OULU 2010
A 564

ACTA UNIVERSITATIS OULUENSIS

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BIOSYNTHESIS OF HYPERICINS AND HYPERFORINS IN *HYPERICUM PERFORATUM* L. (ST. JOHN'S WORT) – PRECURSORS AND GENES INVOLVED

FACULTY OF SCIENCE,
DEPARTMENT OF BIOLOGY,
UNIVERSITY OF OULU

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BIOSYNTHESIS OF HYPERICINS AND HYPERFORINS IN *HYPERICUM PERFORATUM* L. (ST. JOHN'S WORT) – PRECURSORS AND GENES INVOLVED

Academic dissertation to be presented with the assent of the Faculty of Science of the University of Oulu for public defence in Kuusamonsali (Auditorium YB210), Linnanmaa, on 29 October 2010, at 12 noon

UNIVERSITY OF OULU, OULU 2010

Acta Univ. Oul. A 564, 2010

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ISBN 978-951-42-6309-5 (Paperback)
ISBN 978-951-42-6310-1 (PDF)
http://herkules.oulu.fi/isbn9789514263101/
ISSN 0355-3191 (Printed)
ISSN 1796-220X (Online)
http://herkules.oulu.fi/issn03553191/

Cover design
Raimo Ahonen

JUVENES PRINT
TAMPERE 2010

Karppinen, Katja, Biosynthesis of hypericins and hyperforins in *Hypericum perforatum* L. (St. John's wort) – precursors and genes involved

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Acta Univ. Oul. A 564, 2010
Oulu, Finland

Abstract

Hypericum perforatum L. (St. John's wort) is a medicinal plant widely utilized for the treatment of depression. The antidepressant activity is mainly attributed to the phenolic compounds hypericins and hyperforins, which also have a wide range of other pharmacologically interesting properties. The biosynthetic routes leading to hypericins and hyperforins are poorly understood, although a polyketide pathway including type III polyketide synthases (PKSs) has been suggested to be involved. Furthermore, a gene called *hyp-1* is assumed to attend to the final stages of the hypericin biosynthesis. In the present work, the biosynthesis of hypericins and hyperforins in *H. perforatum* was further studied by focusing on the elucidation of the precursors and genes involved.

The incorporation of isotopically labelled branched-chain amino acids into hyperforins was investigated as well as the possibilities to enhance the production of hyperforins in *H. perforatum in vitro* cultures by feeding them with amino acid precursors. Furthermore, two novel cDNAs encoding for type III PKSs were isolated from *H. perforatum*. The functions of these new genes, designated *HpPKS1* and *HpPKS2*, as well as the role of *hyp-1* were elucidated by comparing their expression with the levels of hypericins and hyperforins in *H. perforatum* tissues. The enzymatic activity of the recombinant HpPKS2 protein was also analyzed. To study Hyp-1 at a protein level, a protein extraction method was optimized for tissues of *Hypericum* species.

The results show the incorporation of valine and isoleucine into the acyl side chain of hyperforin and adhyperforin, respectively. Through the biotransformation of the amino acid precursors, it is possible to enhance the levels of adhyperforin, but not hyperforin, in *H. perforatum* shoot cultures, which demonstrates the tight regulation of the hyperforin biosynthesis. A correlation between *HpPKS1* expression and hyperforins was detected in *H. perforatum* tissues. The localization of *HpPKS2* mRNA in dark glands in which hypericins accumulate as well as the octaketide synthase activity of the recombinant HpPKS2 suggest that *HpPKS2* is associated with possible co-operating tailoring enzymes in the biosynthesis of hypericins. The presence of both *hyp-1* mRNA and Hyp-1 protein in distinct places compared with hypericins in *H. perforatum* tissues does not support the idea that Hyp-1 would be involved in the biosynthesis of hypericins in dark glands, although mobility of the Hyp-1 protein was shown to be possible.

The present thesis extends knowledge about the biosynthesis of hypericins and hyperforins in *H. perforatum* by providing new candidate genes for their biosynthesis and by identifying precursors for hyperforins. Moreover, new information was obtained about the role of *hyp-1* in *H. perforatum*.

Keywords: biosynthesis, hyperforins, hypericins, Hypericum perforatum, Hyp-1, St. John's wort, type III polyketide synthases

Acknowledgements

The present work was carried out at the Department of Biology at the University of Oulu. It was financially supported by the Finnish Cultural Foundation, the Alfred Kordelin Foundation, the Finnish Concordia Fund, the Oulu University Scholarship Foundation, the Jenny and Antti Wihuri Foundation, the Niemi Foundation, the Oscar Öflund Foundation, the Tauno Tönning Foundation, the Orion Corporation Research Foundation, the Northern Environmental Research Network (NorNet), the Faculty of Science at the University of Oulu, the Finnish Foundation for Economic and Technology Sciences (KAUTE) and by the Academy of Finland (SUNARE project, grant no. 52741), which are all gratefully acknowledged.

I wish to express my sincere gratitude to my supervisors, Professor Anja Hohtola and Professor Hely Häggman, for their guidance and support throughout the project. Anja is most warmly thanked for providing a challenging topic and encouraging and helping me in every step during the work. I am grateful to Hely for all the help and valuable advice during the work. Professor Eva Čellárová (P. J. Šafárik University in Košice, Slovakia) and Docent Markku Keinänen (University of Eastern Finland, Joensuu) are appreciated for reviewing the thesis. Dr. Mirka Rauniomaa is warmly thanked for revising the language of the thesis and original papers.

I would like to thank Docent Ari Tolonen, Dr. Sampo Mattila, M.Sc. Juho Hokkanen and M.Sc. Aleksanteri Petsalo (Department of Chemistry, University of Oulu) for collaboration with chemical analyses. Special thanks to Ari for guidance with the HPLC-technique. I am also thankful to Professor Peter Neubauer (Technical University of Berlin, Germany) and Docent Erja Taulavuori for their useful advices and valuable contributions to this work. Professor Eva Čellárová and Dr. Jan Košuth (P. J. Šafárik University in Košice, Slovakia) are acknowledged for providing various *Hypericum* species for analyses and for exchanging valuable information and ideas concerning the study of *H. perforatum*. Professor Rik Wierenga, Docent Ulrich Bergmann and laboratory assistant Ville Ratas (Department of Biochemistry, University of Oulu) helped me with protein analyses, for which they are warmly thanked. I am also grateful to Professor Ludger Beerhues (Technical University of Braunschweig, Germany) for providing me the opportunity to visit his laboratory in September 2008.

Several people at the Department of Biology and at the Botanical Gardens are gratefully acknowledged for their valuable help. Gardener Tuula Kangas is

especially thanked for taking care of *Hypericum* plants during the years. Scientific Curators Ritva Hiltunen and Mirja Siuruainen and all the staff of the Botanical Gardens are acknowledged for their helpful attitude. Docent Satu Mänttäri and Dr. Katja Anttila have offered valuable advice concerning immunoblotting analyses and the use of a confocal microscope, for which they are warmly thanked. Acknowledged are also Docent Anneli Kauppi for her kind help in microscopical analyses and Dr. Helmi Kuittinen for advice in phylogenetic analyses. I am extremely grateful for the technical assistance of laboratory amanuensis Tuulikki Pakonen, head laboratory manager Hanna-Liisa Suvilampi as well as laboratory technicians Taina Uusitalo and Tarja Törmänen, who all have kindly helped me with the laboratory work. Thanks to laboratory engineer Niilo Rankka and laboratory technician Matti Rauman for technical help. Office secretaries Sisko Veijola, Marja Liisa Mielikäinen, Ritva Paaso-Dahl and Erja Vaarala as well as amanuensis Minna Vanhatalo are thanked for their help in practical matters.

I wish also to thank all the "sisters and brothers" in the Plant Physiology division who have worked with me during the years. Dr. Laura Jaakola is especially warmly thanked for her encouraging attitude and guidance to the world of molecular biology. Warm thanks also to Docent Anna Maria Pirttilä, Dr. Zsuzsanna György, Dr. Mysore Tejesvi and fellow doctoral students Terttu Kämäräinen-Karppinen, Soile Jokipii-Lukkari, Suvi Sutela, Jaana Vuosku, Janne Koskimäki, Marian Sarala, Marjaana Tahkokorpi, Johanna Metsometsä, Anna-Kaisa Anttila, Jaanika Edesi, Pavlo Ardanov, Abdul Shakoor and Giacomo Cocetta for all the friendly pieces of advice and pleasant company in the lab.

Finally, the support of my family and friends are warmly acknowledged. I owe my greatest thanks to my parents, my family-in-law and especially to Heikki for all their support and encouragement during the years.

Oulu, September 2010 Katja Karppinen

Abbreviations

2-PS	2-pyrone synthase
ACS	acridone synthase
ALS	aloesone synthase
BA	N6-benzyladenine
BAS	benzalacetone synthase
BBS	bibenzyl synthase
BIS	biphenyl synthase
BPS	benzophenone synthase
BUS	isobutyrophenone synthase
CHS	chalcone synthase
CoA	coenzyme A
CTAS	coumaroyltriacetic acid synthase
DAD	diode array detector
DIG	digoxigenin
DMAPP	dimethylallyl diphosphate
ESIMS	electrospray ionization mass spectrometry
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPP	geranyl diphosphate
HEDS	homoeriodictyol/eriodictyol synthase
HKS	hexaketide synthase
HPLC	high-performance liquid chromatography
MS medium	Murashige and Skoog tissue culture medium
MS/MS	tandem mass spectrometry
OKS	octaketide synthase
ORF	open reading frame
PCR	polymerase chain reaction
PCS	pentaketide chromone synthase
PKS	polyketide synthase
PR-10	pathogenesis-related class 10
RACE	rapid amplification of cDNA ends
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
STCS	stilbenecarboxylate synthase
STS	stilbene synthase
UPLC	ultra-performance liquid chromatography
VPS	phlorisovalerophenone synthase

List of original publications

This thesis is based on the following papers, which are referred to in the text by their Roman numerals:

I Karppinen K, Hokkanen J, Tolonen A, Mattila S & Hohtola A (2007) Biosynthesis of hyperforin and adhyperforin from amino acid precursors in shoot cultures of *Hypericum perforatum*. Phytochemistry 68: 1038–1045.

II Karppinen K & Hohtola A (2008) Molecular cloning and tissue-specific expression of two cDNAs encoding polyketide synthases from *Hypericum perforatum*. J Plant Physiol 165: 1079–1086.

III Karppinen K, Hokkanen J, Mattila S, Neubauer P & Hohtola A (2008) Octaketide-producing type III polyketide synthase from *Hypericum perforatum* is expressed in dark glands accumulating hypericins. FEBS J 275: 4329–4342.

IV Karppinen K, Taulavuori E & Hohtola A (2010) Optimization of protein extraction from *Hypericum perforatum* tissues and immunoblotting detection of Hyp-1 at different stages of leaf development. Mol Biotechnol 46: 219–226.

V Karppinen K & Hohtola A (2010) Vasculature-associated expression of *hyp-1*, a gene suggested for the biosynthesis of hypericin in *Hypericum perforatum*. Manuscript.

The author of this thesis has been the primary author in all of the original publications. The author has been responsible for the design of the experiments, carrying out the analyses, with the exception of the HPLC/MS/MS analyses (I) and UPLC/ESIMS analyses (III), handling the data and drafting the manuscripts.

Contents

1 Introduction

1.1 *Hypericum perforatum*

Hypericum perforatum L., commonly known as St. John's wort, is a traditional medicinal plant that has for centuries been utilized in folk medicine for a range of purposes (Nathan 2001, Bilia *et al.* 2002, Butterweck 2003). The plant has attracted attention because of its wound-healing, anti-inflammatory, diuretic and sedative properties and it has been used to treat neurological disorders and traumas (Bombardelli & Morazzoni 1995, Di Carlo *et al.* 2001, Bilia *et al.* 2002, Butterweck 2003). Today, *H. perforatum* is one of the most prescribed and clinically investigated medicinal plant species: alcoholic extracts of the aerial parts of flowering plants are widely utilized for the treatment of mild and moderately severe depression (Butterweck 2003, Müller 2003, Wurglics & Schubert-Zsilavecz 2006, Linde *et al.* 2008). Several clinical studies have demonstrated that St. John's wort extracts are as efficient as conventional synthetic antidepressants but have fewer side-effects (Nathan 2001, Agostinis *et al.* 2002, Bilia *et al.* 2002, Lawvere & Manoney 2005, Linde *et al.* 2008). The side-effects of *H. perforatum* preparations mainly concern photosensitivity when administrated at high doses and interaction with some other pharmaceuticals (Greeson *et al.* 2001, Bilia *et al.* 2002, Lawvere & Manoney 2005, Wurglics & Schubert-Zsilavecz 2006). The plant is also under intensive investigation for its anti-inflammatory, antiviral, antibacterial and antitumoral effects (Schempp *et al.* 2000, Kubin *et al.* 2005, Medina *et al.* 2006) and has been suggested for the treatment of substance dependence (Uzbay 2008).

