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Full Length Research Paper

Molecular analysis of type III polyketide synthase (PKS) gene family from *Zingiber officinale* Rosc

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Enzymes of the type III polyketide synthase family is considered to have significant role in biosynthesis of structurally diverse polyketide scaffolds in *Zingiber officinale*. Genome wide analysis of polyketide synthase gene family in *Z. officinale* identified partial sequences of six members. Comparative sequence analysis showed that four of them ZoPKS2, 3, 4 and 6 were novel forms as revealed by the significant variations at the highly conserved regions. Phylogenetic analysis also showed separate clustering of the novel forms along with the non-chalcone forming PKSs. This sequence identified in the study forms the first and basic information of the PKS gene family from the Zingiberaceae.

Key words: *Zingiber officinale*, CHS, PKS, multigene family, phylogenetic analysis.

INTRODUCTION

Zingiber officinale Rosc. (Ginger), the well known spice contains several nonvolatile constituents responsible for its characteristic pungent flavor as well as pharmacological properties. The medicinal properties of gingerol and other related pungent vanilloid compounds of *Z. officinale* had been studied extensively (Lee and Surh, 1998; Fuhrman et al., 2000). But from the biosynthetic point of view the formation of these compounds remains to be elusive. [6]-Gingerol has been shown to be synhesized from ferulic acid, malonate and hexanoyl moieties by precursor feeding experiments (Denniff et al., 1980). The proposed biosynthesis highlights the presence of novel forms of type III polyketide synthase(s) enzymes in *Z. officinale*. Recent identification of polyketide synthase with the curcuminoid synthase activity further supports the presence of polyketide synthase with possible gingerol synthase activity in *Z. officinale* (Katsuyama et al., 2007).

Enzymes of type III polyketide synthase (PKS) super family including the well studied chalcone synthases (CHSs) play very significant role in the biosynthesis of plant polyketide derivatives and they exist as multi-gene family in most plants (Koes et al., 1987, Ryder et al., 1987). The rice genome has shown to have PKS family with more than 30 members indicating that PKSs can be involved in diverse biochemical process (Katsuyama et al., 2007). The typical PKS reaction as explained for the prototype chalcone synthase involve the condensation of one molecule of 4- coumaroyl CoA with three malonyl CoA leading to the formation of naringenin chalcone.

In addition to the typical CHSs, the PKS super family also includes functionally divergent members like 2-pyrone synthase, benzalacetone synthase, stilbene synthase, valerophenone synthase, etc. They differ in the starter molecule selectivity, number of chain extension and also in the mechanism of cyclisation.

In general PKSs are homodimeric proteins of approximately 40 – 45 kDa with a catalytic triad Cys-His-Asn at the active site. Structural insight into the PKS reaction mechanism as elucidated for the CHS from the *Medicago sativa* indicates the presence of three inter connected cavities; the CoA binding tunnel, a coumaroyl binding pocket and a cyclisation pocket at the active site (Ferrer et al., 1999).

The shape and volume of the active site cavity determines the substrate specificity, chain length, folding and cyclisation and all of these are in turn depend on the amino acid composition of specific PKS. The second exon of PKS contribute to most of the active site residues and has well conserved stretches of amino acid residues in most PKSs. This facilitates the use of degenerate oligonucleotide primer based PCR methods for the genomic exploration of PKS super family from plants. Similar approach was successfully used for the identification of novel members of the PKSs from other plants as in the case of benzalacetone synthase from *Rheum palmatum*

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Table 1. List of polyketide synthase genes selected for the phylogenetic analysis from NCBI with the corresponding accession number

