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Chapter II

Plant Polyketide Synthases

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Abstract:
The Polyketide Synthases (PKSs) are condensing enzymes which form a myriad of polyketide compounds. In plants several PKSs have been identified and studied. This mini-review summarizes what is known about plant PKSs, and some aspects such as specificity, reaction mechanisms, structure, as well as their possible evolution are highlighted.

II.1 Introduction

The polyketide natural products are one of the largest and most diverse groups of secondary metabolites. They are formed by a myriad of different organisms from prokaryotes to eukaryotes. Antibiotics and mycotoxins produced by fungi and actinomycetes, and stilbenoids and flavonoids produced by plants are examples of polyketide compounds. They have an important role in medicine, due to their activities such as antimicrobial, antiparasitic, antineoplastic and immunosuppresive (Rawlings, 1999; Sankawa, 1999; Whiting, 2001).

II.2 Polyketide Synthases

The Polyketide Synthases (PKSs) are a group of enzymes that catalyzes the condensation of CoA-esters of acetic acid and other acids to give polyketide compounds. They are classified according to their architectural configurations as type I, II and III (Hopwood and Herman, 1990; Staunton and Weissman, 2001; Fischbach and Walsh, 2006). The type I describes a system of one or more multifunctional proteins that contain a different active site for each enzyme-catalyzed reaction in polyketide carbon chain assembly and modification. They are organized into modules, containing at least acyltransferase (AT), acyl carrier protein (ACP) and β-keto acyl synthase (β-kS) activities. Type I PKSs are sub-grouped as iterative or modular; usually present in fungal or bacterial systems, respectively (Moore and Hopke, 2001; Moss *et al.*, 2004). The type II is a system of individual enzymes that carry a single set of iteratively acting activities and a minimal set consists of two ketosynthase units (α- and β-KS) and an ACP, which serves as an anchor for the growing polyketide chain. Additional PKS subunits such as ketoreductases, cyclases or aromatases define the folding pattern of the polyketo intermediate and further post-PKS modifications, such as oxidations, reductions or glycosylations are added to the polyketide (Rix *et al.*, 2002; Hertweck *et al.*, 2007). The only known group of organism that employs type II PKS systems for polyketide biosynthesis is soil-borne and marine Gram-positive actinomycetes. The type III is present in bacteria, plants and fungi (Austin and Noel, 2003; Seshime *et al.*, 2005; Funa *et al.*, 2007); they are essentially condensing enzymes that lack ACP and act directly on acyl-CoA substrates.

II.3 Plant Polyketide Synthases

In plants several type III PKSs have been found and all of them participate in the biosynthesis of secondary metabolites (Table 1 and Figure 1); chalcone synthase (CHS), 2-pyrone synthase (2-PS), stilbene synthase (STS), bibenzyl synthase (BBS), homoeriodictyol/eriodictyol synthase (HEDS or HvCHS), acridone synthase (ACS), benzophenone synthase (BPS), phlorisovalerophenone synthase (VPS), isobutyrophenone synthase (BUS), coumaroyl triacetic acid synthase (CTAS), benzalacetone synthase (BAS), *C*-methyl chalcone synthase (PstrCHS2), anther-specific chalcone synthase-like (ASCL) and stilbene carboxylate synthase (STCS) are some examples from this group (Atanassov *et al.*, 1998; Austin and Noel, 2003; Eckermann *et al.*, 2003; Klingauf *et al.*, 2005; Wu *et al.*, 2008). As CHS and STS are the most studied enzymes, this group is often called the family of the CHS/STS type. It is known that plant PKSs share 44-95% amino acid sequence identity and utilize a variety of different substrates ranging from aliphatic-CoA to aromatic-CoA substrates, from small (acetyl-CoA) to bulky (*p*-coumaroyl-CoA) substrates or from polar (malonyl-CoA) to nonpolar (isovaleroyl-CoA) substrates giving to the plants an extraordinary functional diversification.

Table I. Examples of type III polyketide synthases, preferred substrates and reaction products.

