# Search for Genes Involved in Anthraquinone Biosynthesis in *Galeruca tanaceti*

## Polyketide synthases (PKS)

Two major biosynthetic pathways are described for production of anthraquinones. The shikimate pathway is used to synthesise anthraquinone with substituents like hydroxyl or methyl groups only on one ring, whereas the polyketide pathway is used to produce anthraquinones with both rings substituted (Han et al. 2001) (see Chapter 1, Fig. 1B + C). The 1,8-dihydroxylated anthraquinones chrysazin and chrysophanol and their respective precursors, the anthrones dithranol and chrysarobin, found in Galerucini leaf beetles like the tansy leaf beetle (*Galeruca tanaceti*) (Hilker et al. 1992; Pankewitz and Hilker 2006) are typical candidates for biosynthesis *via* the polyketide pathway. Enzymes responsible for polyketide biosynthesis are polyketide synthases (PKS).

So far, PKS have been found in a wide range of organisms. A lot of bacteria, fungi and plants possess polyketides and the respective enzymes (Staunton and Weissman 2001). As described in Chapter 7, PKS can be divided into three major types of polyketide synthases (Staunton and Weissman 2001). Type I PKS are multifunctional enzymes containing domains for different catalytic activities (Hopwood 1997). Bacterial type I PKS possess multiple sets of domains (modules), and each module catalyses only one elongation step and the following reduction step on a $C_2$ residue. A typical polyketide produced by a type I PKS in bacteria is, for example, the antibiotic erythromycin. In contrast, fungal type I PKS act iteratively meaning that each elongation step is carried out on the same module. Fungal products produced by PKS I are, for example, norsorolinic acid which is the intermediate for the mycotoxin aflatoxin or 6-methylsalicylic acid. In contrast, type II PKS are multi-enzyme complexes containing each catalytic site on a separate polypeptide, synthesising mostly aromatic products such as actinorhodin. Type III PKS are homodimeric enzymes

acting iteratively and contain only a ketoacyl synthase (KS) (Staunton and Weissman 2001). A typical type III PKS product in bacteria is, for example, flaviolin. Type III PKS in plants are, for example, responsible for the production of chalcones as precursors of flavonoid biosynthesis. In fungi, so far no metabolite of type III PKS origin has been found, but it is suggested that plant flavonoid-like metabolites are synthesised (Seshime et al. 2005). However, a lot of studies reported findings that do not match this classification of PKS into three types (Shen 2003). Type I PKS in bacteria are not restricted to noniterative activity, since in some bacteria species a type I PKS acting iteratively was found. Also the occurrence of type III PKS is not restricted to plants. This type was additionally found in bacteria and recently also in fungi (see Chapter 7). As described in Chapter 7, aromatic compounds like anthraquinones can possibly be produced by all three types of PKS (Hopwood 1997; Austin and Noel 2003).

The results described in Chapter 2 and 3 support the hypothesis that anthraquinones in the tansy leaf beetle are produced by the beetle itself. With chemical methods using NMR techniques it could clearly be demonstrated that the anthraquinone chrysophanol is produced by an eukaryotic organism meaning that either the beetle itself or an endosymbiotic fungus produces the anthraquinones (Chapter 4; Bringmann et al. 2006). Summarising all the results described in Chapters 2 - 4, a production of the polyketidic anthraquinones by the beetle itself is very likely, since (a) neither bacterial nor fungal DNA was found in the eggs which are thought to be the vertical transfer site of putative endosymbiotic microorganisms in the tansy leaf beetle, and (b) the beetles still produce anthraquinones after treatment with antibiotics and antimycotics.

Genes encoding PKS should be present in the tansy leaf beetle – either within the beetle genome, as argued above – or in endosymbiotic microrganisms. For example, the polyketide pederin was supposed to be produced by a bacterial symbiont in the staphylinid beetles *Paederus* spec., and indeed, bacteria similar to *P. aeruginosa* were detected. The search for PKS genes resulted in isolation of a PKS cluster. Flanking regions of this cluster showed a prokaryotic architecture supporting the previous findings (Piel 2006). So far, no PKS genes have been detected in the genome of an insect or other animals. Thus, the search for PKS genes is difficult, because no animal PKS gene sequences are available for the design of primers to amplify putative parts of PKS genes in the tansy leaf beetle. A

detailed discussion on possible PKS genes that might be present in insects and some implications on polyketides and their origin are given in Chapter 7.