Species	Accession number
Sorghum bicolor CHS	AY069951
Camellia sinensis CHS	D26593
Ipomoea alba CHS	AY257214
Lilium hybrid cv. 'Acapulco' CHS	AF169798
Dictamnus albus CHS	AJ850132
Vitis vinifera CHS	AF020709
Callistephus chinensis CHS	Z67988
Daucus carota CHS	AJ006780
Petroselinum crispum CHS	V01538
Glycine max CHS	DQ239918
Arabis alpina CHS	AAF23559
Rorippa amphibia CHS	AAG43348
Cardamine amara CHS	AAF23560
Bromheadia finlaysoniana CHS	O23731
Petunia x hybrida CHS	CAA32733
Sinapis alba CHS	CAA34460
Hordeum vulgare CHS	CAA41250
Triticum aestivum CHS	AY286098
Zea mays CHS	AAW56963
Cardamine flexuosa CHS	AAT96398
Ipomoea trifida CHS	AAY58168
Ipomoea batatas CHS	Q9MB40
Bromheadia finlasoniana CHS	O23729
Oryza sativa CHS	ABA94123
Oryza sativa CHS	CAA61955
Brassica napus CHS	AAC31911
Medicago sativa CHS	L02901
Psilotum nudum VS**	AB022683
Hydrangea macrophylla CTAS**	AB011468
Humulus lupulus VS**	AB047593
Gerberahybrida 2PS**	Z38097
Streptomyces griseus RppA**	AB018074
Escherichi coli FABH+	M96793
Rheum palmatum BAS**	AF326911
*Phalaenopsis sp.*BBS**	X79903

CHS- typical chalcone synthase; ** Non-chalcone forming polyketide synthases; + Out group used for the phylogenetic analysis

and type III polyketide synthase from *Wachendorfia thyrsiflora* (Abe et al., 2001; Brand et al., 2001).

In the present study, genome wide analysis of PKS super family was carried out in *Z. officinale* by PCR based method. This is the first report of the sequence information of PKS family containing putative chalcone forming and the non-chalcone forming members from *Z. officinale*.

MATERIALS AND METHODS

Z. officinale Rosc. var. Riode Janeiro collected from the Agriculture College, Vellayani, Thiruvananthapuram, Kerala was used for the experiment. DNA isolation was carried out from young leaves by CTAB method (Murray and Thompson, 1980). Inosine containing degenerate oligonucleotide primers based on the highly conserved sequences of known PKSs were used for amplification of a core fragment using genomic DNA as the template (Abe et al., 2001). The sequences of the primers used for the study were: CHS1 5'-RARGCIITIMARGARTGGGGICA-3', CHS2 5'-GCIAARGAYITI-GCIGARAAYAA-3', CHS35'-CCCMWITCIARICCITCICCIGTIGT-3' and CHS4 5'-TCIAYIGTIARICCIGGICCRAA-3'.

The PCR reaction (50 µL) contained 100 ng of genomic DNA, 30 pmoles of each primer, 1.25 units of *Taq* DNA polymerase (Promega), 1.5 mM $MgCl_2$ and 200 µM dNTPs. PCR was carried out for 35 cycles in a Bio-Rad iCycler using the following conditions: initial denaturation was given for 3 min at 94°C, the cyclic conditions were denaturation at 94°C for 30 sec, annealing at 42°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 7 min. Primary PCR was carried out using the primers CHS1 and CHS4 followed by nested PCR with primers CHS2 and CHS3 using 1 µL of the primary PCR product as the template.

The PCR product about (560 bp) was analyzed in a 2% agarose gel and the gel-purification was carried out using the GFX gel band purification system (Amersham). The purified fragment was ligated to pGEMT easy vector (Promega) and propagated in *Escherichia* strain JM109 (Promega). Plasmid isolation and purification was done using the Wizard plus SV Minipreps DNA purification system (Promega). Screening of 70 clones was conducted by sequencing using Big Dye terminator sequencing kit (Perkin Elmer) in an ABI-310 Prism automated sequencer. Sequence similarity was analyzed by the Multi Align programme (Corpet, 1988).

The nucleotide sequences were translated to amino acid sequences using Bioedit programme (Hall, 1999). For the phylogenetic analysis, the sequences were first aligned at the amino acid level using Clustal W (Thompson et al., 2004). A total of 41 sequences (Table 1) of the PKS super family including the six sequences from *Z. officinale* were used for the reconstruction of phylogeny. This also included *Escherichia coli* FABH as the out group and also seven other PKSs as representatives of the newly emerging non chalcone forming or CHS- like PKSs.

