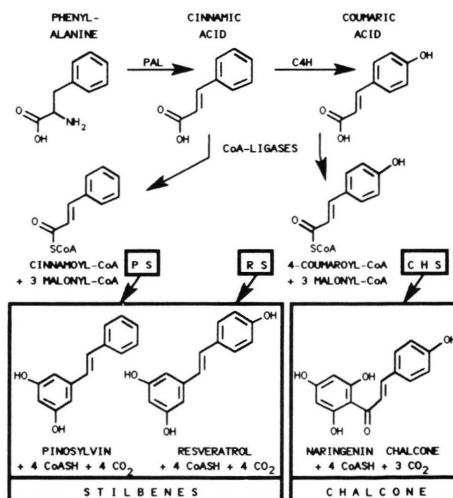


Fig. 2. Biosynthetic pathway from phenylalanine to pinosylvin, resveratrol, and naringenin chalcone. Note that pinosylvin and resveratrol synthases release four molecules of CO₂, while chalcone synthase liberates only three. Enzyme abbreviations: PAL = phenylalanine ammonia-lyase, C4H = cinnamate 4-hydroxylase (monooxygenase), PS = pinosylvin synthase, RS = resveratrol synthase, CHS = chalcone synthase.

investigations of interest for basic research. Stilbene synthases are also of potential interest in agriculture: they produce phytoalexin-active substances in one step from precursors which are present in all plants. However, most of the important crop plants do not synthesize stilbenes, and it is interesting to speculate whether expression of these enzymes in heterologous hosts will contribute to increased resistance against pathogens.

Resveratrol synthase and chalcone synthase

Chalcone synthase is a key enzyme in flavonoid biosynthesis (see [14] for review), a pathway leading to products quite different from stilbenes. It uses the same substrates as resveratrol synthase and catalyzes the same type of enzyme reaction; this and other properties of the proteins suggested that they are related. The final products, however, are different (chalcone *versus* stilbene), and chal-

cone synthase releases three molecules of CO₂ in contrast to the four liberated by resveratrol synthase (Fig. 2). The molecular analysis of cDNAs and genomic sequences does indeed indicate that the genes have a common evolutionary origin. The availability of cloned sequences now allows a much more detailed analysis of the similarities and differences than was possible so far.

A. Sequence comparison

The first analysis of resveratrol synthase sequences [13] revealed 70–75% identity on the protein level with anyone of the known chalcone synthases, and similar figures were obtained for the DNA sequences. Even the gene structures are very similar: all known chalcone synthase genes contain an intron which splits a cysteine codon at a conserved position, and the resveratrol synthase genes studied so far have an intron at precisely the same

