Endophytes
as alternative paclitaxel sources:

chemistry and genetics of *Taxomyces andreanae* and the endophytic flora of *Wollemia nobilis*

Agata Staniek
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Proefschrift

Promotores: Prof. dr. O. Kayser
Prof. dr. W.J. Quax

Beoordelingscommissie: Prof. dr. W. Baer-Dubowska
Prof. dr. E. Leistner
Prof. dr. R. Verpoorte

i just want to stay curious

Heath Ledger

Contents

Chapter 1

Introduction & scope of the thesis

Pharmaceutical Biology perceives plants as 'bio-factories' of potentially valuable therapeutic compounds. Even now, in the era of modern chemistry, technology and automation, it is impossible to underestimate the impact of plant-derived natural products as pharmaceutically relevant lead compounds. In fact, world's most universally used drug Aspirin[TM] is a derivative of salicylic acid originally obtained from white willow (*Salix alba*).

Plants, like all other organisms, live immersed in a thriving community of microbes. The diversity of fungi, oomycetes and bacteria with which plants co-exist can bring both plague and benefit. However, parasitic and symbiotic associations are merely the two extreme outcomes of a continuum of interorganismal interactions. Remarkably little is understood about plant--microbe interplay that is, at first glance, symptomless. Complex communities of poorly studied plant-associated microbes, endophytes could prove to be a yet untapped reservoir of natural products bearing pharmaceutical potential. The more we understand how plants tame, thwart and succumb to their 'bugs' and *vice versa*, the more likely we will be able to extract new resources for potentially novel and successful disease treatments.

The following section of the hereby presented thesis (**chapter 2**) gives an extensive overview of the current state of knowledge about endophytes. Starting with the disambiguation of the very definition of endophytic organisms, it gives account of their impact on pharmaceutical, as well as agricultural arenas. Further, it focuses on the nature of the interactions between endophytes and their plant hosts, taking heed of the importance of evolutionary genetics and ecological factors. Moreover, the genetic background of endophytic biosynthetic pathways is discussed with an emphasis put on recent advances in functional genomics as a driving force for a better understanding of endophytic microbes and for their further exploiting as a source of therapeutically relevant compounds presumed to push forward the frontiers of drug discovery.

Paclitaxel, the world's first billion dollar anticancer medication, was originally derived from the inner bark of Pacific yew (*Taxus brevifolia*). While an array of reports on alternative, endophytic paclitaxel producers seemed to have caused quite a controversy over the past two decades, we carried out an in-depth investigation of *Taxomyces andreanae* – the very first

presumed endophytic synthesizer of the valuable antineoplastic diterpenoid (**chapter 3**).

The greatest botanical surprise of the last century, *Wollemia nobilis* is not only one of the oldest plant species on the globe, but was also reported to host a paclitaxel producing endophyte, *Pestalotiopsis guepinii*. Consecutive sections of the thesis deal with the *in vitro* culturing (**chapter 4**), chemistry (**chapter 5**) and endophytic flora (**chapter 6**) of the unique conifer.

Finally, **chapter 7** integrates the results of the studies described in the thesis and presents future perspectives.

Endophytes
exploiting biodiversity for the improvement
of natural product-based drug discovery

Agata Staniek, Herman J. Woerdenbag, Oliver Kayser

Abstract

Endophytes, microorganisms that colonize internal tissues of all plant species, create a huge biodiversity with yet unknown novel natural products, presumed to push forward the frontiers of drug discovery. Next to the clinically acknowledged antineoplastic agent, paclitaxel, endophyte research has yielded potential drug lead compounds with antibacterial, antiviral, antioxidant, insulin mimetic, anti-neurodegenerative and immunosuppressant properties. Furthermore, while being implicated in livestock neurotoxicosis, some endophyte-produced alkaloids have been shown to display insecticidal activity. The endophyte-host relationship is postulated to be a 'balanced antagonism'. Moreover, the plausibility of horizontal gene transfer (HGT) hypothesis is taken into account. Knowledge of the genetic background of endophytic natural product biosynthesis is discussed on the basis of loline alkaloids, ergopeptines, lolitrems and maytansinoids. The current dynamic progress in genomics will contribute to a better understanding of endophytic microbes and to further exploiting them as a source of pharmaceutically relevant compounds.

Introduction

Endophyte (Gr. *endon*, within; *phyton*, plant) – the term was first coined by de Bary (de Bary, 1866) and has become deeply embedded in the literature ever since. At present, endophytic organisms are defined as 'microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects' (Stone *et al*., 2000). First reports describing these microbes date back to the turn of the 19th and 20th century (Freeman, 1904). The most frequently encountered endophytes are representatives of the fungi; however, the existence of many endophytic bacteria has been documented as well.