The phylogenetic relationships were carried out by Neighbor-Joining (NJ) analysis using the Poisson correction implemented in the MEGA3.1 (Kumar et al., 2004). Gaps in the amino acid sequences were treated as missing data. Bootstrapping of 1000 pseudo replicates was used to examine the robustness of the lineages.

RESULTS AND DISCUSSION

It is very likely, that the evolutionary changes on genes of the secondary metabolism are a key factor responsible for the metabolite richness and diversity of *Z. officinale*. Identification and characterization of plant specific or metabolite specific PKSs from other plants support the presence of novel PKS(S) in *Z. officinale* for the biosynthesis of the diketide derived gingerol precursor. This is because the structural and functional requirement for the PKS in *Z. officinale* to accept the feruloyl, malonyl and hexanoyl moieties is considered to be very different when

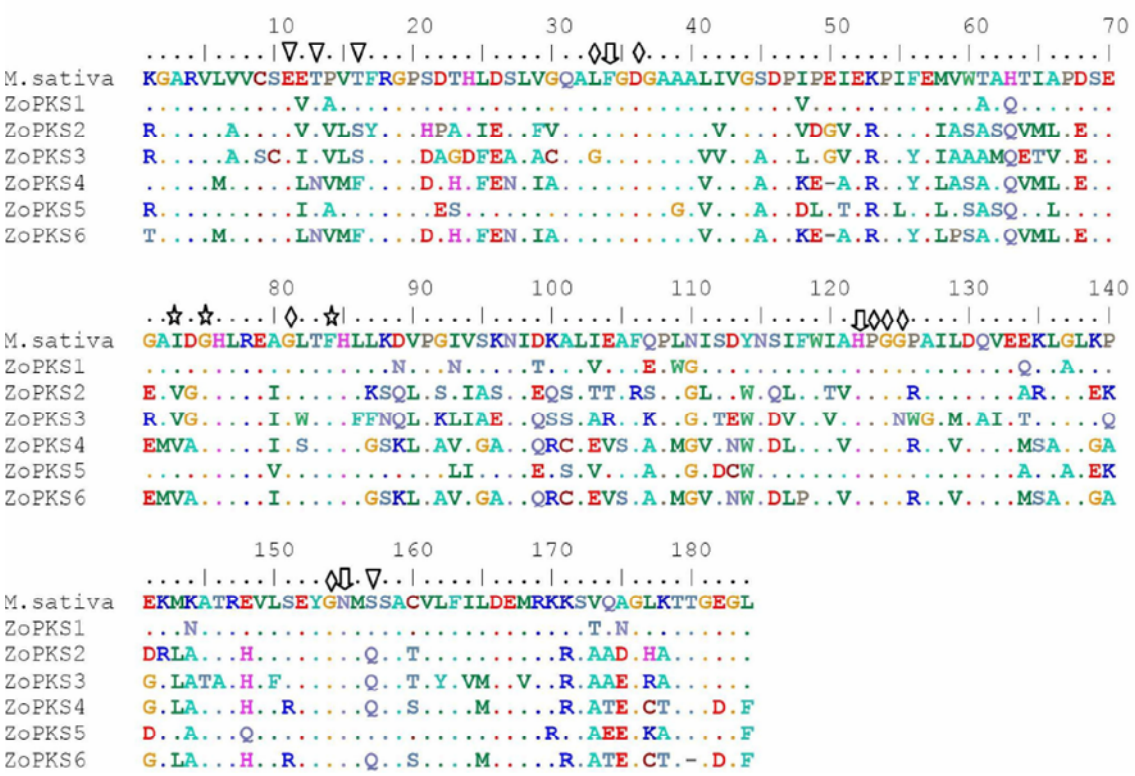

Figure 1. Comparative analysis of PKS family from *Z. officinale* with CHS from *M. sativa*. The nucleotide sequence were translated to corresponding amino acid sequence and was used for the comparison. This region corresponds to the 182-366aa region of typical PKS. The amino acid residues forming the active site (⇩), substrate binding pocket (▽), cyclisation pocket (☆) and pocket to shape the active site (◊) are shown in the alignment.

compared to the typical reaction where coumaroyl and malonyl units is used as the substrates.