Enzyme	Substrates (stater, extender, no. condensations)	Type of ring closure, ring type	Product	References
Plant:				
None cyclization reaction				
Benzalacetone synthase (BAS), EC 2.3.1.-	*p*-coumaroyl-CoA, Malonyl-CoA (1X)		Benzalacetone (**1**)	Borejsza-Wysocki and Hrazdina, 1996; Abe *et al*., 2001; Zheng and Hrazdina, 2008
	Feruloyl-CoA, Malonyl-CoA (1X)		Methoxy-benzalacetone (**12**)	
One cyclization reaction				
Benzalacetone synthase (BAS), EC 2.3.1.-	*N*-methylanthraniloyl-CoA (or anthraniloyl-CoA), Malonyl-CoA (or methyl-malonyl-CoA) (1X)	-, heterocyclic	4-hydroxy-2(1*H*)quinolones (**3**)	Abe *et al*., 2006a
CTAS type		Lactonization, heterocyclic		
C-methylchalcone synthase (PstrCHS2)	Diketide-CoA, Methyl-malonyl-CoA (1X)		Methyl-pyrone (**4**)	Schröder *et al*., 1998
Styrylpyrone synthase (SPS) or Bisnoryangonin synthase (BNS)	*p*-coumaroyl-CoA, Malonyl-CoA (2X)		Bisnoryangonin (**5**)	Beckert *et al*., 1997; Herderich *et al*., 1997; Schröder Group
	Caffeoyl-CoA, Malonyl-CoA (2X)		Hispidin (**6**)	
2-pyrone synthase (2-PS)	Acetyl-CoA, Malonyl-CoA (2X)		Triacetic acid lactone (TAL) (**7**)	Eckermann *et al*., 1998
p-Coumaroyltriacetic acid synthase (CTAS)	*p*-coumaroyl-CoA, Malonyl-CoA (3X)		*p*-coumaroyltriacetic acid lactone (**8**)	Akiyama *et al*., 1999
CHS type		Claisen, aromatic		
Chalcone synthase (CHS), EC 2.3.1.74	*p*-coumaroyl-CoA, Malonyl-CoA (3X)		Naringenin chalcone (**9**)	Whitehead and Dixon, 1983; Ferrer *et al*., 1999
Phlorisovalerophenone synthase (VPS), EC 2.3.1.156	Isovaleroyl-CoA, Malonyl-CoA (3)		Phlorisovalerophenone (**10**)	Paniego *et al*., 1999; Okada and Ito, 2001

Table 1.*Continued.*

Enzyme	Substrates (stater, extender, no. condensations)	Type of ring closure, ring type	Product	References
Isobutyrophenone synthase (BUS)	Isobutyryl-CoA, Malonyl-CoA (3X)		Phlorisobutyrophenone (**11**)	Klingauf *et al*., 2005
Benzophenone synthase (BPS), EC 2.3.1.151	*m*-hydroxybenzoyl-CoA, Malonyl-CoA (3X)		2,3',4,6-tetrahydroxybenzophenone (**12**)	Beerhues, 1996
	Benzoyl-CoA, Malonyl-CoA (3X)		2,4,6-trihydroxybenzophenone (**13**)	Liu *et al*., 2003
Acridone synthase, EC 2.3.1.159 (ACS)	*N*-methylanthraniloyl-CoA, Malonyl-CoA (3X)		1,3-dihydroxy-*N*-methylacridone (**14**)	Junghanns *et al*., 1998; Springo *et al*., 2000
Homoeriodictyol/ eriodictyol synthase (HEDS or HvCHS)	Feruloyl-CoA, Malonyl-CoA (3X)		Homoeriodictyol (**15**)	Christensen *et al*., 1998
	Caffeoyl-CoA, Malonyl-CoA(3X)		Eriodictyol (**16**)	
STS type		Aldol, aromatic		
Stilbene synthase (STS), EC 2.3.1.95	*p*-coumaroyl-CoA, Malonyl-CoA (3X)		Resveratrol (**17**)	Schöppner and Kindl, 1984; Austin *et al*., 2004a
Pinosylvin synthase, EC 2.3.1.146	Cinnamoyl-CoA, Malonyl-CoA (3X)		Pinosylvin (**18**)	Raiber *et al*., 1995; Schanz et al., 1992; Fliegmann *et al*., 1992
Bibenzyl synthase (BBS)	Dihydro-*m*-coumaroyl-CoA, Malonyl-CoA (3X)		3,3',5-trihydroxybibenzyl (**19**)	Reinecke and Kindl, 1994; Preisig-Müller *et al*., 1995
Biphenyl synthase (BIS)	Benzoyl-CoA, Malonyl-CoA (3X)		3,5-dihydroxybiphenyl (**20**)	Liu *et al*., 2007
Stilbenecarboxylate synthase (STCS)	Dihydro-*p*-coumaroyl-CoA, Malonyl-CoA (3X)	Aldol without decarboxylation, aromatic	5-hydroxylunularic acid (**21**)	Eckermann *et al*., 2003; Schröder Group