H. perforatum belongs to the Clusiaceae family. It is currently known that the genus *Hypericum* comprises over 450 species of herbaceous perennials, evergreen and deciduous shrubs as well as trees (Kirakosyan *et al.* 2004, Beerhues 2006, Ayan & Çirak 2008, Bruni & Sacchetti 2009, Karioti & Bilia 2010). The herbaceous perennial *H. perforatum* is native to Europe, Asia and North Africa but has been introduced into many temperate regions of the world, including North and South America, South Africa, Australia and New Zealand (Čellárová *et al.* 1995, Briskin & Gawienowski 2001, Kirakosyan *et al.* 2004, Bruni & Sacchetti 2009). In these regions, the plant has often become an aggressive weed (Mitich 1994, Čellárová *et al.* 1995, Bruni & Sacchetti 2009). The plant typically grows on sunny hillsides, dry meadows and fields, forest clearings, along

roadsides and other similar habitats (Mitich 1994, Bombardelli & Morazzoni 1995, Čellárová *et al.* 1995, Bruni & Sacchetti 2009).

The erect stem of St. John's wort reaches a height of up to 100 cm (Bombardelli & Morazzoni 1995, Maleš *et al.* 2006). The plant has opposite, sessile leaves and five-petalled bright yellow flowers that are arranged in terminal corymbs (Čellárová *et al.* 1995, Maleš *et al.* 2006). The majority of the small, brown seeds are developed apomictically (Čellárová *et al.* 1995, Matzk *et al.* 2001). *H. perforatum* is a highly variable species. At least three subspecies are recognized based on the morphology of the leaves (Maleš *et al.* 2006, Bruni & Sacchetti 2009, Filippini *et al.* 2010) and, although the plant is usually a tetraploid ($2n = 4x = 32$), differences in chromosome number have also been detected (Brutovská *et al.* 2000b, Matzk *et al.* 2001, Maleš *et al.* 2006, Bruni & Sacchetti 2009). Originally, *H. perforatum* may have evolved from *H. maculatum* (or their common ancestor) via autopolyploidization (Brutovská *et al.* 2000a).

The presence of different types of secretory structures, including dark glands, translucent glands and various secretory canals, is characteristic to *H. perforatum* (Curtis & Lersten 1990, Fornasiero *et al.* 1998, Ciccarelli *et al.* 2001, Onelli *et al.* 2002). The dark glands appear as black or dark-red multicellular nodules that are visible in leaf margins, stems, flower petals and stamens (Fornasiero *et al.* 1998, Zobayed *et al.* 2006). They consist of a core of large cells surrounded by a sheath of one to three flat cell layers (Curtis & Lersten 1990, Onelli *et al.* 2002). In some parts of the flowers, the dark glands also form elongated dark canals (Curtis & Lersten 1990, Zobayed *et al.* 2006). The translucent glands cause leaves to look perforated. The structures are schizogenous cavities delimited by an inner layer of flattened secretory cells and an outer layer of large parenchymatous cells (Curtis & Lersten 1990, Ciccarelli *et al.* 2001, Soelberg *et al.* 2007). The translucent glands are found in sepals and petals of flowers as well as in leaves, where they are scattered throughout the leaf lamina (Ciccarelli *et al.* 2001). Additionally, secretory canals of three different types have been described for *H. perforatum* tissues (Ciccarelli *et al.* 2001).

1.2 Secondary metabolites of *H. perforatum*

H. perforatum contains a wide range of biologically active secondary metabolites that belong to different chemical groups. Naphthodianthrones (hypericins), phloroglucinols (hyperforins), xanthones, essential oils, phenolic acids and a broad array of flavonoids, including flavonols, biflavones and procyanidins, have

been identified in extracts of the plant (Greeson *et al.* 2001, Bilia *et al.* 2002, Butterweck 2003, Patočka 2003, Tatsis *et al.* 2007). Particularly, naphthodianthrones, phloroglucinols and flavonoids are present in *H. perforatum* tissues. Several pharmacological studies have confirmed that these compounds are the main contributors to the antidepressant activity of the species through various mechanisms of action (Nathan 2001, Butterweck 2003, Butterweck *et al.* 2003, Medina *et al.* 2006). Additionally, Indian St. John's wort plants have been found to contain unusually high quantities of xanthones exhibiting antidepressant properties (Muruganandam *et al.* 2000).

1.2.1 Naphthodianthrones

The presence of red-pigmented naphthodianthrone derivatives is characteristic to several species of the genus *Hypericum* (Kitanov 2001, Crockett *et al.* 2005, Ayan & Çirak 2008, Bruni & Sacchetti 2009, Karioti & Bilia 2010). The naphthodianthrones detected in *H. perforatum* include hypericin and its 2-methoxy derivative pseudohypericin as well as their precursors protohypericin and protopseudohypericin (Fig. 1). Pseudohypericin is the main naphthodianthrone in *H. perforatum* and is generally present in the plant at two- to four-fold higher levels than hypericin, depending on the plant variety (Greeson *et al.* 2001, Patočka 2003, Karioti & Bilia 2010). Also trace amounts of the oxidation products of pseudohypericin, namely cyclopseudohypericin and isopseudohypericin, have been detected in *H. perforatum* extracts (Patočka 2003, Kirakosyan *et al.* 2004, Naït-Si & Fourneron 2005). However, it is not clear whether these compounds exist naturally in the plant or derive from pseudohypericin during the extraction procedure (Wirz *et al.* 2001, Naït-Si & Fourneron 2005). In addition to the representatives of the genus *Hypericum*, hypericin has also been found in some fungi and animal species (Seigler 1998, Falk 1999, Kusari *et al.* 2008, Kusari *et al.* 2009).

Hypericins are most abundantly present in the petals and stamens of flowers but are also found in high amounts in *H. perforatum* leaves (Repčák & Mártonfi 1997, Zobayed *et al.* 2006). Levels of 0.03 to 0.3 percent of hypericins have been found in leaves and 1 up to 14 percent in flower buds (Tekel'ová *et al.* 2000, Sirvent *et al.* 2003, Zobayed *et al.* 2006).

Hypericins accumulate in the dark glands of *H. perforatum* (Briskin & Gawienowski 2001, Pasqua *et al.* 2003, Zobayed *et al.* 2006, Hölscher *et al.* 2009). These glands have also been proposed to be the sites of their biosynthesis

(Zobayed *et al.* 2006, Kornfeld *et al.* 2007). The localization of hypericins in the glandular structures is considered to have evolved as a mechanism that avoids the potential toxicity of these compounds to plant tissues (Walker *et al.* 2001, Agostinis *et al.* 2002, Onelli *et al.* 2002, Bruni & Sacchetti 2009). Although the function of hypericins in *Hypericum* species is still not clear, it has been proposed that they serve a defensive role against herbivores and plant pathogens (Fields *et al.* 1990, Fornasiero *et al.* 1998, Agostinis *et al.* 2002, Onelli *et al.* 2002, Sirvent & Gibson 2002, Sirvent *et al.* 2003, Bruni & Sacchetti 2009).

Hypericin is possibly the most powerful natural photosensitizer so far described (Falk 1999, Agostinis *et al.* 2002, Miskovsky 2002, Karioti & Bilia 2010). The ingestion of large quantities of *Hypericum* plants by grazing animals has been shown to cause phototoxic symptoms known as hypericism, a condition of severe sensitivity to light (Seigler 1998, Falk 1999, Bourke 2000, Patočka 2003). Due to its photodynamic properties, hypericin has pharmaceutical potential as an antiviral, antiretroviral and antitumoral agent (Falk 1999, Agostinis *et al.* 2002, Miskovsky 2002, Taher *et al.* 2002, Kubin *et al.* 2005, Wurglics & Schubert-Zsilavecz 2006, Karioti & Bilia 2010). Especially its use in photodynamic therapy (PDT) in the treatment of variety of tumors is under intensive investigation (Cole *et al.* 2008, Kacerovská *et al.* 2008). Hypericin is also today used as a fluorescent diagnostic tool in the detection of tumor cells (Kubin *et al.* 2008, Thong *et al.* 2009). Contribution of hypericin to the antidepressant activity of *H. perforatum* has also been shown (Bilia *et al.* 2002, Patočka 2003, Kubin *et al.* 2005, Wurglics & Schubert-Zsilavecz 2006).

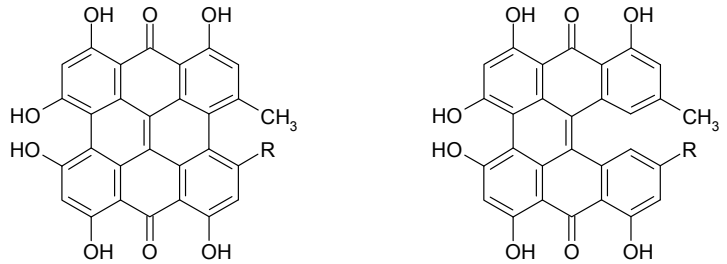


Fig. 1. The main naphthodianthrones of *H. perforatum*.

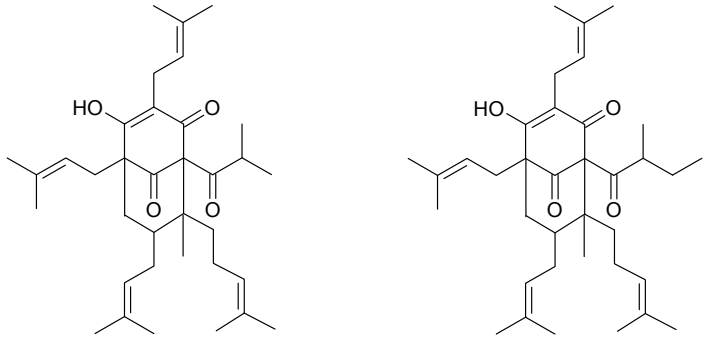
1.2.2 Phloroglucinols

Different types of phloroglucinol derivatives are widely distributed in the genus *Hypericum* (Patočka 2003, Crockett *et al.* 2005, Dall'Agnol *et al.* 2005, Beerhues 2006). The major phloroglucinols found in *H. perforatum* are polyprenylated acylphloroglucinol hyperforin and its homologue adhyperforin (Fig. 2). Hyperforin generally exists in a plant in two- to ten-fold higher amounts than adhyperforin (Greeson *et al.* 2001, Patočka 2003). Their biosynthetic intermediates hyperfirin and adhyperfirin (Tatsis *et al.* 2007, Hölscher *et al.* 2009) as well as their homologues secohyperforin and secoadhyperforin can also be detected as minor constituents in *H. perforatum* (Kirakosyan *et al.* 2004, Charchoglyan *et al.* 2007). Additionally, trace amounts of various oxygenated analogues of hyperforin have been found in extracts of the species (Trifunović *et al.* 1997, Brolis *et al.* 1998, Verotta *et al.* 1999, Verotta *et al.* 2000, Shan *et al.* 2001, Vajs *et al.* 2003, Wu *et al.* 2004, Hashida *et al.* 2008). At present, it is not known whether the analogues are natural compounds or degradation products of hyperforin that are generated during the extraction procedure (Verotta *et al.* 2000, Vajs *et al.* 2003, Kirakosyan *et al.* 2004, Beerhues 2006).

Hyperforins occur most abundantly in *H. perforatum* flowers, especially in pistils and subsequently forming fruits, but they are also highly present in leaves (Repčák & Mártonfi 1997, Tekel'ová *et al.* 2000, Sirvent *et al.* 2003, Beerhues 2006). A hyperforin content of 6.9 percent in flower buds, 8.5 percent in unripe fruits and 1.5 percent in leaves has been reported (Tekel'ová *et al.* 2000, Sirvent *et al.* 2003). Hyperforins have been shown to accumulate in the translucent glands of *H. perforatum* (Soelberg *et al.* 2007, Hölscher *et al.* 2009). It has also been suggested that hyperforins would be biosynthesized in the parenchymatous secretory cells delimiting the cavity of translucent glands (Soelberg *et al.* 2007). The benefit of hyperforins to the plant still remains unclear, but they may serve as a defensive compound against herbivores and other pests (Sirvent & Gibson 2002, Sirvent *et al.* 2003, Beerhues 2006).

Hyperforin is an unstable compound when exposed to heat, light or oxygen (Beerhues 2006, Medina *et al.* 2006). For this reason, its bioactivity has been difficult to study and, until recently, it was not considered as a pharmacologically active constituent of *H. perforatum* (Beerhues 2006, Wurglics & Schubert-Zsilavecz 2006). Today, hyperforin is considered as the main bioactive compound responsible for the antidepressant effects of St. John's wort (Müller 2003, Patočka 2003, Medina *et al.* 2006, Wurglics & Schubert-Zsilavecz 2006). Hyperforin has

been shown to contribute to the antidepressant properties of the species by inhibiting the re-uptake of a number of neurotransmitters (Müller 2003, Medina *et al.* 2006). Similar activities have also been detected in its homologue adhyperforin (Jensen *et al.* 2001, Müller 2003). Evidence for the usefulness of hyperforin for other pharmacological applications has also been found. In addition to antidepressant activity, hyperforin exhibits other neurological effects (Medina *et al.* 2006) as well as antitumoral (Schempp *et al.* 2002, Hostanska *et al.* 2003, Donà *et al.* 2004, Medina *et al.* 2006), anti-inflammatory (Schempp *et al.* 2000, Schempp *et al.* 2003, Medina *et al.* 2006), antibacterial (Reichling *et al.* 2001, Medina *et al.* 2006) and antiprotozoal effects (Verotta *et al.* 2007).



hyperforin adhyperforin

Fig. 2. The main phloroglucinols of *H. perforatum*.