Studies on catalytically divergent members of the PKS super family show that changes on functionally significant key amino acid residues enable them to produce various products by altered reaction mechanism or substrate specificity. So a comparative sequence analysis of PKSs can give gateway information for making predictions about their putative function or at least to differentiate them as typical chalcone forming or novel forms. Structural studies on CHS from *M. sativa* show that four amino acid residues (Cys164, Phe 215, His303 and Asp336) are conserved at the active site of all members of the CHS family (Ferrer et al., 1999). Conserved amino acids are also present at the coumaroyl CoA binding pocket (Ser133, Glu192, Thr194, Thr197 and Ser338) and cyclisation pocket (Thr132, Met137, Phe215, Ile254, Gly256, Phe265 and Pro375).

In addition to this, certain amino acids (Pro138, Gly163, Gly167, Leu214, Asp217, Gly262, Pro304, Gly305, Gly306 Gly335, Gly374, Pro375 and Gly376) are also essential to shape the active site of CHS. A strong conservation of amino acid residues at these positions can lead to the typical chalcone forming reaction and the variation results in non-chalcone type products.

Amplification of the core fragment of PKS gene family from *Z. officinale* resulted in the formation of 562 bp product corresponding to the second exon. More specifically this represents the 182 - 366 amino acid regions of the typical PKS (the length of full protein is generally 389aa). Also this region contains most of the residues forming the catalytic machinery of PKSs. Comparative sequence analysis of PKS clones from *Z. officinale* identified six sequentially different clones and are represented as ZoPKS1-6. The sequence were submitted to NCBI under the following accession numbers AY509195 (ZoPKS1), AY873969 (ZoPKS2), DQ851164 (ZoPKS3), DQ089697 (ZoPKS4), DQ851166 (ZoPKS5) and DQ851165(ZoPKS6).

A detailed comparative analysis of all the six PKSs show that the two PKSs like the ZoPKS1 and ZoPKS5 satisfy all the requirements both at position and also by the nature of amino acid residues for the typical chalcone synthases (Figure 1). So these two forms are very likely to have typical CHS function in *Z. officinale*. But other PKSs like ZoPKS2, ZoPKS3, ZoPKS4 and ZoPKS6 have very remarkable variation on amino acid residues corresponding to the substrate binding region and other functionally significant areas suggesting novel or non-chalcone type function which make them to be consider-