To design primers for putative PKS genes in the tansy leaf beetle, we chose sequences of published PKS genes. Our choice was based on the criteria outlined below.

(1) We looked mostly for PKS genes encoding enzymes catalysing the biosynthesis of polyketidic aromatic compounds (if possible, aromatics similar to anthraquinones).

(2) Furthermore, primers were used that have previously been described for amplification of PKS genes from microorganisms associated to insects. The reasons for the choice of the individual primers used are mentioned below.

(3) The ketosynthase (KS) domain is highly conserved when compared to other domains/ enzymes within a PKS cluster making it easier to use PCR primers and to amplify fragments of potential PKS genes (Hopwood 1997; Lee et al. 2001). Additionally, the KS region is the only domain/ enzyme present in all three types of PKS and is therefore used also in this study to detect PKS genes in eggs and larvae of the anthraquinone containing tansy leaf beetle.

(4) Primers for all three types of PKS genes were used to search for PKS genes in *G. tanaceti* since no proof is available that aromatic polyketides such as anthraquinones are produced by a specific type of PKS, as outlined above.

The first search for PKS in *G tanaceti* was done with genomic DNA from eggs. Eggs were chosen because PKS genes should be present in the eggs when the beetle is producing the anthraquinones itself, and even if the anthraquinones are produced by a so far undetectable microorganisms, we expect the endosymbionts also in the eggs, as argued above and in Chapter 1. Prior to DNA preparation the eggs were surface sterilised with sodium hypochloride, and DNA was extracted using the method described in Chapter 2. These DNA samples were used for different PCRs with the primers mentioned in table 1.

**Table 1.** Primers used to amplify a part of the ketosynthase (KS) domain/ enzyme of different types of polyketide synthases (PKS). S = G/ C, Y = C/ T, W = A/ T, R = A/ G, M = A/ C, K = G/ T, B = C/ G/ T, D = A/ G/ T, N = A/ C/ G/ T, I = Inosin. [a] Primer designed using b = bacterial, f = fungal, pl = plant, I = type I PKS, II = type II PKS, III = type III PKS.

| Primer (PKS type)[a] | Sequences (5´ to 3´) | Paired with | Reference |
|---|---|---|---|
| KSDPQQF (b I) | MGN GAR GCN NWN SMN ATG GAY CCN CAR CAN MG | KSHGTGR | Piel 2002 |
| KSHGTGR (b I) | GGR TCN CCN ARN SWN GTN CCN GTN CCR TG | KSDPQQF | |
| LC1 (f I) | GAY CCI MGI TTY TTY AAY ATG | LC2c | Bingle et al. 1999 |
| LC2c (f I) | GAR ATG CAY GGI ACI GGI AC | LC1 | |
| LC3 (f I) | GCI GAR CAR ATG GAY CCI CA | LC5c | Bingle et al. 1999 |
| LC5c (f I) | GAR GCI CAY GCI ACI TCI AC | LC3 | |
| KS1b (f I) | GGR CCC AGY III AIY RWT GAY ACI GCI TG | AT1b | Couch & Gaucher 2004 |
| AT1b (f I) | GCR WAK TCI CCM ARA CTA TGI CC | KS1b | |
| PKSII_for (b II) | TSG CST GCT TCG AYG CSA TC | PKSII_rev | Metsä-Ketelä et al. 2002 |
| PKSII_rev (b II) | TGG AAN CCG CCG AAB CCG CT | PKSII_for | |
| KSIII_fwd (b III) | TCG CTS CTS TCG AAC GGC CTS TTC GGC GAC GCS CTS TCG GC | KSIII_rev | Cortés et al. 2002 |
| KSIII_rev (b III) | CTC SGC GGT GAT SCC GGG SCC GAA GCC SGC GAT SAG GC | KSIII_fwd | |
| 174S-PKS (pl III) | GCI AAR GAY ITI GCI GAR AAY AA | 368A-Pks | Abe et al. 2005 |
| 368A-Pks (pl III) | CCC MWI TCI ARI CCI TCI CCI GTI GT | 174S-PKS | |
| 112S-PKS (pl III) | RAR GCI ITI MAR GAR TGG GGI CA | 380A-PKS | Abe et al. 2005 |
| 380A-PKS (pl III) | TCI AYI GTI ARI CCI GGI CCR AA | 112S-PKS | |
| dPKS1-f1 (f I) | TCN NNN NNN NNN GAY ACR GCN TGY TC | UPM | |
| dPKS1-f2 (f I) | GCN DMN GGN TAY KSN CGN GSN GAR | UPM | |
| dPKS1-f3 (f I) | DTN GAR ATG CAY GGN ACN GGN AC | UPM | |
| dPKS1-f4 (f I) | ACR GCN TGY TCN TCN TCN YTN GTN | UPM | |
| dPKS1-r1 (f I) | NCC NGC NNN NGT NCC NGT NCC RT | UPM | |
| dPKS1-r2 (f I) | NGC YGT RTC NNN NNN NNN NGA NGG NC | UPM | |
| UPM | CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACA ACG CAG AGT | | |