1.3 Biosynthetic pathways of hypericins and hyperforins

The biosynthetic routes leading to hypericins and hyperforins are still poorly understood, but both of the compound groups are presumed to be biosynthesized via a polyketide pathway in which type III polyketide synthases (PKSs) act as key enzymes (Adam *et al.* 2002, Bais *et al.* 2003, Klingauf *et al.* 2005, Beerhues 2006, Zobayed *et al.* 2006).

The biosynthetic pathway leading to hypericins most likely starts with the condensation of one molecule of acetyl coenzyme A (CoA) with seven molecules of malonyl-CoA to form a linear octaketide chain that subsequently undergoes specific cyclization to form emodin anthrone (Falk 1999, Bais *et al.* 2003, Kirakosyan *et al.* 2004, Zobayed *et al.* 2006, Karioti & Bilia 2010, Michalska *et al.* 2010). The reaction is carried out by a type III PKS having octaketide synthase

(OKS) activity. Emodin anthrone is further oxidized to emodin probably by the activity of emodinanthrone oxygenase (Karioti & Bilia 2010). Condensation of emodin anthrone and emodin followed by dehydration yields emodin dianthrone, which subsequently undergoes phenolic oxidation that leads to the formation of protohypericin (Falk 1999, Bais *et al.* 2003, Kirakosyan *et al.* 2004, Zobayed *et al.* 2006, Karioti & Bilia 2010). All these reactions have been suggested to be catalyzed by a phenolic coupling protein called Hyp-1 (Bais *et al.* 2003, Michalska *et al.* 2010). Oxidation of the methyl group of protohypericin has been presumed to yield protopseudohypericin (Karioti & Bilia 2010). Conversion of the protoforms to hypericin and pseudohypericin can take place under the influence of light, but Hyp-1 has also been suggested to be involved in these reactions (Bais *et al.* 2003, Kirakosyan *et al.* 2004, Zobayed *et al.* 2006). The chemical synthesis of hypericin has been achieved by following the pattern of the biosynthesis, using emodin anthrone as a precursor (Falk 1999, Kubin *et al.* 2005, Karioti & Bilia 2010). An overview of the biosynthesis of the main hypericins of *H. perforatum* is presented in Fig. 3.

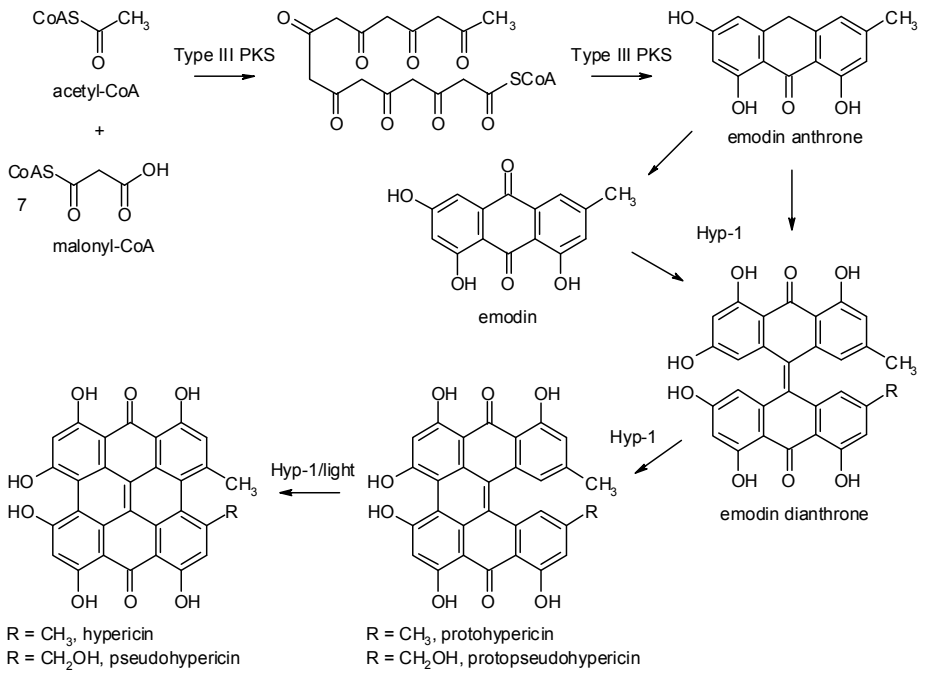


Fig. 3. Proposed biosynthetic pathway of hypericins.

The biosynthesis of hyperforins is divided into two sections that comprise the formation of the acylphloroglucinol nucleus and the subsequent attachment of prenyl side chains to it. The nucleus of hyperforin derives from the condensation of one molecule of isobutyryl-CoA with three molecules of malonyl-CoA to yield a linear tetraketide intermediate that is further cyclised, leading to the formation of phlorisobutyrophenone (Adam *et al.* 2002, Klingauf *et al.* 2005, Beerhues 2006). A type III PKS called isobutyrophenone synthase (BUS) is responsible for the catalyzation of the reaction (Klingauf *et al.* 2005, Beerhues 2006). In the biosynthesis of adhyperforin, 2-methylbutyryl-CoA instead of isobutyryl-CoA is utilized as a starter substrate (Klingauf *et al.* 2005, Charchoglyan *et al.* 2007). The nucleus of hyperforins is subsequently prenylated stepwise, using isoprenoid units, derived via a non-mevalonate pathway, as prenyl donors (Adam *et al.* 2002, Boubakir *et al.* 2005, Beerhues 2006). The first prenylation step is catalyzed by a prenyltransferase using dimethylallyl diphosphate (DMAPP) as a prenyl donor (Boubakir *et al.* 2005, Beerhues 2006). Furthermore, two molecules of DMAPP and one molecule of geranyl diphosphate (GPP) are finally joined to the acylphloroglucinol nucleus to yield hyperforin and adhyperforin (Adam *et al.* 2002, Beerhues 2006, Soelberg *et al.* 2007). For the biosynthesis of secohyperforins, only DMAPP has been suggested to be utilized as a prenyl donor (Charchoglyan *et al.* 2007). An overview of the biosynthesis of the main hyperforins found in *H. perforatum*, based on current knowledge, is presented in Fig. 4. The chemical synthesis of hyperforin has been found difficult and thus remains undescribed (Nicolaou *et al.* 2005, Beerhues 2006).

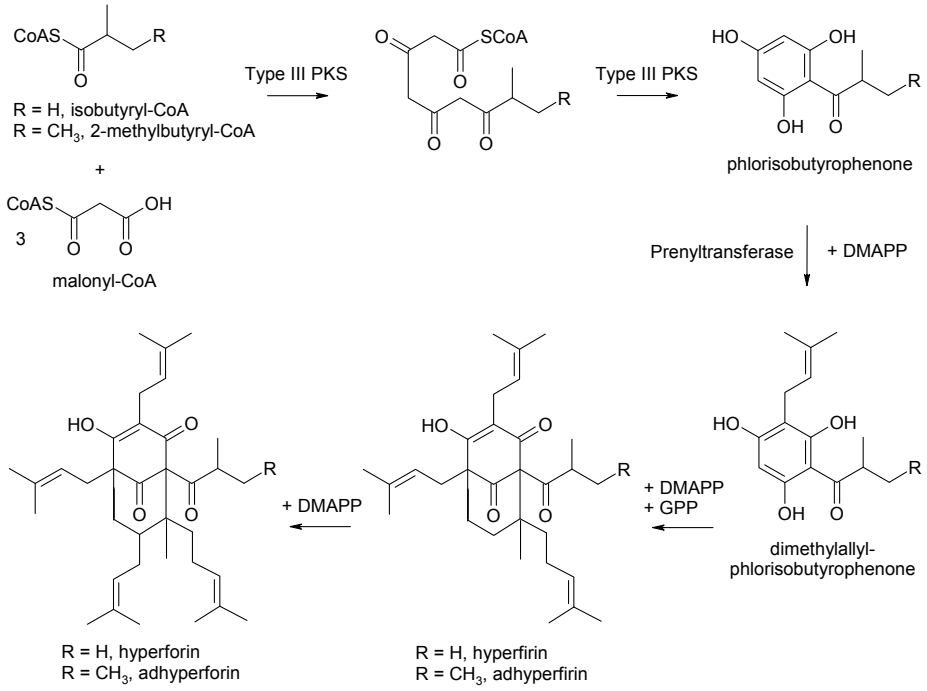


Fig. 4. Proposed biosynthetic pathway of hyperforins.

1.3.1 Type III PKSs

Type III PKSs, also referred to as chalcone synthase (CHS) superfamily enzymes, are the key enzymes in the biosynthesis of a large variety of secondary metabolites in plants (Austin & Noel 2003, Flores-Sanchez & Verpoorte 2009, Hertweck 2009, Abe & Morita 2010), but the enzymes have also been discovered in some bacteria and fungi (Seshime *et al.* 2005, Gross *et al.* 2006). Type III PKSs are structurally and mechanistically distinct from type I and type II PKSs that are found in bacteria and fungi (Staunton & Weissman 2001, Austin & Noel 2003, Flores-Sanchez & Verpoorte 2009, Hertweck 2009). Type III PKSs are simple homodimeric enzymes of 40–45 kDa subunits with highly conserved cysteine-histidine-asparagine catalytic triad present in their active center (Ferrer *et al.* 1999, Austin & Noel 2003, Shi *et al.* 2008, Flores-Sanchez & Verpoorte 2009). The enzymes catalyze a sequential decarboxylative condensation of malonyl-CoA units to an aromatic or aliphatic CoA-thioester starter unit to form a linear

polyketide chain that is further cyclized and aromatized in the same active site (Staunton & Weissman 2001, Austin & Noel 2003, Shi *et al.* 2008, Flores-Sanchez & Verpoorte 2009). The reaction sequence shares similarities with fatty acid synthases of primary metabolism from which the enzymes are likely to have developed during evolution (Austin & Noel 2003, Flores-Sanchez & Verpoorte 2009).

Gene encoding for CHS that catalyzes the first committed step in the flavonoid biosynthesis, the formation of naringenin chalcone, was the first type III PKS gene isolated (Reimold *et al.* 1983, Austin & Noel 2003). CHS represents both the most frequently occurring and the most investigated member of the type III PKS family at a structural and mechanistical level (Ferrer *et al.* 1999, Austin & Noel 2003). All the functionally divergent type III PKSs have been suggested to arise via gene duplication followed by mutations of the *chs* gene (Tropf *et al.* 1994, Helariutta *et al.* 1996, Durbin *et al.* 2000, Schröder 2000, Austin & Noel 2003, Flores-Sanchez & Verpoorte 2009). Today, an increasing number of functionally variable type III PKSs have been described for different plant species. The functional diversity of type III PKS enzymes derives from small differences in the active site that influence their preference for starter substrate, the number of condensation reactions performed with malonyl-CoA and the mechanism of cyclization and aromatization of linear polyketide intermediates (Schröder 2000, Austin & Noel 2003, Shi *et al.* 2008, Flores-Sanchez & Verpoorte 2009). In some cases, the reaction intermediates have also been found to be modified by interaction with other enzymes (Welle *et al.* 1991, Schröder 1997, Schröder 2000, Austin & Noel 2003). The currently known functionally divergent type III PKSs are presented in Table 1. Features of the different enzymes have been described in several reviews (Schröder 1997, Schröder 2000, Austin & Noel 2003, Shi *et al.* 2008, Flores-Sanchez & Verpoorte 2009, Abe & Morita 2010).

Table 2. Functionally divergent type III PKSs in plants.