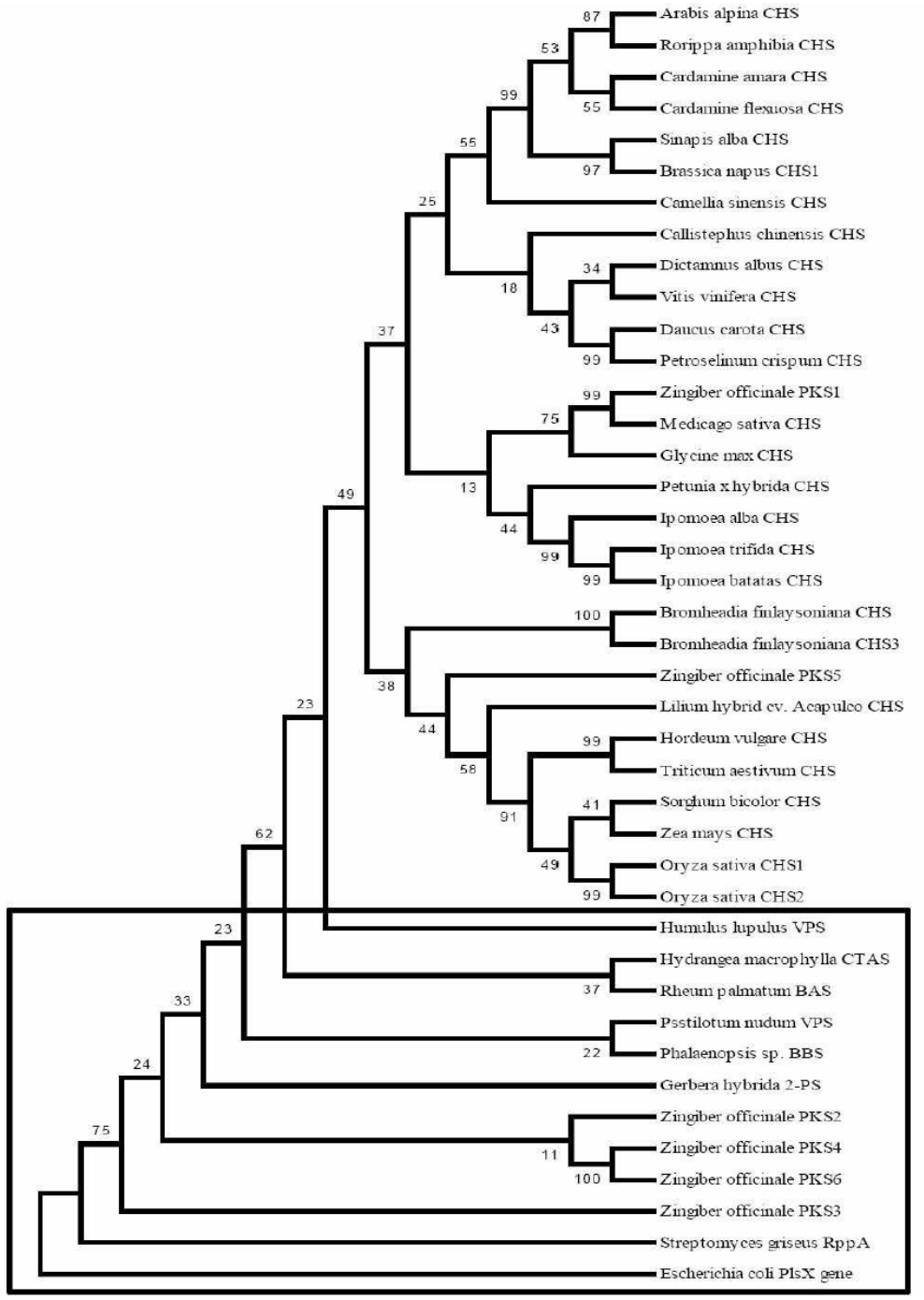

Figure 2. Phylogenetic analysis of PKS family from *Z. officinale* with other PKSs. Among the different PKSs from *Z. officinale*, two of them like the *Z. officinale* PKS1 and 5 forms cluster with the typical chalcone forming PKSs and rest of them clusters with non chalcone forming PKSs.

ed novel PKSs.

The amino acid residues present at the active site of all known members of PKS super family like Phe (215), His (303) and Asn (336) are conserved in all six forms.

Among the amino acids required for the Coumaroyl CoA binding like the Glu (192), Thr (194), Thr (197) and Ser (338) the PKS family of *Z. officinale* shows remarkable variations.

The Threonine at 194 are replaced by Asparagine in ZoPKS4 and ZoPKS6 and Threonine at 197 is replaced by Serine in ZoPKS2 and ZoPKS3 and by Phenylalanine in ZoPKS4 and ZoPKS6. Serine at 338 positions is re-

placed by Glutamine in ZoPKS2, 3, 4 and 6. Among the amino acids for the cyclisation (Phe (215), Ile (254), Gly (256), Phe (265) and Pro 375), Ile at 254 position is replaced by Valine in the ZoPKS2, ZoPKS3, ZoPKS4 and ZoPKS6.

Among the residues, which shape the active site geometry, all the amino acid residues are conserved in all the six forms except Leu 214 and Gly 306. These are replaced by Glycine at 214 position and asparagines at 306 positions in ZoPKS3. All the numbering and comparison was made in relation to the information from the structural details of CHS from *M.sativa.* The results obtained from the phylogenetic analysis also reveal distinct clustering of PKSs from *Z. officinale*.