All primers used amplify a part of the KS region of polyketide synthases. The primer pair KSDPQQF/ KSHGTGR was used by Piel (2002) to amplify a part of bacterial type I PKS responsible for pederin biosynthesis in staphylinid beetles. The pederin PKS was the first and only polyketide synthase identified so far in an endosymbiont of a beetle. The primer pairs LC1/ LC2c and LC3/ LC5c were used by Bingle and coworkers (1999) to amplify a fragment of the KS region of fungal type I PKS and were also used for detection of PKS genes in fungi associated with insects and nematodes (Lee et al. 2001). The KS1/ AT1 primers are the only primers used that do not only amplify the KS region, but also a part of the adjacent acyltransferase (AT) gene in fungal type I PKS. Primers were designed in the relatively conserved active sites of both enzymatic domains (Couch and Gaucher 2004). Couch and Gaucher used this primer pair to detect an emodin anthrone PKS catalysing a step in the biosynthetic pathway leading to sulochrin. Since the anthraquinone emodin is structurally very similar to the anthraquinones found in Galerucini, these primers were chosen. Type II PKS are known from bacteria, and therefore only one primer pair was used for this type of PKS, since a prokaryotic anthraquinone producer in *G. tanaceti* can be excluded by the chrysophanol NMR measurements mentioned above. PKS II_for/ PKS II_rev primers bind to KS sequences of *Streptomyces* species producing aromatic polyketides (Metsä-Ketelä et al. 2002). Because of the ability to produce aromatic compounds, these primers were selected. Three primer pairs were chosen for the amplification of type III PKS. First the KS III_fwd/ KS III_rev combination described by Cortés and coworkers (2002) was used amplifying a bacterial type III PKS producing a deep red aromatic polyketide naphthoquinone in *Saccheropolyspora erythraea*. The primer pairs 174S-PKS/ 368A-PKS and 112S-PKS/ 380A-PKS have been designed for type III PKS in plants. Abe et al. (2005) described a so called octaketide (like the anthraquinones) synthase in aloe (*Aloe arborescens*). This octaketide synthase is responsible for the production of “chrysophanol anthrone” (Abe et al. 2005).

With none of the above described primer combinations (Tab. 1) using DNA from tansy leaf beetle eggs a PKS gene could be detected.