Enzyme	Reference
2-pyrone synthase (2-PS)	Helariutta *et al.* 1995, Helariutta *et al.* 1996, Eckermann *et al.* 1998
acridone synthase (ACS)	Junghanns *et al.* 1995, Lukačin *et al.* 1999, Wanibuchi *et al.* 2007
aloesone synthase (ALS)	Abe *et al.* 2004
benzalacetone synthase (BAS)	Berejsza-Wysocki & Hrazdina 1996, Abe *et al.* 2001, Zheng & Hrazdina 2008
benzophenone synthase (BPS)	Beerhues 1996, Schmidt & Beerhues 1997, Liu *et al.* 2003
bibenzyl synthase (BBS)	Reinecke & Kindl 1993, Preisig-Müller *et al.* 1995
biphenyl synthase (BIS)	Liu *et al.* 2004, Liu *et al.* 2007a, Liu *et al.* 2010
C-methylchalcone synthase	Schröder *et al.* 1998
chalcone synthase (CHS)	Reimold *et al.* 1983, Ferrer *et al.* 1999, Liu *et al.* 2003
coumaroyltriacetic acid synthase (CTAS)	Akiyama *et al.* 1999
curcuminoid synthase (CUS)	Katsuyama *et al.* 2007
hexaketide synthase (HKS)	Springob *et al.* 2007, Jindaprasert *et al.* 2008
homoeriodictyol/eriodictyol syntase (HEDS)	Christensen *et al.* 1998
isobutyrophenone synthase (BUS)	Klingauf *et al.* 2005
octaketide synthase (OKS)	Abe *et al.* 2005a, Karppinen *et al.* 2008, Mizuuchi *et al.* 2009
pentaketide chromone synthase (PCS)	Abe *et al.* 2005b
phlorisovalerophenone synthase (VPS)	Paniego *et al.* 1999, Okada & Ito 2001, Yamazaki *et al.* 2001
stilbenecarboxylate synthase (STCS)	Eckermann *et al.* 2003
stilbene synthase (STS)	Schröder *et al.* 1988, Fliegmann *et al.* 1992, Austin *et al.* 2004

1.4 Production of secondary metabolites in plant tissue cultures

Commercial St. John's wort preparations show inconsistency in their contents of bioactive compounds, which has raised doubts concerning the safety and efficacy of the products (Wurglics *et al.* 2001, Kirakosyan *et al.* 2004, Couceiro *et al.* 2006, Bruni & Sacchetti 2009). The amounts of hypericins and hyperforins in extracts prepared from field- or greenhouse-grown plants are greatly influenced by many factors, such as the time of harvest (Southwell & Bourke 2001, Walker *et al.* 2001, Gray *et al.* 2003, Couceiro *et al.* 2006), geographical origin of the plants (Walker *et al.* 2001) as well as the presence of biotic or abiotic contaminants (Sirvent & Gibson 2002, Murch *et al.* 2003, Bruni *et al.* 2005, Tirillini *et al.* 2006). The levels of the compounds have also been shown to vary

between genetic variants (Couceiro *et al.* 2006) and be affected by several environmental factors, such as CO_2 concentration (Mosaleeyanon *et al.* 2005), temperature (Zobayed *et al.* 2005, Couceiro *et al.* 2006) and the availability of water (Gray *et al.* 2003, Zobayed *et al.* 2007), nitrogen as well as light (Briskin & Gawienowski 2001, Germ *et al.* 2010).

The use of *in vitro* culture systems has been reported as an alternative strategy for the commercial production of plant material in the pharmaceutical industry to satisfy the growing demand of natural products as well as to obtain less variable preparations (Bourgaud *et al.* 2001, Ramachandra Rao & Ravishankar 2002, Verpoorte *et al.* 2002, Kirakosyan *et al.* 2004, Zhou & Wu 2006, Karuppusamy 2009). For this purpose, *in vitro* propagation protocols were described also for *H. perforatum* (Čellárová *et al.* 1992, Zdunek and Alfermann 1992). Since the first reports, numerous protocols have been described and levels of hypericins and hyperforins have been monitored in the cultures (*e.g.* Murch *et al.* 2000, Bais *et al.* 2002, Košuth *et al.* 2003, Pasqua *et al.* 2003, Kirakosyan *et al.* 2004, Gadzovska *et al.* 2005, Karppinen *et al.* 2006a). Large-scale *in vitro* production of *H. perforatum* in bioreactors has also been reported on (Zobayed *et al.* 2003, Zobayed & Saxena 2003, Zobayed *et al.* 2004, Karppinen *et al.* 2006a, Karppinen *et al.* 2006b, Karppinen *et al.* 2006c, Cui *et al.* 2010). However, generally the use of large-scale plant tissue cultures has only had limited success because the yields of secondary compounds in the cultures have been too low for commercialization (Verpoorte *et al.* 2002, Kirakosyan *et al.* 2004, Zhou & Wu 2006).

In addition to the optimization of growth conditions and the selection of high-producing cell lines, elicitation, biotransformation of precursors and genetic engineering have been employed as strategies to improve the biosynthesis of secondary compounds in plant tissue cultures (Bourgaud *et al.* 2001, Ramachandra Rao & Ravishankar 2002, Verpoorte *et al.* 2002, Zhou & Wu 2006, Karuppusamy 2009). For this purpose, an array of investigations has been conducted to enhance the levels of hypericins and hyperforins in tissue cultures of *H. perforatum* through the addition of elicitors (*e.g.* Kirakosyan *et al.* 2000, Walker *et al.* 2002, Kirakosyan *et al.* 2004, Gadzovska *et al.* 2007, Liu *et al.* 2007b, Pavlíc *et al.* 2007) and potential precursors (Liu *et al.* 2007c) to the cultures. The development of an efficient transformation method has also been under investigation for certain *Hypericum* species in order to improve the biosynthesis of therapeutically useful compounds through genetic engineering (Di Guardo *et al.* 2003, Vinterhalter *et al.* 2006, Franklin *et al.* 2007, Komarovská *et*

al. 2009). Among the species, *H. perforatum* still remains highly recalcitrant towards *Agrobacterium*-mediated transformation methods (Franklin *et al.* 2007, Franklin *et al.* 2008). Recently, the importance of understanding the biosynthetic pathways and their regulation has been emphasized as a prerequisite for the efficient manipulation of desired secondary metabolites in *in vitro* cultures (Verpoorte *et al.* 2002, Kirakosyan *et al.* 2004, Zhou & Wu 2006, Bruni & Sacchetti 2009, Karuppusamy 2009).

2 Aims of the study

The main objective of the study was to gain knowledge about the biosynthesis of hypericins and hyperforins in *H. perforatum*. Due to the importance of the plant for the pharmaceutical industry, the biosynthetic pathways and possibilities for alternative production methods of its bioactive compounds are of particular interest. Today, only a small amount of information is available on the biosynthesis of the bioactive compounds. The present work focused on elucidating precursors and candidate genes involved in the biosynthesis of the polyketide compounds hypericins and hyperforins in *H. perforatum*. For these purposes, research was carried out

a) to elucidate precursors of hyperforins and to investigate their usefulness in the production of hyperforins by biotransformation in *H. perforatum in vitro* cultures (I),
b) to isolate novel type III PKS encoding genes from the polyketide pathway of *H. perforatum* (II),
c) to analyze the expression and function of the novel type III PKS genes and their possible involvement in the biosynthesis of hypericins and hyperforins in *H. perforatum* (II, III),
d) to optimize a protein extraction method for *Hypericum* tissues to enable protein level analyses of gene expression (IV) and
e) to elucidate the role of *hyp-1* in the biosynthesis of hypericins at the transcript and protein level in *H. perforatum* tissues (IV, V).

3 Materials and methods

3.1 Plant material and initiation of *in vitro* cultures

Hypericum perforatum L. plants of Finnish origin were collected from the Botanical Gardens of the University of Oulu, Finland. At an early stage of flowering, the plants were harvested and dissected into various organs: roots, stems, leaves and flower buds (II, III, IV, V). The leaves were further dissected into leaf margins and leaf interior parts (II, V). Plants were also grown in a greenhouse in peat pots for three months before they were dissected (V). The excised organs were immediately either fixed for *in situ* RNA hybridization analysis as described in papers III and V or frozen in liquid nitrogen and subsequently stored at –80 °C until used for RNA isolation (II, V), protein extraction (IV, V) or determination of the contents of hypericins and hyperforins (II, V).

In amino acid feeding experiments (I), *in vitro* liquid cultures were employed. To establish the cultures, seeds that originated from a wild population (Helsinki, Finland) were surface sterilized, and then germinated on a plant growth regulator-free half strength MS medium (Murashige & Skoog 1962). Shoot multiplication of the plantlets was obtained on MS medium supplemented with 0.1 mg l^{-1} N^6-benzyladenine (BA). Liquid shoot cultures were initiated by cutting the multiplicated shoots into segments and transferring them into 50-ml Erlenmeyer flasks containing 20 ml of MS media supplemented with 0.1 mg l^{-1} BA and 0.1 g l^{-1} ascorbic acid. For the initiation of compact callus aggregate cultures, the segments were transferred into liquid MS media containing 0.5 mg l^{-1} BA, 0.1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 g l^{-1} ascorbic acid. Subcultures were carried out every 5–8 days by cutting the tissues into small pieces and placing them into a fresh culture medium. The flasks were shaken at 130 rpm under a 16 h photoperiod at 84 µmol m^{-2} s^{-1} irradiance at 22 °C.

3.2 Amino acid feeding experiment

Amino acids were added to 12-day-old liquid cultures. L-[U-$^{13}C_5$]Valine, L-[U-$^{13}C_6$]isoleucine or L-[U-$^{13}C_6$]leucine was supplied to the cultures in a final concentration of 2 mM. At least four replicate flasks were employed. L-Valine, L-isoleucine, L-leucine or L-threonine was added in a final concentration of 0–5

mM, and three replicate flasks were employed. The control cultures were left untreated. The cultures fed with ^{13}C labelled amino acids were harvested after 5 days of incubation for analyses with high-performance liquid chromatography (HPLC) combined with a tandem mass spectrometry (MS/MS) to quantify the effect of feeding on different molecular species of hyperforin and adhyperforin. The cultures fed with unlabelled amino acids were harvested after 5 days of incubation for analyses with HPLC combined with a diode array detector (DAD) to determine the effect of precursor feeding on the hyperforin and adhyperforin levels.

3.3 RNA isolation and cDNA synthesis

Total RNA was isolated separately from different organs according to the protocol described by Jaakola *et al.* (2001). cDNA was synthesized from the total RNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with commercial random primers according to the manufacturer's instructions. The cDNA was purified from genomic DNA with a gel extraction method (Jaakola *et al.* 2004).

3.4 Isolation of type III PKS encoding genes

Fragments of PKS genes were amplified from leaf cDNA by a polymerase chain reaction (PCR) with partially degenerated oligonucleotide primers (II). The primers were designed based on the sequence homologies found in the previously isolated type III PKS encoding genes. The sequences of the primers were 5'-RGC MAT YRA DGA RTG GGG-3' (sense primer) and 5'-TTY TCN GCN ADR TCC TTS G-3' (antisense primer). The PCR was performed with DyNazyme^{TM} II DNA polymerase (Finnzymes, Espoo, Finland). The PCR conditions were 94 °C for 5 min, followed by 40 cycles at 94 °C for 1 min, 50 °C for 2 min and 72 °C for 2 min, and final extension at 72 °C for 10 min. The PCR products were separated by electrophoresis on a 1% (w/v) ethidium bromide-stained agarose gel. A band of expected size was excised from the gel and sequenced. The nucleotide sequences were determined by using an ABI 3730 DNA sequencer (Applied Biosystems, Foster City, CA, USA) with a BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). Full-length cDNA sequences of the new PKS genes, designated *HpPKS1* and *HpPKS2*, were obtained with 3'- and 5'-rapid amplification of cDNA ends (RACE) method using a SMART^{TM} RACE cDNA

Amplification Kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.

3.5 Sequence analysis

Sequences of the *HpPKS1* and *HpPKS2* genes were analyzed using bioinformatic tools that can be found on the websites http://www.expasy.org/ and http://www.ncbi.nlm.nih.gov/. All sequence alignments were performed with the CLUSTAL W program. To study relationships of type III PKS family proteins, a phylogenetic tree was constructed by the neighbor-joining method, using the software of MEGA 2.0. The reliability of the tree was measured by a bootstrap analysis with 1000 replicates.

3.6 Gene expression analysis

Expression of the *HpPKS1*, *HpPKS2* (II) and *hyp-1* (V) genes was studied by quantitative real-time PCR. For analyses, the cDNA was prepared separately for the different organs of *H. perforatum*. Relative quantification was performed using a LightCycler 2.0 instrument (Roche Molecular Biochemicals, Mannheim, Germany) with a DyNAmo™ Capillary SYBR® Green qPCR Kit (Finnzymes) according to the manufacturer's instructions. The gene-specific primers used for real-time PCR are shown in Table 2. For the quantification of the PCR products, 18S ribosomal RNA (*18S rRNA*) gene (II, V) or glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene (V) was used as a control. The ratio of gene-specific expression to control gene signal was defined as relative expression. Samples from three independent plants were employed for analyses.

The cell type expression of the *HpPKS2* (III) and *hyp-1* (V) genes was studied with *in situ* RNA hybridization. Fixed organs (roots, stems, leaves and flower buds) were dehydrated and infiltrated in paraffin. The paraffin-embedded samples were sectioned to 8 µm thickness by using a microtome and spread on glass slides. The sections were incubated in xylene to remove paraffin and then rehydrated. *In situ* RNA hybridization analyses were performed with digoxigenin (DIG)-labelled RNA probes. A *HpPKS2* fragment of 205 bp and a *hyp-1* fragment of 312 bp were amplified by PCR as described in the original papers (III, V) and ligated to pGEM-T Easy vector (Promega, Madison, WI, USA). DIG-labelled sense and antisense RNA probes were prepared from the linearized plasmids by *in vitro* transcription using a DIG RNA Labelling Kit (Roche) according to the

manufacturer's instructions. *In situ* RNA hybridization steps for *HpPKS2* are described in paper III and for *hyp-1* in paper V.

The tissue sections were examined under a light microscope (Nikon Optiphot-2; Nikon Corporation, Tokyo, Japan) or scanned with a confocal laser scanning microscope (LSM-5 Pascal, Zeiss, Jena, Germany).

Table 3. Gene-specific primers used for gene expression analysis by quantitative real-time PCR.