Table 1.*Continued.*

Enzyme	Substrates (stater, extender, no. condensations)	Type of ring closure, ring type	Product	References
More than 2 cyclization reactions				
Miscellaneous type		-, heterocyclic or aromatic		
Pentaketide chromone synthase (PCS)	Acetyl-CoA, Malonyl-CoA (4X)		5,7-dihydroxy-2-methylchromone (**22**)	Abe *et al*., 2005a
Hexaketide synthase (HKS)	Acetyl-CoA, Malonyl-CoA (5 X)		6-(2',4'-dihydroxy-6'-methyl-phenyl)-4-hydroxy-2-pyrone (**23**)	Springob *et al*., 2007; Jindaprasert *et al*., 2008
Aloesone synthase (ALS)	Acetyl-CoA, Malonyl-CoA (6X)		Aloesone (**24**)	Abe *et al*., 2004a
Octaketide synthase (OKS)	Acetyl-CoA, Malonyl-CoA (7X)		SEK4 (**25**) and SEK4b (**26**) (octaketides)	Abe *et al*., 2005b
Bacteria				
PKS18	Lauroyl-CoA, Malonyl-CoA (1X)	Pyrone type ring-folding	Lauroyl triketide pyrone (**27**), Lauroyl tetraketide pyrone (**28**)	Saxena *et al*., 2003; Sankaranarayanan *et al*., 2004
Monoacetylphloroglucinol synthase (PhlD)	Malonyl-CoA (3X)	CHS type ring-folding	phloroglucinol (**29**)	Achkar *et al*., 2005; Zha *et al*., 2006
3,5-dihydroxyphenylacetate synthase (DHPAS), (DpgA)	Malonyl-CoA (4X)	STS type ring-folding	3,5-dihydroxyphenylacetic acid (**30**)	Li *et al*., 2001; Pfeifer *et al*., 2001
1,3,6,8-tetrahydroxynaphthalene synthase (THNS, RppA)	Malonyl-CoA (5X)	-, two cyclization reactions	1,3,6,8-tetrahydroxynaphthalene (**31**), THN	Funa *et al*., 1999; Funa *et al*., 2002
Fungi				
2'-oxoalkylresorcylic acid synthase (ORAS)	Stearoyl-CoA, Malonyl-CoA (4X)	STS type ring-folding without decarboxylation	2,4-dihydroxy-6-(2'-oxononadecyl)-benzoic acid (**32**)	Funa *et al*., 2007