In the phylogenetic analysis ZoPKS1 and ZoPKS5 forms cluster with typical chalcone synthases (Figure 2). But the rest of PKS form a cluster with non-chalcone forming PKS. This also supports the novelty of the PKSs and also the probable functional divergence. Gene duplication and subsequent selection might have played an important role in occurrence of sequentially diverse PKSs in *Z. officinale*. As a plant rich in structurally diverse metabolites of the polyketide origin novel forms of PKSs can be greatly expected in *Z. officinale.*

The current study shows that the PKS gene family in *Z. officinale* contains at least six members and sequence data generated in the study forms a basis for further studies. The identification of benzalacetone synthase from *R. palmatum* and type III polyketide synthase from *W. thyrsiflora* proves importance of novel PKSs in plant metabolism. The novel forms of PKSs identified in the current study is having significant value in relation to ginger and gingerol and it is very probable that some members of the novel PKSs can have gingerol synthase activity but has to be confirmed by further experiments.

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REFERENCES

Abe I, Takahashi Y, Morita H, Noguchi H (2001) . A novel polyketide synthase that plays a crucial role in the biosynthesis of phenyl butanones in *Rheum palmatum.* Eur. J. Biochem. 268 :3354-3359.

Brand S, Holscher D, Schierborn A, Svatos A, Schroder J , Schneider B (2006) .A type III polyketide synthase from *Wachendorfia thyrsiflora* and its role in diarylheptanoid and phenylphenalenone biosynthesis, Planta. 224: 413-28.1.

Lee E, Surh YJ (1998) Induction of apoptosis in HL-60 cells by pungent vaniloids, [6]-gingerol and [6]-paradol. Cancer Lett. 134 ,163-168.

Corpet F (1988). Multiple sequence alignment with hierarchical clustering. Nucl. Acids Res. 16 : 10881- 10890.

Denniff P, Macloed I , Whiting DA (1980). Studies in the biosynthesis of [6]-gingerol, pungent principle of ginger (*Zingiber officinale*). J. Chem. Society Perkin Trans. 1: 2637-2644.

Fuhrman B, Rosenblat M, Hayek T (2000). Ginger extract consumption reduces plasma cholesterol, inhibits LDL oxidation and attenuates development of atherosclerosis in atherosclerotic, apolipoprotein E-deficient mice. J. Nutr. 130: 1124-1131.

Ferrer JL, Jez JM, Bowman ME, Dixon RA, Noel JP (1999). Structure of chalcone synthase and the molecular basis of plant polyketide biosynthesis. Nat Struct Biol. 6 : 775-784.

Hall TA (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids Symp. Ser. 41: 95-98.

Katsuyama Y, Matsuzawa M, Funa N, Horinouchi S (2007). In vitro biosynthesis of curcuminoids by type III polyketide synthase from Oryza sativa.JBC. 52: 37702-37709.

Koes RE, Spelt CE, Mol JNM, Geratas AGM (1987). The chalcone synthase multigene family of *Petunia hybrida* (V30) Sequence homology, chromosomal location and evolutionary aspects. Plant Mol. Biol. 10: 375-385.

Kumar S, Tamura K, Nei M (2004) .MEGA3: Integrated software for Molecular Evolutionary Genetics analysis and sequence alignment. Briefings in Bioinformatics. 5 :150-163.

Murray MG, Thompson WF (1980) .Rapid isolation of high molecular weight plant DNA, Nucleic Acid Res. 8: 4321- 4325.

Ryder TB, Hedrick SA, Bell JN, Liang X, Clouse SD, Lamb CJ (1987). Organisation and differential activation of a gene family encoding the plant defense enzyme CHS in *Phaseolus vulgaris*. Mol. Gen. Genet. 210: 219-233.

Schroder J (1997). A family of plant specific polyketide synthases:facts and predictions. Trends Plant Sci. 2: 373- 378.

Thompson JD, Higgins DG, Gibson TJ (1994) .CLUSTALW: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position specific gap penalties and weight matrix choice. Nucleic Acids Res. 22 :4673-4680.