Since no anthraquinone production was found in the overwintering eggs of *G. tanaceti* (Chapter 5), further search for PKS genes was done with RNA isolated from larvae of the tansy leaf beetle. First, total RNA was extracted from 100 *G tanaceti* larval fat bodies using the Invertebrate RNA kit (Peqlab Biotechnologie GmbH). The mRNA was

subsequently extracted with Oligotex® mRNA Mini Kit (Qiagen GmbH), and the Smart™ RACE cDNA amplification kit was used to convert the mRNA into 5´-RACE ready cDNA and 3´-RACE ready cDNA. This cDNA was used for PCR using the above described primer pairs LC1/ LC2c, LC3/ LC5c, KS1b/ AT1b, PKSII_for/ PKSII_rev, 174S-PKS/ 368A-PKS and 112S-PKS/ 380A-PKS (Tab. 1). Additionally, 3´-RACE reactions were carried out using the primers dPKS1-f1-4, and 5´-RACE using the primers dPKS1-r1 and dPKS1-r2 in combination with the universal primer UPM binding on the sequence introduced by Smart RACE kit components (Tab. 1). These primers were created using relatively conserved regions in the alignment presented by Kroken et al. (2003) of sequences from type I PKS. The alignment contained the above described KS domain of the PKS (Kroken et al. 2003).

Also when analysing cDNA from larvae of the tansy leaf beetle, no PKS genes could be found. Since anthraquinones like chrysophanol are produced in larvae (Bringmann et al. 2006), and such anthraquinones are typical for the polyketide pathway, genes encoding PKS should be present in larvae. The so far unsuccessful search for PKS genes might be due to the fact that the primers designed on the base of known bacterial, fungal or plant KS were too specialised and therefore could not bind to potential PKS genes in the tansy leaf beetle.

## Anthrone oxygenases

To detect genes encoding enzymes responsible for the biosynthesis of anthraquinones and anthrones, not only the above mentioned PKS genes need consideration. After synthesis of the polyketide chain several other enzymes, so called post-PKS enzymes, are modifying the polyketide chain (Rix et al. 2002). A class of these post-PKS enzymes are oxygenases. A very interesting oxygenase in respect to anthraquinone biosynthesis is the anthrone oxygenase (AknX) identified in *Streptomyces galilaeus* (Chung et al. 2002). This anthrone oxygenase catalyses the conversion of the polyketide aklanonic acid anthrone to aklanonic acid (Fig. 1A). Chung et al. (2002) could demonstrate that the anthrone oxygenase also can use emodinanthrone as substrate, and the anthrone is effectively oxidised to the corresponding anthraquinone emodin (Fig. 1B). Since emodin and the anthraquinones chrysazin and chrysophanol found in Galerucini are structurally closely related, an

anthrone oxygenase might also catalyse in the beetles the oxidation of anthrones to anthraquinones. The anthrones dithranol and chrysarobin are present in eggs of Galerucini (Hilker et al. 1992; Pankewitz and Hilker 2006). Their oxidation leads to chrysazin and chrysophanol, respectively.

Two problems are present when searching for similar anthrone oxygenases in the tansy leaf beetle: (1) This anthrone oxygenase is found in the prokaryotic *Streptomyces galilaeus* having another composition of DNA with a high GC content, and no other sequence of such an oxygenase is known in animals, and (2) the oxidation step from anthrones to anthraquinones is not necessarily catalysed by an oxygenase, since a spontaneous oxidation without any enzyme is also possible.