-, undefined

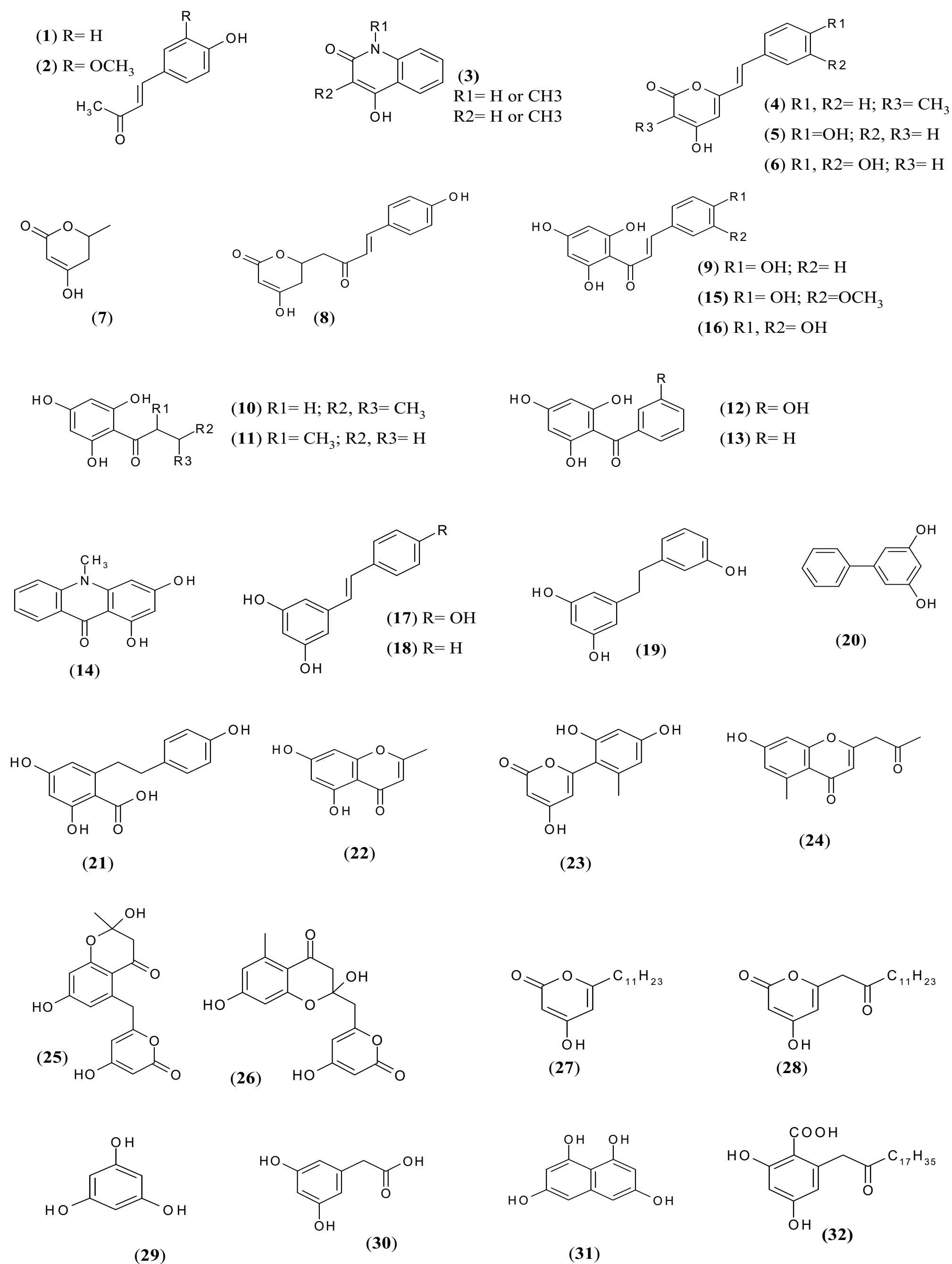

Figure 1. Some compounds biosynthesized by type III PKS.

II.3.1 Type of cyclization reaction

Divergences by the number of condensation reactions (polyketide chain elongation), the type of the cyclization reaction and the starter substrate are characteristic of the type III PKSs (Schröder, 2000). Based on the mechanism of the cyclization they are classified as CHS-, STS- and CTAS-type (Figure 2).