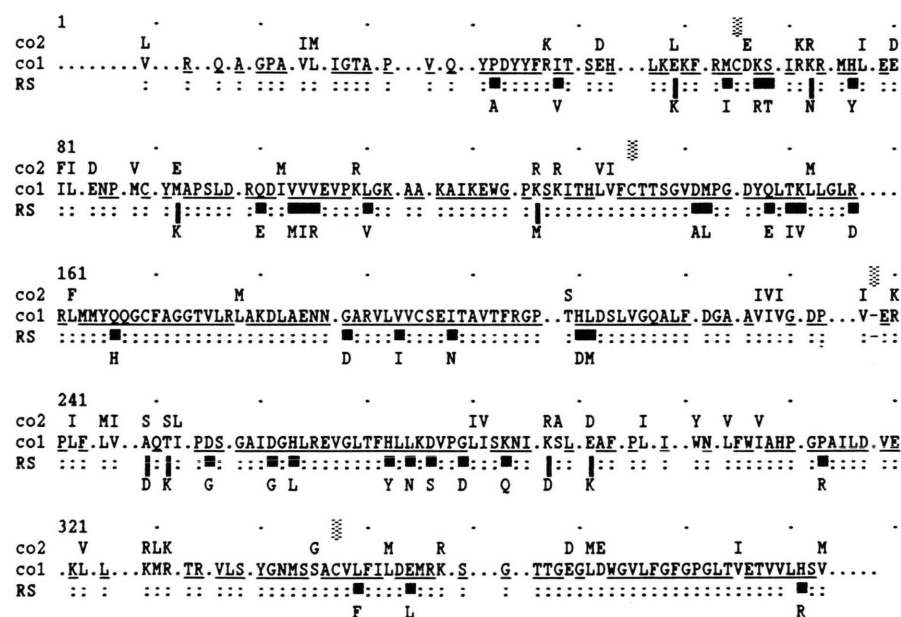


Fig. 3. Comparison of chalcone synthase and resveratrol synthase. co1 and co2 present the consensus sequence for chalcone synthase. co1: underlined letters show the amino acids strictly conserved in all enzymes; dots are positions of variable amino acids; co2 and not underlined letters in co1 represent the consensus sequence extended by inclusion of presumably conservative exchanges. Resveratrol synthase (RS): colons indicate identity with the extended chalcone synthase consensus. The differences are marked as ■ (deviation from strict consensus) or | (deviation from extended consensus), and the amino acids are shown in the line below. Some additional details marked with *** a) position 65: cysteine split by the intron conserved in chalcone synthase and in resveratrol synthase genes; b) amino acid in position 238: absent in most chalcone and in resveratrol synthase; it is found only in chalcone synthases from *Arabidopsis thaliana* and *Sinapis alba*; c) positions 135 and 347: these cysteines are likely candidates for the active site of the condensing activity (see Fig. 6). CHS sequences include data from 11 different plants [15–19]; the RS sequences are from [13] and unpublished work.

position. Indeed, in the absence of additional information it would not seem easy to distinguish the two genes or proteins at the sequence level.

It is therefore fortunate that chalcone synthases have been cloned from many different sources, including monocotyledonous and dicotyledonous plants. Comparisons showed immediately that the proteins are highly conserved with 81–90% identity between enzymes from different sources [15]. This allowed the formulation of a consensus sequence which covers 64% of the protein sequence, a figure which increases to 78% if the consensus is extended to include presumably conservative amino acid exchanges. Additional sequences published subsequently have confirmed the validity of this approach [17–19].

Fig. 3 shows a comparison of the consensus with the known sequences of resveratrol synthases. Most of the strictly conserved amino acids are also found in this stilbene synthase. Resveratrol synthase deviates from the chalcone synthase consensus in 35 positions (14% of the consensus). A closer inspection reveals that some of these may be considered to be conservative exchanges which are possibly of little or no functional significance. If the extended consensus is included in the comparison, an additional number of systematic differences become apparent. Interestingly, they are mostly in charged amino acids, and this may well be of functional importance, for example in the folding of enzyme-bound intermediates prior to formation of the different ring systems.

B. Similarities and differences of the enzyme reactions

Both proteins catalyze the stepwise chain elongation of 4-coumaroyl-CoA with carbon units from malonyl-CoA, followed by the formation of a new aromatic ring by cyclization of an enzyme-bound intermediate. The reaction mechanism has been investigated by *in vitro* experiments with chalcone synthase [20–22], and Fig. 4 summarizes some of these aspects and a comparison with resveratrol synthase. Although precise conformations of the enzyme-bound intermediates are not known, the structures shown here are consistent with the formation of the by-products dihydropyrone and bis-noryangonin. The latter is of particular interest because it has in the new ring sys-

tem a configuration which is similar to that found in resveratrol. This suggests that up to this point (two acetate units added) the reaction intermediates are able to assume conformations which are very similar for both enzymes. If so, the conformations after addition of the third acetate unit are likely to be different (Fig. 4), because the configuration of the enzyme-bound intermediates presumably plays an important role in determining whether a ring system of the stilbene or of the chal-