Gene	Primers (5'-3')	Reference
HpPKS1	TGT ACG TCT CAT CCA GTG AGC (forward) ACA CCA CCG TAA CAG CCT AAG (reverse)	II
HpPKS2	TAC ACA AGG AGA CAC CCA AGC (forward) GGA ACT GGC AAC ATA GCA TCC (reverse)	II
hyp-1	CAG GCT GTT TAA GGC ATT GGT C (forward) GGG ATG TCC ATC AAC GAA AGT G (reverse)	V
18S rRNA	AAA CGG CTA CCA CAT CCA AG (forward) CAA CCC AAA GTC CAA CTA CG (reverse)	II, V
GAPDH	ATG GAC CAT CAA GCA AGG ACT G (forward) GAA GGC CAT TCC AGT CAA CTT C (reverse)	V

3.7 Recombinant protein production

For the characterization of the HpPKS1 (unpublished data), HpPKS2 (III) and Hyp-1 (V) proteins, expression plasmids for recombinant protein production were constructed. The coding region of *HpPKS2* was amplified from leaf cDNA by PCR as described in paper III. For the amplification of the coding region of *HpPKS1* from leaf cDNA, a forward primer 5'-CTC TGT AGT GCC TGC CGG ATC C**AT G**TC TAA CT-3' (the translation start codon is in bold and the *Bam*HI site is underlined) and a reverse primer 5'-TGT GGT ATG GTA CC**T CA**T AGG CAT AGG CTT CG-3' (the translation stop codon is in bold and the *Kpn*I site is underlined) were utilized for PCR that was conducted under the same conditions as described for *HpPKS2*. The coding region of *hyp-1* was amplified from leaf cDNA by PCR as described in paper V.

The PCR products were purified, digested with restriction enzymes and ligated into pQE30 expression vector (Qiagen, Hilden, Germany) as described in

paper III. The constructed recombinant plasmids were transferred to *Escherichia coli* host strain M15 [pREP4] (Qiagen) for the production of the HpPKS1, HpPKS2 or Hyp-1 protein. The transformed *E. coli* cells harboring one of the recombinant plasmids were grown in Luria-Bertani liquid medium in the presence of ampicillin and kanamycin at 30 °C. Recombinant protein production was initiated by adding isopropyl thio-ß-D-galactoside (IPTG). The cultures for HpPKS1 and HpPKS2 protein production were incubated at 16 °C for 20 h before the *E. coli* cells were harvested by centrifugation. Incubation at 37 °C for 4 h was used in Hyp-1 protein production. Purification of the recombinant HpPKS1, HpPKS2 and Hyp-1 proteins were performed under native conditions using nickel-nitrilotriacetic acid (Ni-NTA) resin based chromatography as described in detail in paper III. The molecular weights and purities of the recombinant proteins were evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Furthermore, the molecular weight of HpPKS2 was determined by size exclusion chromatography using a Superdex 200 column (GE Healthcare, Buckinghamshire, UK). Elution profiles were detected by a refractive index detector (Shodex; Showa Denko, Tokyo, Japan) and a MiniDAWN Treos light scattering system (Wyatt Technology Corp., Santa Barbara, CA, USA).

3.8 Enzyme assays

Protein concentration was measured according to Bradford (1976), and 100 µg of purified recombinant HpPKS2 protein was employed for enzyme assays. The enzyme was incubated with potential starter substrates (acetyl-CoA, isobutyryl-CoA, benzoyl-CoA or hexanoyl-CoA) and extending substrate (malonyl-CoA) at 30 °C for 90 min. The biosynthetic products were extracted with ethyl acetate and, after evaporation, dissolved in methanol for identification with ultra-performance liquid chromatography (UPLC) combined with electrospray ionization mass spectrometry (ESIMS).

3.9 Protein extraction

A method suitable for high-quality protein extraction from *H. perforatum* tissues was optimized during the present study. The qualitatively and quantitatively optimized protocol based on sodium borate extraction and phenol/methanolic ammonium acetate precipitation is described in detail in paper IV. The protein

concentration of the extracted samples was measured according to a study by Bradford (1976).

3.10 SDS-PAGE and immunoblotting

SDS-PAGE was carried out for the denatured protein samples using a 0.75 mm thick 12% separation gel and 3% stacking gel. A Mini-Protean II electrophoresis system (Bio-Rad, Hercules, CA, USA) was used for separation. After electrophoresis, the proteins were either visualized with Coomassie Brilliant Blue or transferred for immunoblotting onto polyvinylidene difluoride membrane (PVDF) with a Mini Trans Blot Electrophoretic Transfer Cell (Bio-Rad). The immunological detection of the Hyp-1 protein was carried out with an affinity-purified rabbit polyclonal antibody raised against a synthetic peptide (VKLEAVGGGSKGKIC). The steps of Hyp-1 immunodetection are described in detail in papers IV and V.

3.11 Chemical analyses

HPLC/MS/MS analyses were carried out to quantify different molecular species of hyperforin and adhyperforin after feeding of *in vitro* liquid cultures with isotopically labelled amino acids. For this purpose, the method described by Tolonen *et al.* (2002) was used with small modifications, as described in paper I.

The hyperforin and adhyperforin contents were determined from *H. perforatum in vitro* liquid cultures after feeding with unlabelled amino acids, using the HPLC-DAD-method according to Tolonen *et al.* (2003) with small modifications, as described in paper I.

The quantification of hypericin, protohypericin, pseudohypericin, protopseudohypericin, hyperforin and adhyperforin from different *H. perforatum* organs was determined with the HPLC-DAD-method according to Tolonen *et al.* (2003) with small modifications, as described in papers II and V.

The biosynthetic products catalyzed by the recombinant HpPKS2 protein were identified with UPLC/ESIMS as described in paper III.

3.12 Statistical analyses

Statistical analyses were performed by a one-way analysis of variance (ANOVA) using the SPSS program, version 13.0 (SPSS Inc., Chicago, IL, USA).

4 Results

4.1 Biotransformation of branched-chain amino acids to hyperforins

The possible utilization of branched-chain amino acids as precursors in the biosynthesis of hyperforins was studied by feeding isotopically labelled valine, isoleucine and leucine into *H. perforatum in vitro* shoot cultures (I). Upon the addition of L-[U-$^{13}C_5$]valine and L-[U-$^{13}C_6$]isoleucine to the cultures, the ^{13}C labels were found to be incorporated into the acyl side chains of the biosynthesized hyperforin and adhyperforin, respectively. L-[U-$^{13}C_6$]Leucine was not efficiently incorporated into either the hyperforin or the adhyperforin structure. The incorporation rate of L-[U-$^{13}C_6$]isoleucine into adhyperforin was found to be higher than the incorporation rate of L-[U-$^{13}C_5$]valine into hyperforin.

Possibilities to enhance the levels of hyperforin and adhyperforin in *H. perforatum in vitro* shoot cultures by precursor feeding were tested (I). The supplementation of unlabelled L-isoleucine or L-threonine, a precursor of isoleucine, was found to increase significantly the production of adhyperforin in the shoot cultures. The highest induction was obtained with the addition of 2 mM of L-isoleucine that stimulated a 3.7-fold increase in the adhyperforin content. The supplementation of 3 mM L-threonine induced a 2.0-fold increase in the amount of adhyperforin. No significant stimulating effect on either the hyperforin or the adhyperforin production was detected by supplementing L-valine or L-leucine at different concentrations.

Fast-growing compact callus aggregate cultures of *H. perforatum* showing lower levels of tissue differentiation than shoot cultures were also utilized for biotransformation studies. However, no stimulating effect of either L-isoleucine or L-valine on the adhyperforin or hyperforin levels was detected in these cultures (I).

4.2 Isolation and sequence analysis of cDNAs encoding for type III PKSs from *H. perforatum*

Two cDNAs encoding for type III PKSs, in addition to CHS and benzophenone synthase (BPS) cDNAs, were isolated by a homology-based method with degenerated primers from *H. perforatum* (II). These two previously

uncharacterized genes were designated *HpPKS1* and *HpPKS2*. The full-length cDNA of *HpPKS1* was 1573 bp long, containing an open reading frame (ORF) of 1161 bp encoding for a 386 amino acid protein. The calculated molecular mass of the deduced protein was 42.4 kDa. The full-length cDNA of *HpPKS2* was 1559 bp containing an ORF of 1182 bp encoding for a 393 amino acid protein with a calculated molecular mass of 43.1 kDa. The deduced amino acid sequences of the HpPKS1 and HpPKS2 showed a 36% identity with each other.

Both HpPKS1 and HpPKS2 exhibited sequence homology at an amino acid level with the previously identified type III PKS family proteins (II). Most of the highly conserved amino acid residues including the catalytic triad cysteine-histidine-asparagine, characteristic to all type III PKSs, were conserved in both of the sequences. The deduced amino acid sequence of the full-length HpPKS1 showed at its highest an 80% identity with some putative CHS-like proteins with a so-far unknown function. HpPKS2 showed at its highest only a 56% identity with previously cloned CHS-family proteins. HpPKS1 and HpPKS2 showed only 23% and 21% deduced amino acid sequence identities, respectively, with *Streptomyces griseus* RppA, the bacterial type III PKS. Similarly, only 23% and 22% identities, respectively, were found with *Aspergillus oryzae* csyA, the fungal type III PKS.

In the constructed phylogenetic tree (Fig. 5), HpPKS1 grouped with functionally divergent non-chalcone-producing type III PKSs (unpublished data). The same was true for HpPKS2 (III, Fig. 5). However, the enzymes were positioned on sub-branches of their own without a particularly close relationship with any of the presently known, functionally characterized enzymes. The deduced amino acid sequence of HpPKS1 showed a 33–40% identity and HpPKS2 a 43–50% identity with the functionally divergent non-chalcone-forming type III PKSs.

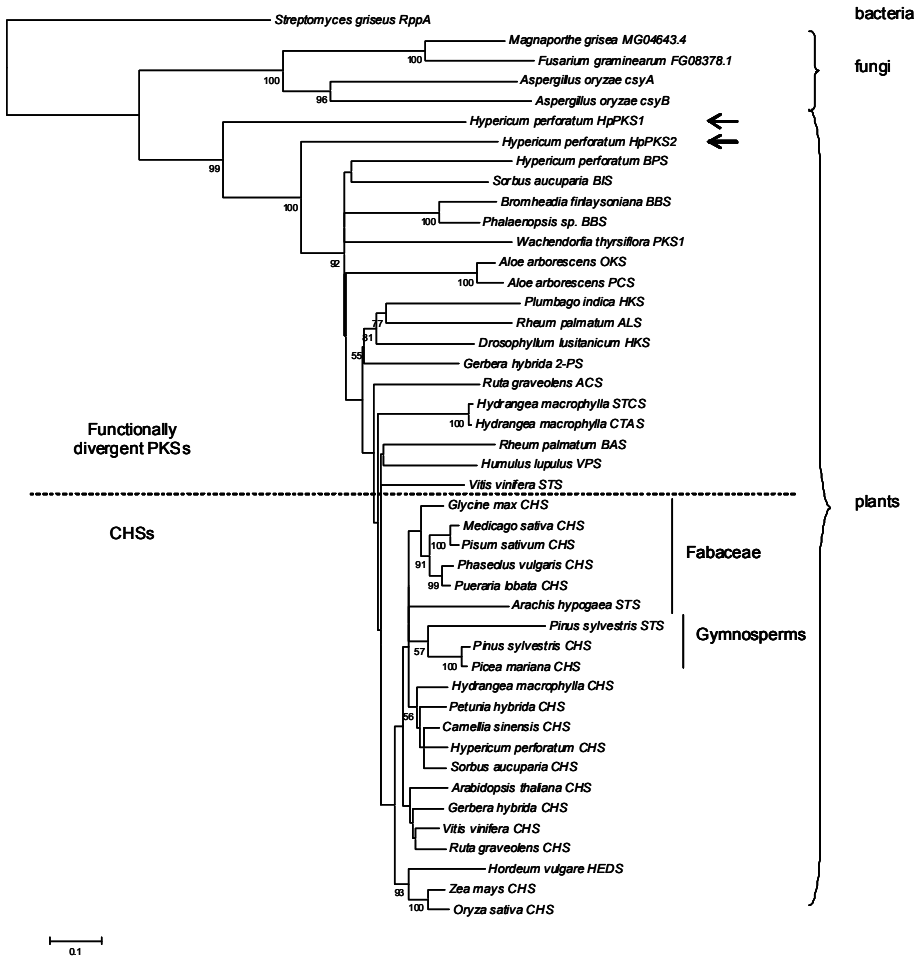


Fig. 5. Phylogenetic analysis of HpPKS1 and HpPKS2 with other type III PKS proteins.

4.3 Expression of *HpPKS1* and *HpPKS2* genes in *H. perforatum* tissues

The *HpPKS1* and *HpPKS2* genes were found to exhibit differential expression patterns in *H. perforatum* organs (II). The expression of *HpPKS1* was highest in flower buds, compared with the lower expression levels found in stems, leaf interior parts and leaf margins. Only a relatively weak expression of *HpPKS1* was detected in *H. perforatum* roots. The *HpPKS2* expression was highest in flower

buds and also relatively high in leaf margins containing dark glands, compared with the weak expression found in stems, roots and leaf interior parts free of dark glands.