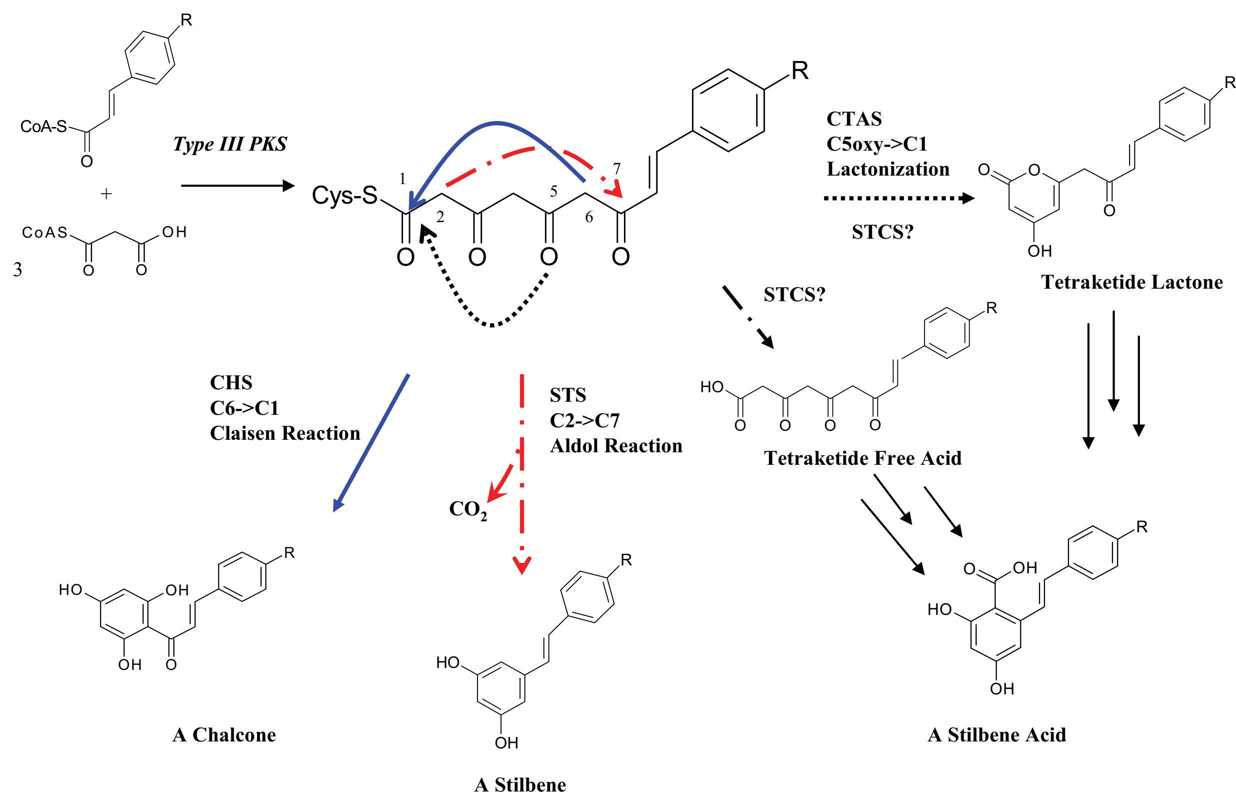

Figure 2. Type of cyclization by plant PKS. R, OH, H. Modified from Austin *et al*., 2004a.

In the CHS-type the intramolecular cyclization from C6 to C1 is called Claisen condensation; this mechanism for the carbon-carbon bond formation is not only used for the biosynthesis of polyketides, but also for fatty acids (Heath and Rock, 2002). In the STS -type the cyclization is from C2 to C7, with an additional decarboxylative loss of the C1 as CO_2, this reaction is an Aldol type of condensation. In the CTAS-type there is a heterocyclic lactone formation

between oxygen from C5 to C1, called lactonization. Regarding the biosynthesis of stilbene carboxylic acids, Eckermann *et al.* (2003) reported the expression of a PKS with STCS activity from *Hydrangea macrophylla* L. and it was proposed to be an Aldol condensation without decarboxyation of the C1. The same group reported expression of STCSs in *Marchantia polymorpha* (Schröder Group). Although, the formation of the stilbenecarboxylate represented 40-45% of the product mixture pyrone formation was predominant. It has been suggested that the formation of a tetraketide free acid or lactone is the product of the STCS and undergoes spontaneous cyclization to yield the stilbenecarboxylate. Aromatization and reduction could be additional steps to stilbenecarboxylic acid formation (Akiyama *et al.*, 1999; Schröder Group). Some examples of metabolites which could be formed by a STCS-type PKS in *Cannabis sativa* (Fellermeier and Zenk, 1998; Fellermeier *et al.*, 2001), *Ginkgo biloba* (Adawadkar and ElSohly, 1981), liverworts species (Valio and Schwabe, 1970; Pryce, 1971), *Amorpha fruticosa* (Mitscher *et al.*, 1981), *Gaylussacia baccata* (Askari *et al.*, 1972), *Helichrysum umbraculigerum* (Bohlmann and Hoffmann, 1979), *Syzygium aromatica* (Charles *et al.*, 1998) and *H. macrophylla* (Asahina and Asano, 1930; Gorham., 1977) are shown in figure 3. Together with the different types of cyclization mentioned above some PKSs only catalyze condensation reactions without a cyclization reaction. BAS, which has been isolated from raspberries and *Rheum palmatum* (Borejsza-Wysocki and Hrazdina, 1996; Abe *et al.*, 2001), catalyzes a single condensation of malonyl-CoA to *p*-coumaroyl-CoA starter to form *p*-hydroxybenzalacetone. In *Oryza sativa* curcuminoid synthase (CUS) condenses two *p*-coumaroyl-CoAs and one malonyl-CoA to form bisdemethoxycurcumin (Katsuyama *et al.*, 2007) and for the initial step in diarylheptanoid biosynthesis from *Wachendorfia thyrsiflora* a PKS was identified (Brand *et al.*, 2006).