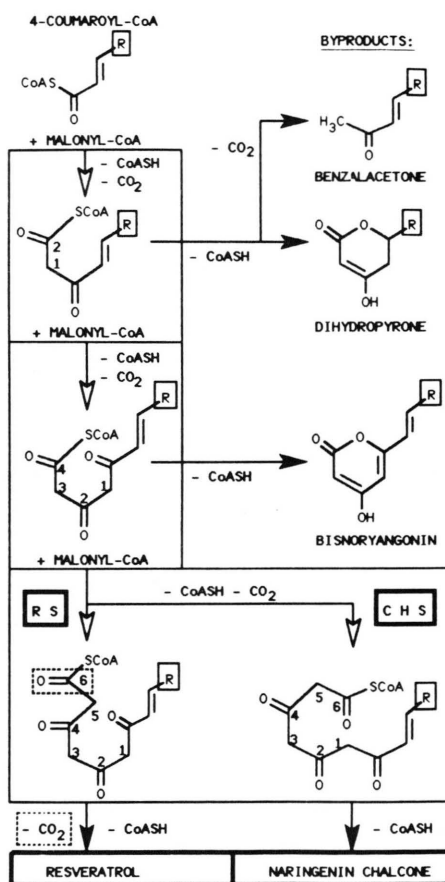


Fig. 4. Hypothetical intermediates in the biosynthesis of resveratrol and naringenin chalcone, and by-products of chalcone synthase under certain *in vitro* conditions [20–22]. The starter molecule 4-coumaroyl-CoA is elongated stepwise by sequential addition of three acetate units *via* malonyl-CoA; finally an enzyme-bound intermediate is folded into conformations which allow the ring closure either to resveratrol or to the chalcone. The stilbene synthase reaction is accompanied by the simultaneous removal of an additional CO₂ (C6 in the numbering of this figure; dotted boxes). R = phenolic ring system of 4-coumaroyl-CoA.

cone type is formed. The resveratrol synthase reaction usually involves the concomitant removal of an additional CO_2 (C6 in the numbering of Fig. 4). The existence of stilbene carboxylic acids (*e.g.* hydrangeic acid, Fig. 1) shows that this is, however, not an essential part of all stilbene synthase reactions.

The results indicate that the two proteins are indeed closely related in structure as well as in function, and it seems likely that both use similar scaffolds for substrate binding and the condensing reaction. The key which controls the formation of different ring systems must be hidden in the protein sequences, and if the model proposed in Fig. 4 is correct, relatively minor differences may be sufficient. The three-dimensional structures of the two enzymes are not yet known, and therefore it is difficult to estimate the significance of the differences discovered in the amino acid comparison. Also, the comparison would be much improved (i) by additional information from sequences of resveratrol synthases from other plants and (ii) by information on pinosylvin synthases, *i.e.* stilbene synthases which prefer cinnamoyl-CoA over 4-coumaroyl-CoA as substrates. Regardless of these present limitations it seems very likely that at least some of the deviations from the consensus are of functional importance. This allows a question of fundamental significance to be asked: Would it be possible to change a resveratrol synthase into a chalcone synthase by amino acid exchanges which approach and finally complete the consensus sequence? Such experiments are feasible with site-directed mutagenesis of the DNA sequences, and the number of necessary changes does not appear prohibitive. The results will provide interesting insights into the function of these proteins, and possibly of others which also use malonyl-CoA in similar types of reactions. There are no other systems in plants which are available for this type of analysis.

Synthesis of 6'-deoxychalcone by cooperation of chalcone synthase and a reductase

Recent findings [23–25] reveal an interesting aspect to the activities of chalcone synthase. The enzyme can cooperate with an NADPH-dependent reductase to synthesize a deoxychalcone which lacks one of the oxygen functions characteristic for

naringenin chalcone. Precursor studies suggest that the chalcone itself is not the substrate for the reductase, but that the reduction takes place on one of the intermediates, *i.e.* after addition of either two or three acetate units, as shown in the hypothetical model in Fig. 5. This reductase appears to be the key for an important branchpoint: its discovery solves one of the long-standing puzzles in the biosynthesis of certain phytoalexins which are derived from deoxychalcone-type precursors, for example the glyceollins in soybean (see [26] for recent review). The enzyme has been purified from soybean (*Glycine max* L.) cell cultures [25], and induction studies show that it is induced concomitantly with chalcone synthase after elicitor challenge [27]. Interestingly, the purified reductase from soybean can produce deoxychalcone with chalcone synthases from other plants, even with the enzyme from parsley, a plant which does not synthesize deoxyflavonoids [27]. It seems likely that further study of the cooperation between these enzymes will provide further insights into the reactions of chalcone synthase. Although a similar