The precise cell type location of the *HpPKS2* transcripts in *H. perforatum* organs was investigated by *in situ* RNA hybridization using DIG-labelled RNA probes (III). The *HpPKS2* mRNA was localized to leaf margins, flower petals and stamens in the tissue sections of leaves and flower buds of *H. perforatum*. The signal was specifically restricted to multicellular structures known as dark glands and dark canals. In the cross-sections of leaves and flower buds, these glands and canals could be seen to consist of a core of large cells that was surrounded by one to three flat cell layers. The *HpPKS2* transcripts were found to be present in both of the large cells and also in some of the innermost flat cells of the nodules and canals.

4.4 Expression of *hyp-1* gene in *H. perforatum* tissues

The expression of *hyp-1* was explored in *H. perforatum* organs (V). The *hyp-1* transcripts were abundantly found throughout the leaf tissue, with no significant difference observed in the expression levels between leaf margins and leaf interior parts. The expression was also high in intact roots but low in stems, flower buds and old parts of roots.

In situ RNA hybridization with DIG-labelled RNA probes localized the *hyp-1* mRNA in the area of vascular tissues in stems, roots and leaves. The signal was present in both the phloem and the xylem area. In the xylem, the signal was associated with xylem parenchyma cells in both the secondary and the primary xylem. In the phloem, the signal was associated with parenchyma cells, small companion cells and cells surrounding type A secretory canals. In roots, the *hyp-1* transcripts were also found in pericycle cells. The parenchyma cells in the stem cortex and especially in leaves also showed *hyp-1* expression. The signal in leaves was associated with both palisade and spongy parenchyma cells. No expression was present in cells of dark glands in the leaves (V).

4.5 Detection of Hyp-1 protein in *H. perforatum* tissues utilizing optimized protein extraction protocol

To enable the immunoblotting detection of the Hyp-1 protein, a protein extraction protocol was qualitatively and quantitatively optimized for highly phenolic *H.*

perforatum tissues (IV). From the tested extraction buffers and precipitation methods, the extraction with sodium borate buffer at pH 9 or 10 followed by phenol extraction and methanolic ammonium acetate precipitation was found to produce relatively high yields of high-quality proteins from all organs of *H. perforatum*. The protocol also showed to be suitable for protein extraction and electrophoretic analysis from other tested *Hypericum* species (IV, Fig. 6).

The optimized protein extraction protocol was utilized for immunoblotting to detect Hyp-1 protein in *H. perforatum* organs (IV, V). The highest levels of Hyp-1 protein were found in leaves, stems and intact roots, while flower buds and old parts of roots contained lower amounts of Hyp-1 protein. Leaf margins and leaf interior parts contained equal amounts of Hyp-1 protein (V). The presence of Hyp-1 was highest in leaves that were at a mature stage compared to young and senescent leaves (IV).

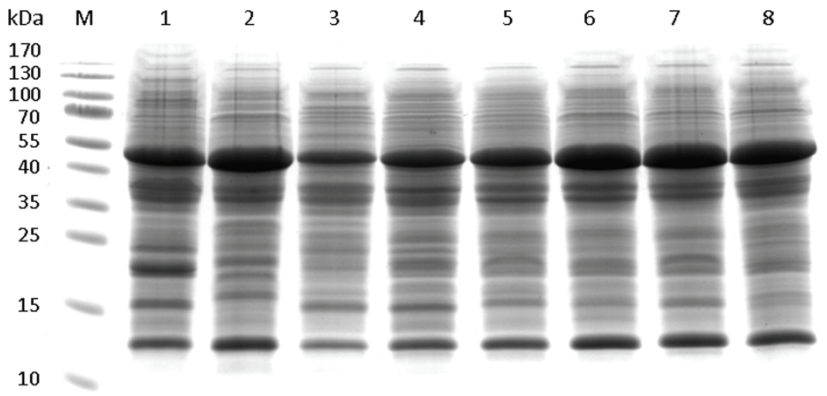


Fig. 6. SDS-PAGE evaluation of proteins from different species of genus *Hypericum* extracted with the method optimized in this study. Lane 1, *H. bupleuroides*; lane 2, *H. kalmianum*; lane 3, *H. balearicum*; lane 4, *H. tomentosum*; lane 5, *H. tetrapterum*; lane 6, *H. pulchrum;* lane 7, *H. erectum;* lane 8, *H. canariense;* lane M, protein molecular mass marker, with sizes (kDa) indicated on the left. Equal amounts of proteins were resolved on 12% polyacrylamide gel and visualized with Coomassie Brilliant Blue.

4.6 Presence of hypericins and hyperforins in *H. perforatum* tissues

The contents of hypericins and hyperforins were measured from *H. perforatum* organs in order to compare their presence with the expression of the *HpPKS1*,

HpPKS2 and *hyp-1* genes and levels of Hyp-1 protein. The highest amounts of both hypericins (hypericin + protohypericin) and pseudohypericins (pseudohypericin + protopseudohypericin) were found in flower buds and leaf margins. Only low levels were present in stems, leaf interior parts and roots (II, V). The contents of hyperforin and adhyperforin were also highest in flower buds, while lower values were measured in leaf interior parts, leaf margins and stems. The hyperforin and adhyperforin levels were lowest in roots (II).

In sections of leaves and flower buds of *H. perforatum*, the red hypericins were located in dark glands and dark canals present in leaf margins, flower petals and stamens. The hypericins were detected in large cells at the core of the glands but also in some of the innermost flat sheath cells (III).

4.7 Characterization of recombinant HpPKS1, HpPKS2 and Hyp-1 proteins

For the characterization of the HpPKS1 (unpublished data), HpPKS2 (III) and Hyp-1 (V) proteins, they were produced as recombinant proteins in *E. coli*. The purified recombinant HpPKS1 protein had a molecular mass of about 40 kDa on SDS-PAGE gel (Fig. 7), while the purified recombinant HpPKS2 protein was 43 kDa in size (III). Based on a size exclusion chromatography analysis, the HpPKS2 enzyme was a homodimer of about 86 kDa (unpublished data). The molecular mass of the purified recombinant Hyp-1 protein was about 18 kDa (V).

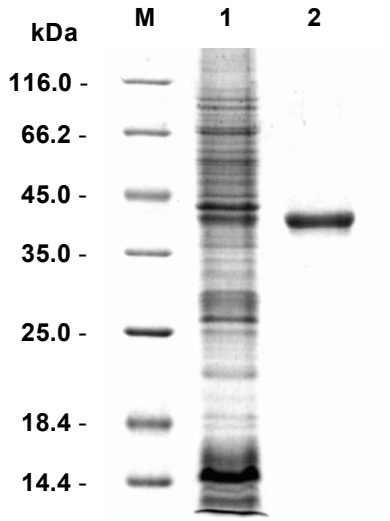


Fig. 7. SDS-PAGE analysis of recombinant HpPKS1 expressed in *E. coli*. Lane 1, soluble proteins from *E. coli* after induction with IPTG; lane 2, purified recombinant HpPKS1 protein; lane M, protein molecular mass marker, with sizes (kDa) indicated on the left.

4.8 Functional analysis of HpPKS2 recombinant protein

In order to clarify the role of *HpPKS2* in *H. perforatum*, the catalytic activity of the recombinant HpPKS2 protein was studied (III). Under *in vitro* conditions, the purified recombinant enzyme accepted various CoA-thioesters, such as acetyl-CoA, isobutyryl-CoA, benzoyl-CoA and hexanoyl-CoA, as starter substrates. The condensation of the starter substrates with malonyl-CoA led to a variety of tri- to octaketide products, including α-pyrones, chromones, phloroglucinols as well as phenylpyrones. OKS activity was only detected when acetyl-CoA was utilized as a starter unit. However, instead of emodin anthrone, the supposed octaketide precursor of hypericins, octaketides SEK4 and SEK4b were detected. The biosynthetic products catalysed by the recombinant HpPKS2 with the tested starter substrates are comprehensively shown in paper III.

5 Discussion

The presence of the biologically active compounds hypericins and hyperforins is characteristic to certain species of the genus *Hypericum*, such as *H. perforatum* (Kitanov 2001, Crockett *et al.* 2005, Ayan & Çirak 2008). Due to the high interest of the pharmaceutical industry for the compounds and the inefficiency of their chemical synthesis (Beerhues 2006, Michalska *et al.* 2010), the biosynthetic pathways and possibilities for alternative production methods, such as *in vitro* production of plant material and enzymatic *in vitro* synthesis of pure compounds, have attracted increasing attention. However, the biosynthesis and regulation of the production of both hypericins and hyperforins are still poorly understood.

5.1 Molecular methods in *Hypericum* research

Despite extensive chemical and pharmacological investigations of extracts of *Hypericum* species, the genus has scarcely been studied at a molecular level. Due to the high amounts of phenolic compounds in *Hypericum* tissues, interfering RNA, DNA as well as protein extraction has made working with the material challenging. Thus, the testing and optimization of molecular methods was necessary during the course of the present study (IV, Vuosku *et al.* 2004). Especially, studies at a protein level have not been conducted widely among *Hypericum* species and there are no earlier reports of immunoblotting analyses or proteomic studies, *i.e.* methods requiring high-quality protein preparations, carried out on organs of *Hypericum* species. In the present study (IV), several protein extraction and purification protocols were tested to find a method that is both qualitatively and quantitatively optimized for high-quality protein extraction from *H. perforatum* organs. The usefulness of the achieved protocol was demonstrated also for other *Hypericum* species, which suggests that in the future the method can be utilized more widely for proteomic studies among the genus *Hypericum*.

5.2 Biosynthetic pathway of hyperforins and role of HpPKS1

Hyperforin biosynthesis via a polyketide pathway in *H. perforatum* was for the first time suggested by Adam *et al.* (2002), based on feeding studies with isotopically labelled glucose and subsequent analysis of hyperforin by nuclear magnetic resonance (NMR) spectroscopy. The authors concluded that the

acylphloroglucinol nucleus of hyperforin could be generated via a polyketide mechanism by a condensation of three malonyl-CoAs with isobutyryl-CoA that could ultimately be derived from valine through α-ketoisovalerate. The suggested condensation of isobutyryl-CoA with three malonyl-CoAs to form hyperforin nucleus was later confirmed by Klingauf *et al.* (2005).

Data from the present study (I) confirm the predictions about the biosynthetic origin of hyperforin via a valine metabolism. The isotopically labelled valine that was fed to the *in vitro* shoot cultures of *H. perforatum* was found to be incorporated into the acyl side chain of the biosynthesized hyperforin. Thus, the results support the view that the acyl side chain of hyperforin is derived from the transamination of valine to α-ketoisovalerate following oxidative decarboxylation to isobutyryl-CoA (Fig. 8A). Additionally, the present study for the first time demonstrates the biosynthetic origin of adhyperforin from isoleucine metabolism (I). After feeding isotopically labelled isoleucine to the *in vitro* shoot cultures of *H. perforatum*, the labels were found to be incorporated into the acyl side chain of adhyperforin. Thus, it can be suggested that the acyl side chain of adhyperforin derives from the transamination of isoleucine to α-ketomethylvalerate and its subsequent oxidative carboxylation to 2-methylbutyryl-CoA, a starter substrate utilized by the type III PKS in the formation of adhyperforin nucleus (Fig. 8B). The described catabolic reactions of valine and isoleucine to isobutyryl-CoA and 2-methylbutyryl-CoA, respectively, probably take place in plant mitochondria (Binder *et al.* 2007).

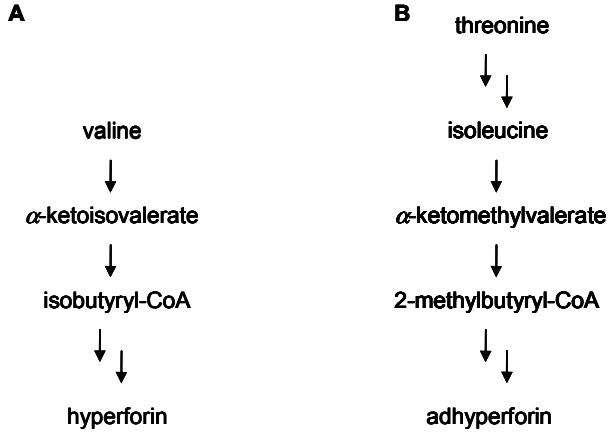


Fig. 8. The proposed catabolic route from branched-chain amino acids to (A) hyperforin and (B) adhyperforin.

The branched-chain amino acids have also earlier been shown to be utilized in the biosynthesis of some plant and bacterial polyketides (Moore & Hertweck 2002). The degradation products of branched-chain amino acids are employed as precursors in the biosynthesis of hop (*Humulus lupulus*) acylphloroglucinols that are produced through a polyketide pathway (Paniego *et al.* 1999, Okada & Ito 2001). Isobutyryl, 2-methylbutyryl and isovaleryl residues of hop bitter acid compounds have been suggested ultimately to derive from valine, isoleucine and leucine, respectively (Drawert & Beier 1976, Goese *et al.* 1999). The results presented in the current study support previous suggestions that bitter acids and hyperforins might be biosynthesized via a similar type of mechanism and that functionally similar type III PKSs might be involved in the biosynthesis of both compound groups (Klingauf *et al.* 2005, Beerhues 2006). A type III PKS showing both BUS and phlorisovalerophenone synthase (VPS) activity has been cloned from lupulin glands of hop (Okada & Ito 2001).