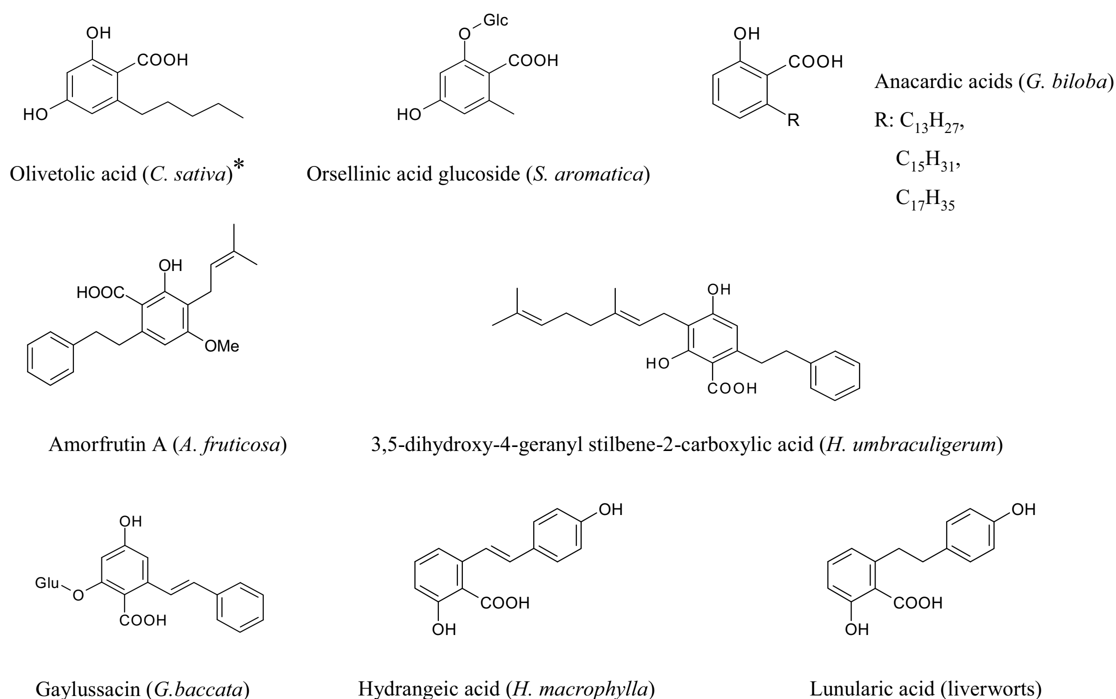

Figure 3. Some examples of alkyl-resorcinolic acids and stilbene carboxylic acids isolated from plants. * Putative intermediate of cannabinoid biosynthesis.

II.3.2 Structure and reaction mechanism

From data bases (NCBI) more than 859 nucleotide sequences have been reported from plant PKSs and several PKS crystalline structures have been characterized (Ferrer *et al.*, 1999; Austin *et al.*, 2004a; Shomura *et al.*, 2005; Jez *et al.*, 2000a; Schröder Group, PDB: 2p0u, MMDB: 45327; Morita *et al.*, 2007; Morita *et al.*, 2008), as well as bacterial type III PKSs (Austin *et al.*, 2004b; Sankaranarayanan *et al.*, 2004). There are no significant differences on the conformation of these crystalline structures, PKSs form a symmetric dimer displaying a $\alpha\beta\alpha\beta\alpha$ five-layered core and in each monomer an independent active site is present. Besides, that dimerization is required for activity and an allosteric cooperation type between the two active sites from the monomers was suggested (Tropf *et al.*, 1995). Furthermore, it was found that the Met 137 (numbering in *M. sativa* CHS) in each monomer helps to shape the active site cavity of the adjoining subunit (Ferrer *et al.*, 1999).

The basic principle of the reaction mechanism consists of the use of a starter CoA-ester to perform sequential condensation reactions with two Carbon units,

from a decarboxylated extender, usually malonyl-CoA. A linear polyketide intermediate is formed which is folded to form an aromatic ring system (Schröder, 1999). In particular, the active site is composed of a CoA-binding tunnel, a starter substrate-binding pocket and a cyclization pocket, and three residues conserved in all the known PKSs define this active site: Cys 164, His 303 and Asn 336. Each active site is buried within the monomer and the substrates enter *via* a long CoA-binding tunnel. The Cys 164 is the nucleophile that initiates the reaction and attacks the thioester carbonyl of the starter resulting in transfer of the starter moiety to the cysteine side chain. Asn336 orients the thioester carbonyl of malonyl-CoA near His303 with Phe215, providing a nonpolar environment for the terminal carboxylate that facilitates decarboxylation and a resonance of the enolate ion to the keto form allows for condensation of the acetyl carbanion with the enzyme-bound polyketide intermediate. Phe215 and Phe265 perform as gatekeepers (Austin and Noel, 2003). The recapture of the elongated starter-acetyl-diketide-CoA by Cys164 and the release of CoA set the stage for additional rounds of elongation, resulting in the formation of a final polyketide reaction intermediate. Later an intramolecular cyclization of the polyketide intermediate takes place (Abe, *et al.*, 2003a; Jez *et al.*, 2000b; Jez *et al.*, 2001a; Lanz *et al.*, 1991; Suh *et al.*, 2000). The GFGPG loop is a conserved region on plant PKSs that provides a scaffold for cyclization reactions (Austin and Noel, 2003; Suh *et al.*, 2000).