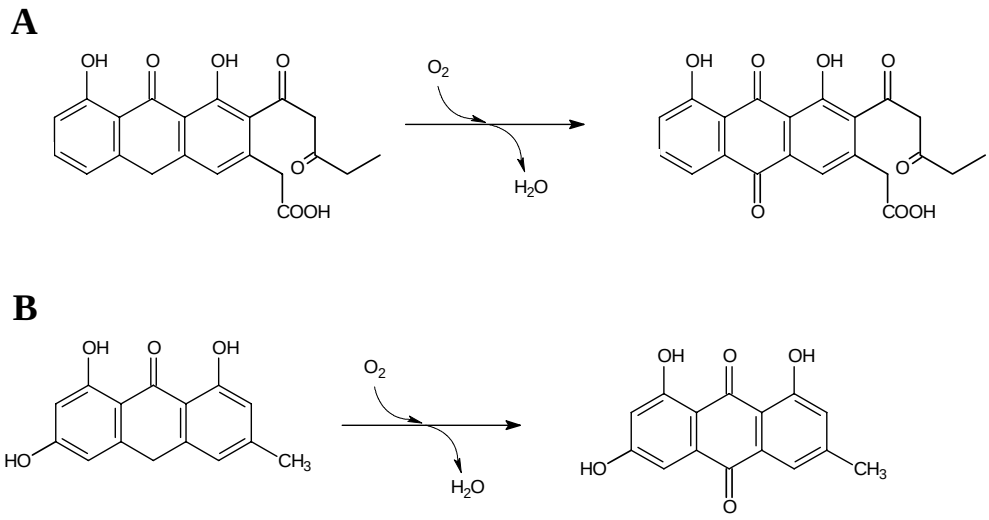


**Figure 1.** Reactions catalysed by the anthrone oxygenase AknX. **A.** Oxidation of aklanonic acid anthrone to aklanonic acid. **B.** Oxidation of emodinanthrone to the anthraquinone emodin (both from Chung et al. 2002).

To design primers for the search for anthrone oxygenases, the known sequence of the AknX gene (AB008466) was aligned with the similar oxygenases *snoaB* (AJ224512) of *S. nogalater* and *frnU* (AF058302) of *S. roseofulvus* using Clustal X (Chung et al. 2002). The gene bank accession numbers of the different genes are given in brackets. Conserved sequence regions were used to create the primers Anthrone_f (5´-TGY DSC AST GGW ASY AST TG-3´) and Anthrone_r (5´-SGS TSK BGS YCW SGG CSC GC-3´) [S = G/ C; Y = C/ T; W = A/ T; K = G/ T; B = C/ G/ T; D = A/ G/ T]. These primers were used both

with genomic DNA extracted from eggs of *G. tanaceti* as well as with 5´-RACE ready cDNA and 3´-RACE ready cDNA obtained from larvae of the tansy leaf beetle.

With none of the two DNA types a PCR product could be yielded. As mentioned above, sequences for primer design of the anthrone oxygenase were only from prokaryotic organisms, but the anthraquinones in the tansy leaf beetles are not produced by prokaryotes (Chapter 4; Bringmann et al. 2006). Therefore, the unsuccessful search for an anthrone oxygenase gene in *G. tanaceti* might be due to the specificity of the used primers not amplifying eukaryotic oxygenases or the lack of such an oxygenase in the beetle's genome.

Future studies should focus on the design of further primers based on eukaryotic PKS genes like the fungal type I PKS and both the fungal and plant type III PKS. Search for PKS genes should continue to use RNA extracted from larvae since it is known that anthraquinones are produced during larval stages. Since the anthraquinone chrysophanol found in fungi, plants and the beetles is formed by the same eukaryotic folding mode (Bringmann et al. 2006), beetles might possess PKS genes similar to the ones in fungi and plants for anthraquinone production. Thus, if a sequence of fungal or plant PKS for chrysophanol synthesis will be detected and described in the literature, primers targeting this PKS should be used with RNA extracted from Galerucini larvae. As described here, the primer design should focus on the KS domain that is relatively conserved when compared to the genes for other enzymes/ enzymatic domains involved in polyketide production. Chapter 7 provides further information on PKS, their relatedness to fatty acid synthases (FAS), and the importance of PKS and FAS for insect polyketide biosynthesis.

## References

Abe, I, Oguro, S, Utsumi, Y, Sano, Y, Noguchi, H (2005). Engineered biosynthesis of plant polyketides: Chain length control in an octaketide-producing plant type III polyketide synthase. J. Am. Chem. Soc. 127:12709-12716.

Austin, MB, Noel, JP (2003). The chalcone synthase superfamily of type III polyketide synthases. Nat. Prod. Rep. 20:79-110.