Of the nearly 300 000 plant species inhabiting our planet, each individual one is host to several to hundreds of endophytes (Tan & Zou, 2001), creating an enormous biodiversity: a myriad of undescribed species, a rich source of novel natural products therefrom and an unknown genetic background of all the interdependencies thus implied.

Several reviewers addressed the issue (e.g. Tan & Zou, 2001; Strobel & Daisy, 2003; Owen & Hundley, 2004; Schulz & Boyle, 2005; Gunatilaka, 2006), shedding light onto selected aspects of the nature of endophytes. The aim of the hereby presented study is to render a comprehensive overview of the current knowledge on the subject. Considerable emphasis is put on the role and potential of metabolic engineering (Tyo *et al*., 2007) and combinatorial biosynthesis (Floss, 2006), as well as on their impact on exploiting the astounding diversity of the endophytic world for its pharmaceutical potential.

Endophytes as biological factories of functional metabolites

Endophytic natural products as drugs and novel drug leads

It seems impossible to underestimate the impact of natural products on the drug discovery process. Even with lack of support from the major pharmaceutical companies, arguing that the natural product screening paradigm established in the late 1980s and early 1990s was no longer compatible with the HTS (high-throughput screening) approach, the significance of secondary metabolites as a source of novel drugs and drug-leads is still alive and well. Over 20 natural-product-derived drugs have been launched onto the worldwide market from 2001 to 2005 and around 140 have undergone various stages of clinical development in all major therapeutic areas (Butler, 2005; Lam, 2007).

The recent development and implementation of new technologies offers unique opportunities in the screening of natural products and will re-establish them as a major source for drug discovery. Improved natural product sourcing, aiming at narrowing the focus from all available sources to a single most prolific and reliable one, points to microorganisms as the ultimate, readily renewable, reproducible and inexhaustible source of novel structures bearing pharmaceutical potential. It is believed that up to 99% of microorganisms have yet to be discovered (Davis *et al*., 2005). This undefined richness of microbial world encompasses the plethora of endophytic entities occupying utterly millions of unique biological niches (higher plants) in various, many a time unusual, environments (Strobel, 2006).

Functional metabolites of endophytic origin have already demonstrated a considerable potential to impact the pharmaceutical arena (Tan & Zou, 2001; Strobel, 2003; Strobel & Daisy, 2003; Strobel *et al*., 2004; Gunatilaka, 2006). A few examples are presented in this section, with the focus on their presumed therapeutic significance.

As the anti-infective branch is experiencing a shortage of lead compounds progressing into clinical trials, new antibacterial templates with novel mechanisms of action should have advantages over known antibiotics, especially in the fight against multi-drug resistant bacteria and emerging pathogens. Guanacastepene (Figure 1), a novel diterpenoid produced by a fungus isolated from the branch of *Daphnopsis americana* growing in Guanacaste, Costa Rica, might prove a representative of a potentially new class of antibacterial agents, showing activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant *Enterococcus faecium* (Singh *et al*., 2000).
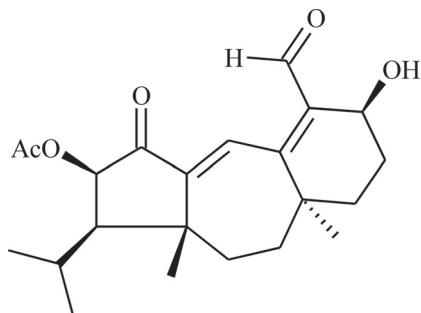

Figure 1. Guanacastepene

Although apparently the potential for the discovery of endophytic compounds having antiviral activity is in its infancy, probably due to absence of appropriate antiviral screening systems in most compound discovery programmes, some promising fungal metabolites have been found. Initially, two novel tridepside human cytomegalovirus (hCMV) protease inhibitors, cytonic acids A and B were isolated from *Cytonaema* sp. inhabiting the internal tissues of *Quercus* sp. (Guo *et al*., 2000). Further studies of the microbial flora characteristic of oak trees resulted in the isolation of a potentially valuable fungal specimen from the leaves of *Quercus coccifera*. This endophyte proved to be a synthesizer of hinnuliquinone (Figure 2) – a potent inhibitor of the HIV-1 protease (Singh *et al*., 2004).
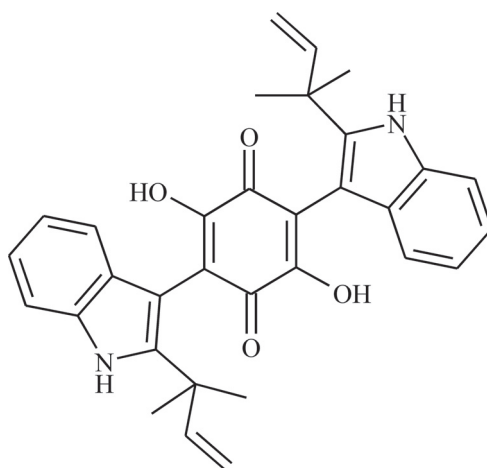
Figure 2. Hinnuliquinone