The involvement of a type III PKS in the biosynthesis of hyperforin has been verified by detecting BUS activity, catalyzing the condensation of one molecule of isobutyryl-CoA with three molecules of malonyl-CoA to form a hyperforin nucleus, in extracts of *H. calycinum* cell cultures (Klingauf *et al.* 2005). However, the gene encoding for the enzyme has not yet been isolated from the *Hypericum* species. Instead, some other type III PKS encoding sequences have earlier been found in certain species of the genus *Hypericum.* From *H. androsaemum,* cDNAs encoding for CHS and BPS have been isolated (Liu *et al.* 2003). CHS has also been cloned from *H. perforatum* (GenBank accession number AF461105). From *in vitro* shoot cultures of *H. hookerianum,* an aromatic type III PKS with a so-far unknown function has also been isolated (GenBank accession number EF186910).

In a search for new genes encoding for type III PKSs from *H. perforatum,* two previously uncharacterized cDNAs, designated *HpPKS1* and *HpPKS2,* were isolated in the present study (II). The sequence homology of the genes with the previously cloned type III PKSs, especially in the highly conserved amino acid residues, classify both of the genes as new members of the type III PKS family. Also the sizes of the produced recombinant proteins coincide with those reported earlier for type III PKS subunits (Schröder 2000, Shi *et al.* 2008, Flores-Sanchez & Verpoorte 2009). However, the deduced amino acid sequences of HpPKS1 and HpPKS2 were not particularly closely related to any of the functionally characterized enzymes to allow any predictions of their function in *H. perforatum*. In a phylogenetic tree, HpPKS1 and HpPKS2 grouped with the functionally divergent PKSs, indicating that they are not CHSs and thus do not attend to

flavonoid biosynthesis in *H. perforatum*. The low sequence similarity with the previously cloned type III PKSs can also explain why these cDNAs have not been isolated earlier from the genus *Hypericum*.

The expression of the HpPKS1 gene was found to correlate with the hyperforin and adhyperforin contents in *H. perforatum* organs (II). The *HpPKS1* expression and also the amounts of hyperforins were high in flower buds, compared with those in leaf interior parts, leaf margins, stems and especially roots. The distribution of hyperforin and adhyperforin in *H. perforatum* organs agrees with earlier reports on their occurrence in the plant (Repčák & Mártonfi 1997, Tekel'ová *et al.* 2000, Sirvent *et al.* 2003, Beerhues 2006). As the expression of *HpPKS1* was high in the aerial parts, especially in the reproducible parts of the plant, it is possible that the gene is involved in the accumulation of protective compounds against herbivores. Suggestions that hyperforins might function as a defensive compound against herbivores and other pests have also been made (Sirvent & Gibson 2002, Sirvent *et al.* 2003, Beerhues 2006). The expression pattern of *HpPKS1* could imply that if the hyperforins are biosynthesized at the same place where they accumulate in *H. perforatum*, *e.g.* translucent glands as suggested recently (Soelberg *et al.* 2007, Hölscher *et al.* 2009), then *HpPKS1* is a candidate gene encoding for the key enzyme in the biosynthesis of hyperforins. However, further investigations including functional analysis of the recombinant HpPKS1 protein, more detailed gene expression studies and possibly knock-out studies are needed to reveal the role of *HpPKS1* in *H. perforatum*.

5.3 Biotransformation and regulation of production of hyperforins

In vitro cultivation of *H. perforatum* has gathered great attention as an optional method for the production of less variable pharmaceutical preparations. In addition to the development of *in vitro* propagation protocols, investigations have been made in order to enhance the levels of pharmaceutically important compounds in the cultures. Strategies for improving the accumulation of both hypericins and hyperforins have mostly included the optimization of growth conditions and the addition of elicitors to the cultures (*e.g.* Kirakosyan *et al.* 2000, Kirakosyan *et al.* 2004, Gadzovska *et al.* 2007, Liu *et al.* 2007b, Pavlíc *et al.* 2007). Trials to enhance levels of hypericins by feeding an emodin precursor have also been conducted without success (Liu *et al.* 2007c). So far, no attempts at enhancing the production of hyperforins with proven precursors by biotransformation have been reported.

In the present study (I), the exogenous feeding with L-isoleucine as well as L-threonine, the precursor of isoleucine, was shown to enhance the adhyperforin production in *H. perforatum in vitro* shoot cultures. The results indicate that the endogenous isoleucine pool in *H. perforatum* shoots is low and a simple supplementation of a biosynthetic precursor is sufficient to elevate the adhyperforin production up to a certain level (Bourgaud *et al.* 2001). By contrast, the addition of exogenous L-valine could not enhance the hyperforin content in shoot cultures which shows that endogenous valine is not the limiting factor of hyperforin biosynthesis but that regulation takes place at the level of enzyme supply at some stage of the biosynthesis. At the moment, it is not known whether the same enzymes are responsible for the formation of both hyperforin and adhyperforin. It is possible that the same type III PKS and prenyltransferases are involved in their biosynthesis and the availability of precursors affects the production ratio of hyperforin and adhyperforin.

In the present study (I), the fast-growing compact callus aggregate cultures of *H. perforatum*, showing lower level of tissue differentiation and lower amounts of hyperforins compared with shoot cultures (Karppinen *et al.* 2006a), were not capable of biotransforming L-isoleucine into adhyperforin. The result indicates that a high level of tissue differentiation is required in the biosynthesis of adhyperforin and thus the biosynthesis of hyperforins may be associated with differentiated structures like many other plant secondary metabolites (Dörnenburg & Knorr 1995, Verpoorte *et al.* 2002). In agreement with this prediction, hyperforins have recently been found to accumulate in translucent glands of *H. perforatum* (Soelberg *et al.* 2007, Hölscher *et al.* 2009). Results from the current biotransformation studies show tight regulation of biosynthesis of hyperforins in *H. perforatum*. Therefore, further research concerning the biosynthesis of these compounds is needed in order to enhance efficiently their production in *H. perforatum* tissues for commercial purposes.

5.4 Role of HpPKS2 in biosynthesis of hypericins

Based on the biosynthesis of structurally related compounds and on the chemical synthesis of hypericin, an anthraquinone called emodin anthrone is predicted to be a biosynthetic precursor of hypericins (Seigler 1998, Falk 1999). The production of emodin anthrone is suggested to proceed via a polyketide pathway in which a type III PKS catalyzes the condensation of one acetyl-CoA with seven malonyl-CoAs to produce an octaketide chain that is further cyclized to form emodin

anthrone (Falk 1999, Bais *et al.* 2003, Kirakosyan *et al.* 2004, Zobayed *et al.* 2006, Michalska *et al.* 2010). So far, such an enzyme activity or the isolation of the gene encoding for the enzyme has not been reported.

The second new type III PKS encoding gene isolated in the present study, *HpPKS2,* showed an expression pattern that correlated with the accumulation of hypericins in *H. perforatum* tissues (II). The result indicates that the gene could be involved in the biosynthesis of hypericins. The *HpPKS2* expression and the contents of hypericins were highest in flower buds and also relatively high in leaf margins that contained dark glands but low in stems and roots as well as in leaf interior parts that were free of dark glands. The high expression in the reproducible parts and also in leaf margins indicates that *HpPKS2* can be involved in the accumulation of protective compounds, such as hypericins, that may act against herbivores (Agostinis *et al.* 2002, Onelli *et al.* 2002, Sirvent & Gibson 2002, Sirvent *et al.* 2003).

The enzymatic function of type III PKSs can seldom be predicted based on their sequence information. This is due to the fact that only a single amino acid substitution near the active site can change the preference of enzymes for starter substrates or a number of performed condensation reactions and lead to variable enzymatic products (Jez *et al.* 2002, Abe *et al.* 2005a, Abe *et al.* 2005b, Abe *et al.* 2006, Klundt *et al.* 2009, Mizuuchi *et al.* 2009). Thus, type III PKSs are commonly produced as recombinant proteins in *E. coli* to study their enzymatic activities under *in vitro* conditions (*e.g.* Liu *et al.* 2003, Abe *et al.* 2005a, Springob *et al.* 2007, Wanibuchi *et al.* 2007, Liu *et al.* 2010). Also a recombinant HpPKS2 protein was produced in *E. coli* to get insight into its enzyme activity and its capability of forming emodin anthrone, the suggested octaketide precursor of hypericins (III).

Incubation of the recombinant HpPKS2 enzyme with potential substrates under *in vitro* conditions gave no concluding results of its catalytic activity *in vivo*. Although the enzyme accepted acetyl-CoA as a starter substrate to form tri- to octaketide products, including octaketides SEK4 and SEK4b, no emodin anthrone was produced (III). However, the formation of octaketides by type III PKSs is rare and earlier OKSs have been characterized only from one plant species. Along with HpPKS2, the longest polyketides known to be produced by type III PKSs, octaketides SEK4 and SEK4b, are also produced by OKSs of *Aloe arborescens* (Abe *et al.* 2005a, Mizuuchi *et al.* 2009). SEK4 and SEK4b are not found in *Aloe* tissues and thus the OKSs are suggested to be involved in the biosynthesis of anthrones and anthraquinones, the compounds structurally resembling emodin

anthrone (Abe *et al.* 2005a, Mizuuchi *et al.* 2009). The formation of shunt products SEK4 and SEK4b by *Aloe* OKSs, rather than the correct cyclization of the produced linear octaketide chain to natural compounds is assumed to be caused by the lack of yet unidentified tailoring enzymes *in vitro* (Abe *et al.* 2005a, Morita *et al.* 2007, Mizuuchi *et al.* 2009). The proposed involvement of tailoring enzymes is supported by the fact that SEK4 and SEK4b are also formed as shunt products by a type II PKS of *Streptomyces coelicolor,* attending to the actinorhodin biosynthesis, when the enzyme is incubated in the absence of downstream tailoring enzymes (Fu *et al.* 1994, Khosla 2009). In fact, many type III PKSs have recently been found incapable of performing expected *in vivo* reactions under *in vitro* conditions. Although accepting the suggested starter substrate and performing a correct number of condensations with malonyl-CoA, the enzymes fail to cyclize the linear polyketide chain to predicted final products (*e.g.* Akiyama *et al.* 1999, Eckermann *et al.* 2003, Abe *et al.* 2005a, Brand *et al.* 2006, Springob *et al.* 2007, Jindaprasert *et al.* 2008, Mizuuchi *et al.* 2009). The phenomena have raised discussion if additional protein partners that stabilize the highly reactive linear polyketide chain, preventing non-specific cyclizations to occur, are more commonly recruited in the biosynthesis of plant polyketides than has earlier been realized (Austin & Noel 2003, Morita *et al.* 2007, Springob *et al.* 2007, Flores-Sanchez & Verpoorte 2009). Thus, the incapability of HpPKS2 to cyclize the produced octaketide chain to emodin anthrone suggests that additional protein partner is involved in the reaction. Type I and type II PKSs commonly utilize tailoring enzymes, such as reductases, cyclases, aromatases, oxygenases, *etc.* for the formation of specifically cyclized polyketide products (Fu *et al.* 1994, Staunton & Weissman 2001, Kurosaki *et al.* 2002, Austin & Noel 2003, Herweck *et al.* 2004, Kalaitzis & Moore 2004). So far, the only characterized example of such co-operative interaction of a type III PKS with a tailoring enzyme in plants is the involvement of polyketide reductase in the biosynthesis of 6′-deoxychalcone (Welle *et al.* 1991, Schröder 2000, Austin & Noel 2003).

Promiscuous substrate specificities under *in vitro* conditions have been reported widely for type III PKSs, also including CHSs (*e.g.* Austin & Noel 2003, Liu *et al.* 2003, Samappito *et al.* 2003, Abe *et al.* 2005a, Brand *et al.* 2006, Springob *et al.* 2007, Wanibuchi *et al.* 2007, Jindaprasert *et al.* 2008). Thus, it was not a surprise that the recombinant HpPKS2 also showed a broad substrate acceptance *in vitro* and was able to utilize, in addition to acetyl-CoA, also isobutyryl-CoA, benzoyl-CoA and hexanoyl-CoA as starter substrates (III). However, with these starter substrates HpPKS2 was not able to catalyze the

formation of octaketides but instead afforded shorter tri- to heptaketide products. The shorter products catalyzed by HpPKS2 were mostly α-pyrones with a linear keto side chain, but also chromones, phloroglucinols and phenylpyrones were detected. These compounds have not been described for *H. perforatum*. The incubation of type III PKSs with non-physiological substrates and in non-optimal assay conditions usually yields shorter derailment products, often pyrones, not found in the plant of their origin (Austin & Noel 2003). It has been suggested that the substrate availability and protein environment, rather than strict substrate specificities, are the major factors in determining the function of these enzymes *in vivo* (Brand *et al.* 2006). In plants, type III PKSs are usually confined to specific organelles and tissues or they may also be present in organized enzymatic complexes that determine their function (Flores-Sanchez & Verpoorte 2009). Thus, it is important to study the location of the enzymes or the corresponding mRNAs in the plant to get a comprehensive view of their function *in vivo*.