Bingle, LEH, Simpson, TJ, Lazarus, CM (1999). Ketosynthase domain probes identify two subclasses of fungal polyketide synthase genes. Fungal Genet. Biol. 26:209-223.

Bringmann, G, Noll, TF, Gulder, TAM, Grüne, M, Dreyer, M, Wilde, C, Pankewitz, F, Hilker, M, Payne, GD, Jones, AL, Fiedler, H-P (2006). Different polyketide folding modes converge to an identical molecular architecture. Nat. Chem. Biol. 2:429-433.

Chung, J-Y, Fujii, I, Harada, S, Sankawa, U, Ebizuka, Y (2002). Expression, purification, and characterization of AknX anthrone oxygenase, which is involved in aklavinone biosynthesis in *Streptomyces galilaeus*. J. Bacteriol. 184:6115-6122.

Cortés, J, Velasco, J, Foster, G, Blackaby, AP, Rudd, BAM, Wilkinson, B (2002). Identification and cloning of a type III polyketide synthase required for diffusible pigment biosynthesis in *Saccharopolyspora erythraea*. Mol. Microbiol. 44:1213-1224.

Couch, RD, Gaucher, GM (2004). Rational elimination of *Aspergillus terreus* sulochrin production. J. Biotechnol. 108:117-178.

Han, Y-S, Van der Heijden, R, Verpoorte, R (2001). Biosynthesis of anthraquinones in cell cultures of the Rubiaceae. Plant Cell Tiss. Org. 67:201-220.

Hilker, M, Eschbach, U, Dettner, K (1992). Occurence of anthraquinones in eggs and larvae of several Galerucinae (Coleoptera: Chrysomelidae). Naturwissenschaften 79:271-274.

Hopwood, DA (1997). Genetic contributions to understanding polyketide synthases. Chem. Rev. 97:2465-2497.

Kroken, S, Glass, NL, Taylor, JW, Yoder, OC, Turgeon, BG (2003). Phylogenomic analysis of type I polyketide synthase genes in pathogenic and saprobic ascomycetes. Proc. Natl. Acad. Sci. U.S.A. 100:15670-15675.

Lee, T, Yun, S-H, Hodge, KT, Humber, RA, Krasnoff, SB, Turgeon, GB, Yoder, OC, Gibson, DM (2001). Polyketide synthase genes in insect- and nematode-associated fungi. Appl. Microbiol. Biotechnol. 56:181-187.

Metsä-Ketelä, M, Halo, L, Munukka, E, Hakala, J, Mäntsälä, P, Ylihonko, K (2002). Molecular evolution of aromatic polyketides and comparative sequence analysis of polyketide ketosynthase and 16S ribosomal DNA genes from various *Streptomyces* species. Appl. Environ. Microbiol. 68:4472-4479.

Pankewitz, F, Hilker, M (2006). Defensive components in insect eggs: Are anthraquinones produced during egg development? J. Chem. Ecol. 32:2067-2072.

Piel, J (2002). A polyketide synthase-peptide synthetase gene cluster from an uncultured bacterial symbiont of *Paederus* beetles. Proc. Natl. Acad. Sci. U.S.A. 99:14002-14007

Piel, J (2006). Bacterial symbionts: Prospects for the sustainable production of invertebrate-derived pharmaceuticals. Curr. Med. Chem. 13:39-50.

Rix, U, Fischer, C, Remsing, LL, Rohr, J (2002). Modification of post-PKS tailoring steps through combinatorial biosynthesis. Nat. Prod. Rep. 19:542-580.

Seshime; Y, Juvvadi, PR, Fujii, I, Kitamoto, K (2005). Discovery of a novel superfamily of type III polyketide synthases in *Aspergillus oryzae*. Biochem. Bioph. Res. Co. 331:253-260.

Shen, B (2003). Polyketide biosynthesis beyond the type I, II and III polyketide synthase paradigms. Curr. Opinion Chem. Biol. 7:285-295.

Staunton, J, Weissman, KJ (2001). Polyketide biosynthesis: A millennium review. Nat. Prod. Rep. 18:380-416.