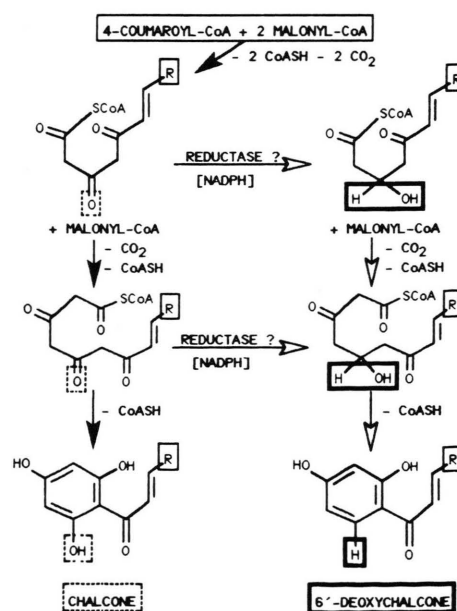
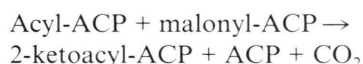


Fig. 5. Model for the cooperation of chalcone synthase with the reductase in the formation of 6'-deoxychalcone. It seems most likely that the reducing step occurs with an enzyme-bound intermediate after reaction of the chalcone synthase with two or three molecules of malonyl-CoA.

interaction of a reductase with a stilbene synthase has not yet been reported, it is worth to note that the stilbene hydrangeic acid (Fig. 1) lacks an oxygen function precisely at the position which would be expected from a reducing step at the same carbon atom as in the chalcone.

Active centers in condensing enzymes

The reactions catalyzed by resveratrol and chalcone synthases involve the stepwise chain elongation of 4-coumaroyl-CoA acetate units *via* malonyl-CoA. The same condensing reaction is realized in the biosynthesis of fatty acids, of 6-methylsalicylic acid [28], and probably in the formation of polyene macrolides (*e.g.* candicidin [29]), long-chain epicuticular waxes (*e.g.* 2-diketones and hydrocarbons [30]) as well as the bitter substances of hops (*e.g.* lupulone and humulone [31]). The most extensively studied of these is the 2-ketoacyl-ACP synthase function in the elongation step of fatty acid synthesis:



In contrast to chalcone and resveratrol synthases, the acyl acceptor and the malonyl donor are activated by attachment to ACP (acyl carrier protein) rather than to coenzyme A, and the condensation products are modified by reduction prior to the next elongation (no reduction steps take place with the two plant-specific enzymes, except in the instance of deoxychalcone formation discussed above). The condensing reactions, however, are quite comparable; they also share two characteristic side reactions (decarboxylation and CO₂ exchange [20]), and chalcone synthase can function at least *in vitro* with butyryl-CoA, hexanoyl-CoA, or benzoyl-CoA as acceptor molecule. Based on these findings it was suggested that 2-ketoacyl-ACP synthase and chalcone synthase share a common evolutionary origin [22].

Active centers for the condensing reaction have been identified by biochemical techniques and/or by comparative analysis of cloned DNA sequences, in enzymes from goose [32], *Saccharomyces cerevisiae* [33], *E. coli* (type I) [34], rat [35, 36], *Cephalosporium caerulens* [38], and recently in 6-methylsalicylic acid synthetase (MSAS) from *Penicillium patulum* (E. Schweizer, University of Erlangen-Nürnberg, personal communication).

The available data suggest that the core of the active site is represented by a short sequence of conserved amino acids surrounding a cysteine (Fig. 6). The only exception is *Cephalosporium caerulens*: this organism produces an antibiotic, cerulenin, which strongly inhibits most 2-ketoacyl-ACP synthase activities [37], and it is thought that the difference explains why the *Cephalosporium caerulens* enzyme is insensitive to its own antibiotic [38]. Obviously, active sites may vary considerably without losing the capacity to catalyze the same reaction. One of the three 2-ketoacyl-ACP synthases (type III) from *E. coli* is insensitive to cerulenin, but the active site has not yet been analyzed. This enzyme is also unusual in at least one other property: it uses acetyl-CoA rather than acetyl-ACP in the condensing reaction [39, 40].