The remarkable functional diversity of the PKSs derives from small modifications in the active site, which greatly influence the selection of the substrate, number of polyketide chain extensions and the mechanism of cyclization reactions. The volume of the active site cavity influences the starter molecule selectivity and limits polyketide length. The 2-PS cavity is one third the size of the CHS cavity. The combination of three amino acids substitutions on Thr197Leu, Gly256Leu and Ser338Ile on CHS sequence changes the starter molecule preference from *p*-coumaroyl-CoA to acetyl-CoA and results in formation of a triketide instead of a tetraketide product (Jez *et al.*, 2000a). From homology modeling studies, it was found that the cavity volume of octaketide synthase (OKS) (Abe *et al.*, 2005b) and aloesone synthase (ALS) (Abe *et al.*, 2004a) is slightly larger than that of CHS; while that of pentaketide chromone synthase (PCS) is almost as large as of ALS (Abe *et al.*, 2005a). The replacing of the residues Ser132Thr, Ala133Ser and Val265Phe fully transformed the ACS to

a functional CHS (Lukacin *et al.*, 2001). The change from His166-Gln167 to Gln166-Gln167 converts the STS from *A. hypogaea* to a dihydropinosilvin synthase (Schröder and Schröder, 1992). It was shown that Gly256, which resides on the surface of the active site, is involved in the chain-length determination from CHS (Jez *et al.*, 2001b); while in ALS Gly256 determines starter substrate selectivity, Thr197 located at the entrance of the buried pocket controls polyketide chain length and Ser338 in proximity of the catalytic Cis164 guides the linear polyketide intermediate to extend into the pocket, leading to the formation of a heptaketide (Abe *et al.*, 2006b).
The cyclization specificities in the active site of CHS and STS are given by electronic effects of a water molecule rather than by steric factors (Austin *et al.*, 2004a). In BAS, the residue Ser338 is important in the steric guidance of the diketide formation reaction and probably BAS has an alternative pocket to lock the coumaroyl moiety for the diketide formation reaction (Abe *et al.*, 2007). Dana *et al.* (2006) analyzed mutant alleles of the *Arabidopsis thaliana* CHS locus by molecular modeling and found that changes in the amino acid sequence on regions not located at or near residues that are of known functional significance can affect the architecture, the dynamic movement of the enzyme, the interactions with others proteins, as well as have dramatic effects on enzyme function.

II.3.2.1 Specificity and byproducts

Probably in *vivo* PKSs are highly substrate-specific and product-specific, as they are confined to specific organelles, tissues or present in organized enzymatic complexes (metabolons). However, *in vitro* PKSs are not very substrate-specific and enzymatic reactions yield derailment byproducts together with the final product in a highly variable proportion. Benzalacetone, bisnoryangonin and *p*-coumaroyltriacetic acid lactone are reaction byproducts from CHS, STS and STCS using *p*-coumaroyl-CoA as starter (Schröder Group). It is known that CHS (Morita *et al.*, 2000; Novak *et al.*, 2006; Raharjo *et al.*, 2004b; Schüz *et al.*, 1983; Springob *et al.*, 2000), STS (Samappito *et al.*, 2003; Zuurbier *et al.*, 1998) and VPS (Okada *et al.*, 2001; Paniego *et al.*, 1999) can use efficiently acetyl-CoA, cinnamoyl-CoA, caffeoyl-CoA, butyryl-CoA, isovaleryl-CoA, hexanoyl-CoA, benzoyl-CoA and phenylacetyl-CoA as starter substrates; moreover, it has been found that CHS (Abe *et al.*, 2003b), OKS (Abe