In the present study (III), the *HpPKS2* transcripts were specifically localized in multicellular structures in leaves and flower buds by *in situ* RNA hybridization analysis. These structures are known as dark glands and dark canals, and they are generally considered as sites for the accumulation of hypericins (Briskin & Gawienowski 2001, Pasqua *et al.* 2003, Zobayed *et al.* 2006). In the present study, the red hypericins were also shown to be situated in these multicellular structures (III). The exact same location of the *HpPKS2* transcripts with hypericins at both organ and cellular levels implies that if the hypericins are biosynthesized in addition to their accumulation in the dark glands and dark canals of *H. perforatum*, then *HpPKS2* is a strong candidate gene encoding for the key enzyme in the biosynthesis of the compounds. Suggestions about the biosynthesis of hypericins in dark glands have been presented already earlier (Onelli *et al.* 2002, Zobayed *et al.* 2006, Kornfeld *et al.* 2007). The first pieces of evidence to support the prediction was given by Zobayed *et al.* (2006) when emodin, an oxidative derivative of emodin anthrone, was detected in high amounts in *H. perforatum* tissues containing dark glands. Kornfeld *et al.* (2007) also suggested that the variation in the levels of accumulated hypericins between two *H. perforatum* germ lines could be due to the differences found in the dark gland structures between the two lines. The variations were found in a shape of the peripheral flat cells of the nodules, and it was suggested that the biosynthesis of hypericins occurs more likely in those cells than in the inner large cells of the nodules. In the present study (III), however, the *HpPKS2* transcripts were found to be located in both the inner large cells and in some of the innermost flat cells of the dark glands.

This indicates that if *HpPKS2* is involved in the biosynthesis of hypericins, then at least the formation of emodin anthrone, the early phase of the biosynthesis of hypericins, may take place in both cell types. Also hypericins were shown to be localized in both cell types in the present study. However, further studies concerning the involvement of HpPKS2 in the biosynthesis of hypericins are needed. Furthermore, the identification of the suggested tailoring enzyme(s) is necassary in order to reveal the functional mechanism of HpPKS2 and other type III PKSs that are not able to cyclize polyketide chain to natural products under *in vitro* conditions.

An endophytic fungus, *Thielavia subthermophila*, isolated from *H. perforatum*, was recently discovered to be capable of biosynthesizing emodin and hypericin (Kusari *et al.* 2008, Kusari *et al.* 2009). However, the fungus has not been suggested to be responsible for the biosynthesis of hypericins in *H. perforatum* because the fungus was found in stems but not in leaves of *H. perforatum.* Moreover, the fungus did not segregate its metabolites, excluding the possibility that hypericin and emodin are transported to dark glands (Kusari *et al.* 2009). Emodin anthrone, pseudohypericin or a gene encoding for Hyp-1, the enzyme suggested to be involved in the final stages of hyperforin formation, were not found in the fungus (Kusari *et al.* 2009). It has therefore been suggested that the fungus produces hypericin independently through a different pathway than its host (Kusari *et al.* 2009, Karioti & Bilia 2010). If HpPKS2 is the enzyme responsible for the first steps of hypericin biosynthesis in *H. perforatum*, it is not of fungal origin but part of the plant biosynthetic route, as shown in the present study by phylogenetic analysis (III).

5.5 Role of Hyp-1 in biosynthesis of hypericins

The final stages of hypericin biosynthesis have been suggested to be conducted by Hyp-1, a phenolic coupling protein belonging to a plant pathogenesis-related class 10 (PR-10) family (Bais *et al.* 2003, Radauer *et al.* 2008, Michalska *et al.* 2010). The gene encoding for Hyp-1 was isolated from undifferentiated cell suspension culture of *H. perforatum* by Bais *et al.* (2003). The recombinant Hyp-1 protein was under *in vitro* conditions able to catalyze a direct conversion of emodin to hypericin and, thus, the enzyme was suggested to be involved in the condensation reaction between emodin and emodin anthrone, followed by dehydration and phenolic oxidation, to yield first protohypericin and finally hypericin in *H. perforatum* tissues (Bais *et al.* 2003). However, the enzymatic reactions of the

recombinant Hyp-1 could not be reproduced in later experiments by Michalska *et al.* (2010) although the size and shape of the Hyp-1 protein active site was found to be compatible for binding two emodin molecules or one hypericin. The role of Hyp-1 in the biosynthesis of hypericins has also been questioned due to the lack of correlation between *hyp-1* expression and the presence of hypericins and dark glands in *H. perforatum* tissues (Bais *et al.* 2003, Košuth *et al.* 2007).

Data in the present study (V) confirm the lack of correlation between the *hyp-1* transcripts and the presence of hypericins in *H. perforatum* tissues. Similarly to the reports of Košuth *et al.* (2007), there was no difference in *hyp-1* expression between leaf margins that contained hypericin accumulating dark glands and leaf interior parts that were free of dark glands. The highest expression of *hyp-1* in *H. perforatum* organs in the present study was found in leaf tissues and intact roots, while considerably lower expression levels were present in stems and old parts of roots. In the current study (V), the *hyp-1* expression was for the first time also measured from the reproductive parts of the plant. Surprisingly, only low levels of *hyp-1* transcripts were present in flower buds, the primary sites for the accumulation of hypericins. The results concerning the *hyp-1* expression in vegetative plant parts are in agreement with the data obtained earlier by Košuth *et al.* (2007) from *ex vitro* plants. However, their results from *ex vitro* plants differentiated significantly from young *in vitro* seedlings, which showed relatively high *hyp-1* expression in roots. These findings indicate that the age of a plant and its organs significantly affects the expression of *hyp-1* in *H. perforatum* tissues. This is also supported by the current study (IV) that demonstrates differential presence of Hyp-1 protein in leaves at different stages of leaf development. Thus, Hyp-1 seems to be developmentally regulated similarly to many PR-10 family proteins (Pinto *et al.* 2005, Liu & Ekramoddoullah 2006, Radauer *et al.* 2008).

The results from the expression studies indicate that if *hyp-1* is the only gene responsible for the final stages of the biosynthesis of hypericins in *H. perforatum*, the regulation of the biosynthesis is complicated and post-transcriptional modification as well as some form of transportation must be involved, as has been suggested already earlier (Bais *et al.* 2003, Zobayed *et al.* 2006, Michalska *et al.* 2010). The *in situ* RNA hybridization analysis mainly located the *hyp-1* transcripts to the mesophyll cells of leaves as well as to the vascular tissues of stems, roots and leaves (V). The expression was present in cells of both the xylem and the phloem, enabling long-distance transportation of the small (17.8 kDa) monomeric Hyp-1 protein (Fernandes *et al.* 2008, Michalska *et al.* 2010). However, data on the Hyp-1 protein levels in *H. perforatum* organs do not support

the claim that the Hyp-1 protein would be transported into and would accumulate in the dark glands. The Hyp-1 protein was not present at any high levels in the tissues of dark glands, but the levels paralleled with the *hyp-1* expression in every organ, except in stems (V). Thus, it is possible that the Hyp-1 protein is mobile but its target is not the dark glands. The possible mobile nature of the Hyp-1 protein needs to be investigated in detail in the future.

For a so-far unknown reason, the expression and the location of many PR-10 proteins has been found in vascular tissues, similarly to *hyp-1* (*e.g.* Breda *et al.* 1996, Walter *et al.* 1996, Kim *et al.* 2004, Pinto *et al.* 2005, Kim *et al.* 2008). Although their function in plants is currently unknown, structural studies have suggested that their role could be involved in the binding and transportation of hydrophobic ligands involved in plant development or in defence-related signalling (Liu & Ekramoddoullah 2006, Radauer *et al.* 2008). Recently, Michalska *et al.* (2010) have suggested that the function of Hyp-1 may also be more likely related to the storage or transport of hypericin than in the actual biosynthesis of hypericin.

Both the accumulation and the biosynthesis of hypericins have been indicated to take place in the dark glands (Zobayed *et al.* 2006, Kornfeld *et al.* 2007). As emodin is also highly present in the dark glands and the transportation of hypericins from other tissues to dark glands seems unlikely (Zobayed *et al.* 2006), it is possible that Hyp-1 is not responsible for the biosynthesis of hypericins in the dark glands of *H. perforatum*. However, tissues lacking dark glands, such as undifferentiated cell cultures, are capable of accumulating hypericins to the vacuoles of cells, indicating that there are two possible sites of accumulation for hypericins in *H. perforatum* (Bais *et al.* 2002, Walker *et al.* 2002, Gadzovska *et al.* 2007). It should be remembered that Bais *et al.* (2003) isolated the *hyp-1* cDNA from undifferentiated cell cultures. Thus, it is possible that there are also two different genes responsible for the biosynthesis of hypericins in two different places. However, more studies are needed to test the hypothesis and to determinate the exact physiological role of Hyp-1 in *H. perforatum*.

During the current study, several genes encoding for PR-10 family proteins showing sequence homology to *hyp-1* were isolated from *H. perforatum* (data not shown). This indicates that *hyp-1* is part of a bigger PR-10 gene family in *H. perforatum*, similarly to many other plant species (Liu & Ekramoddoullah 2006, Handschuh *et al.* 2007, Kim *et al.* 2008). Where these genes are expressed in *H. perforatum* and whether they show any enzymatic activity will be a subject of future studies.

6 Conclusions and future prospects

Despite the enormous pharmacological interests toward hypericins and hyperforins of *H. perforatum* today, there is still only scarce information available about their biosynthesis. This thesis and the original papers elucidate the biosynthesis of both hypericins and hyperforins in *H. perforatum*. Furthermore, an optimized method for extracting high-quality proteins from *Hypericum* tissues suitable for electrophoretic protein analyses is described.

The results of the current study proved for the first time that there is an active biosynthetic pathway from branched-chain amino acids of primary metabolism to hyperforins in *H. perforatum*. Valine was shown to be incorporated into the acyl side chain of the hyperforin nucleus, while isoleucine served as a precursor for the acyl side chain of the adhyperforin nucleus. Furthermore, the proven precursors were for the first time used for the biotransformation of adhyperforin. The adhyperforin levels in *H. perforatum in vitro* shoot cultures were enhanced after the addition of L-isoleucine or L-threonine, a precursor of isoleucine, to the culture media. By contrast, L-valine did not increase the hyperforin content in the shoot cultures, demonstrating the strict regulation of the hyperforin biosynthesis in *H. perforatum*. This shows the importance of further research on the biosynthesis and its regulation in order to enhance efficiently the production of hyperforins in *H. perforatum* tissues.

The first step in isolating the key enzymes, type III PKSs, of the polyketide pathway leading to hypericins and hyperforins was taken. Two previously uncharacterized cDNAs encoding for type III PKSs were isolated from *H. perforatum*. The expression of these novel genes, named *HpPKS1* and *HpPKS2*, were found to correlate with the contents of hyperforins and hypericins, respectively, in *H. perforatum* tissues. The results show that these new genes are good candidates involved in the biosynthesis of hypericins and hyperforins in *H. perforatum*. Furthermore, a closer study of the *HpPKS2* transcripts by *in situ* RNA hybridization demonstrated their presence specifically in the multicellular dark glands in which hypericins accumulate. The functional analysis of the recombinant HpPKS2 showed expected OKS activity using acetyl-CoA as a starter substrate. However, the incapability of HpPKS2 to cyclize the produced octaketide chain to emodin anthrone, the supposed octaketide precursor of hypericins, strongly suggests that additional enzymes are involved in the reaction. Thus, further studies are needed to elucidate the reasons for this failure to reveal the biosynthetic mechanisms of hypericins as well as many other polyketide

compounds whose biosynthesis have been suggested to involve additional tailoring enzymes. In the future, the isolation of the proposed tailoring enzyme(s) from *H. perforatum* and the crystallization of the HpPKS2 protein to reveal its structure and reaction mechanism are of interest. Currently, functional analyses with recombinant HpPKS1 are under way.

No correlation between *hyp-1* expression and the presence of hypericins in *H. perforatum* tissues was found in the current study. We also demonstrated for the first time that the Hyp-1 protein levels were not related to the contents of hypericins in the tissues but paralleled with the mRNA levels in all tissues except in stems. The high Hyp-1 protein level in stems together with the fact that we found *hyp-1* expression to be associated with vascular tissues might be an indication of the mobility of the small Hyp-1 protein. However, our results do not indicate any targeting and accumulation of the Hyp-1 protein into the tissues of dark glands, which would support earlier suggestions that *hyp-1* is responsible for the hypericin biosynthesis in *H. perforatum*. Whether Hyp-1 is involved in the biosynthesis of hypericins in tissues other than dark glands or whether its function is associated only with the binding or transport of hypericin remains to be elucidated in future studies. Furthermore, the roles of the other PR-10 family genes isolated from *H. perforatum* provide interesting research subjects for the near future.

Gathering knowledge on the biosynthesis of hypericins and hyperforins has just recently started and more studies are needed before the pathways are fully solved. The present study provides both new knowledge as well as improved methods to facilitate the elucidation of their biosynthesis. The modern technologies, such as new generation sequencing and developed transformation methods for *Hypericum* species, offer new type of views to the study of the biosynthesis as well as its regulation. Results from these studies will provide new possibilities for alternative production methods for these pharmaceutical compounds that are in high demand.

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ISBN 978-951-42-6309-5 (Paperback)
ISBN 978-951-42-6310-1 (PDF)
ISSN 0355-3191 (Print)
ISSN 1796-220X (Online)

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