The active center of chalcone synthase has not yet been identified experimentally, but the enzyme

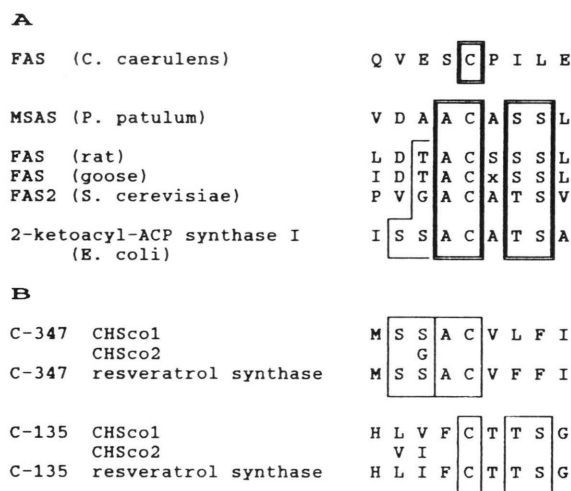


Fig. 6. A. Active centers for the condensing reaction. With the exception of the cerulenin-resistant enzyme from *Cephalosporium caerulens*, the sites share some conserved amino acids surrounding a cysteine. B. Two candidate sequences for the active center of chalcone and resveratrol synthase; sequence elements similar to the active centers in the other enzymes are boxed. FAS (*C. caerulens*): fatty acid synthetase from *Cephalosporium caerulens* [38]; MSAS: 6-methylsalicylic acid synthetase (E. Schweizer, personal communication); FAS (rat): fatty acid synthetase from liver and mammary gland [35, 36]; FAS (goose): fatty acid synthetase from uropygial gland [32]; FAS2 (*S. cerevisiae*): fatty acid synthetase 2 (subunit α) from *Saccharomyces cerevisiae* [33]; 2-ketoacyl-ACP synthase I (*E. coli*): [34]; CHScol and CHScol2: extended chalcone synthase consensus sequence (Fig. 3).

is sensitive to cerulenin [41]. This prompted a search for sequence elements which are similar to the active site identified in the other condensing enzymes, and two candidates were detected in chalcone and resveratrol synthase [34] (cysteines in positions 135 and 347 in Fig. 3; see alignment with the core active sites in Fig. 6). Both are in highly conserved regions, and it would seem difficult to prefer one over the other on the basis of sequence alignments. Kauppinen and colleagues refined the comparison further by including, as far as known, the sensitivity of the enzymes against cerulenin, and they proposed that the sequences at Cys-347 conform more closely to the prediction for a cerulenin-sensitive center (see [34] for a detailed discussion). It would be useful to confirm this prediction by biochemical experiments. Beyond these similarities, chalcone and resveratrol synthases do not reveal obvious homologies with the other condensing enzymes, suggesting that the relation on the amino acid level may be confined to a few conserved amino acids of the core active center. Perhaps this is not too surprising, if one considers not only the similarities of the condensing reactions, but also the pronounced differences between the sum of the reactions catalyzed by fatty acid synthetase and by resveratrol or chalcone synthase.

Outlook

It seems very likely that this type of comparative analysis combining the information from bio-

chemical and molecular studies will be very useful in the near future, especially in the plant field. It contributes not only to discovering similarities and differences, but it also stimulates hypotheses which can be tested with various types of experiments. Plant DNA sequences, for example, often reveal apparent gene families for specific functions, but usually it is very difficult to determine if all of the proteins deduced from DNA sequences are functional. In such cases, one of the supporting criteria could be whether the sequences predict an intact active site or not (see [34] for discussion of an example).

However, the prospects are particularly promising for stilbene and chalcone synthases, because they provide a unique system for studying questions fundamental to molecular recognition and enzyme catalysis. Why is it that two such similar enzymes, which use identical substrates to construct an identical intermediate, produce ultimately different products? How can such enzymes be manipulated to control the final product?

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