et al., 2006c), STS and BAS (Abe *et al.*, 2002) could use methylmalonyl-CoA as extender substrate. Morita *et al.* (2001) reported the biosynthesis of novel polyketides by a STS using halogenated starter substrates of cinnamoyl-CoA and *p*-coumaroyl-CoA, as well as analogs in which the coumaroyl moiety was replaced by furan or thiophene. The formation of long-chain polyketide pyrones by CHS and STS using CoA esters of C_6-, C_8-, C_{10}-, C_{12}-, C_{14}-, C_{16}-, C_{18}-, and C_{20}- fatty acids has been demonstrated (Abe *et al.*, 2005c; Abe *et al.*, 2004b). Recently, a type III PKS from *Huperzia serrata* with a versatile enzymatic activity was reported (Wanibuchi *et al.*, 2007). This PKS can accept from aromatic to aliphatic CoA as starter substrates, including the bulky starter substrates *p*-methoxycinnamoyl-CoA and *N*-methylanthraniloyl-CoA to produce chalcones, benzophenones, phloroglucinols, pyrones and acridones. It was suggested that this enzyme possesses a larger starter substrate-binding pocket at the active site, giving a substrate multiple capacity. The crystallization of this PKS was also reported (Morita *et al.*, 2007).

II.3.2.2 Homology and Evolution

Type III PKSs have around 400 amino acid long polypeptide chains (41-44 kDa) and share from 44 to 95% sequence identity. The PKS reactions share many similarities with the condensing activities in the biosynthesis of fatty acids in plants and microorganisms as well as of microbial polyketides. It has been recognized that all three types of PKSs likely evolved from fatty acid synthases (FASs) of primary metabolism (Austin and Noel, 2003; Schröder, 1999). All PKSs, like their FASs ancestors, possess a β-KS activity that catalyzes the sequential head-to-tail incorporation of two-carbon acetate units into a growing polyketide chain; while FAS performs reduction and dehydration reactions on each resulting β-keto carbon to produce an inert hydrocarbon, PKS omits or modifies some of these latter reactions, thus preserving varying degrees of polar chemical reactivity along portions of the growing linear polyketide chain. The use of CoA-ester rather than of ACP-ester is a long line of evolution that separates type III PKSs from the other PKSs. It has been suggested that STS, 2-PS and CHS isoforms have evolved from CHS by duplication and mutation (Durbin *et al.*, 2000; Eckermann *et al.*, 1998; Helariutta *et al.*, 1996; Lukacin *et al.*, 2001; Tropf *et al.*, 1994). Several phylogenetic analyses (Abe *et al.*, 2001; Abe *et al.*, 2005c; Liu *et al.*, 2003;

Springob *et al*., 2007; Wanibuchi *et al*., 2007) have revealed that the CHS/STS type family is grouped into subfamilies according to their enzymatic function. Hypothesis about evolution of the plant PKSs and its ecological role in the biosynthesis of secondary metabolites have been suggested (Moore and Hopke, 2001; Seshime *et al*., 2005; Jenke-Kodama et al., 2008).

II.4. Concluding remarks

The type III PKSs appears widespread in fungi and bacteria, as well as in plants. Enormous progress has been made in understanding the reaction mechanism of type III PKSs, several crystalline structures have been identified and some reaction mechanisms, e.g. CHS and STS, have been deciphered; however, from others, like STCS, it is still unclear. Systems, such as microorganism (Beekwilder *et al*., 2006; Katsuyama *et al*., 2007; Watts *et al*., 2004; Watts *et al*., 2006; Xie *et al*., 2006), mammal cells (Zhang *et al*., 2006) and plants (Schijlen *et al*., 2006), for the production of plant polyketides have been developed. Improvement of plant microbial resistence (Hipskind and Paiva, 2000; Hui *et al*., 2000; Serazetdinova *et al*., 2005; Stark-Lorenzen *et al*., 1997; Szankowski *et al*., 2003), quality of crops (Husken *et al*., 2005; Kobayashi *et al*., 2000; Morelli *et al*., 2006; Ruhmann *et al*., 2006) or sometimes to give plant specific traits such as color (Aida *et al*., 2000; Courtney-Gutterson *et al*., 1994; Deroles *et al*., 1998; Elomma *et al*., 1993; van der Krol *et al*., 1988) or sterility (Fischer *et al*., 1997; Höfig *et al*., 2006; Taylor and Jorgensen, 1992) are also reported by expression or antisense expression from plant PKSs. Further (novel) polyketides will be produced in the future as well as more PKSs and polyketides will be discovered in nature (Wilkinson and Micklefield, 2007).

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