Two isobenzofuranones, pestacin and isopestacin (Figure 3), with structural similarity to the flavonoids – a well-established group of free-radical-scavengers, proved to exceed the anti-oxidant activity of trolox (a vitamin E derivative) by at least one order of magnitude, as measured by the total oxyradical scavenging capacity (TOSC) assay. These new potent antioxidants were obtained from *Pestalotiopsis microspora*, an endophyte of *Terminalia morobensis* inhabiting the Sepik River drainage of Papua New Guinea (Strobel *et al*., 2002; Harper *et al*., 2003).
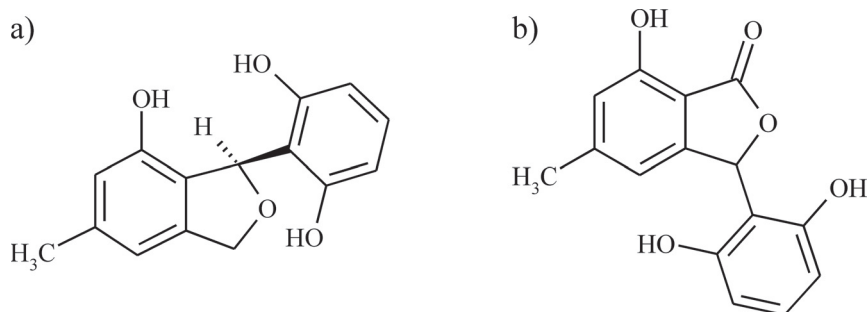
Figure 3. Pestacin (a) & isopestacin (b)

Pseudomassaria sp., a fungal endophyte recovered from leaves of an undetermined plant collected near Kinshasa, Democratic Republic of Congo, was shown to be a biofactory of nonpeptidal insulin mimetic, L-783,281 (Figure 4). Its discovery prompted quite a revolutionary notion in the therapy of diabetes, namely: an orally administered activator of the human insulin receptor (Zhang *et al.*, 1999).
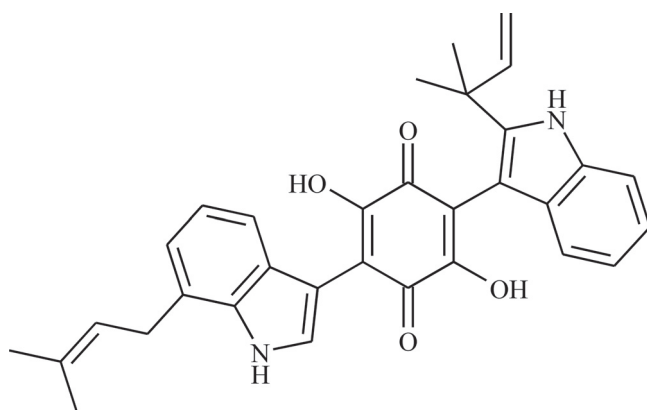
Figure 4. L-783,281

Moreover, this intriguing endophytic metabolite was reported capable of stimulating the *Trk* family of tyrosine kinase receptors, leading to the activation of multiple signalling cascades, culminating in neuroregenerative effects, including neuronal survival and neurite outgrowth (Wilkie *et al.*, 2001). Although the cytotoxicity of the compound seems to preclude its direct therapeutic application, it is a prototype for small molecule insulin and neurotrophin mimetics, and may lead to the development of pharmaceuti-

cally significant compounds for the treatment of diabetes and neurodegen-erative disorders.

Following the track of search for new immunosuppressants from endo-phytes resulted in the isolation of subglutinol A (Figure 5) and B. These diterpene pyrones from *Fusarium subglutinans*, harboured by the perennial twining vine *Tripterygium wilfordii*, showed substantial immunosuppressive activity while causing none of the detrimental cytotoxic effects characteristic of cyclosporine A (Lee *et al*., 1995; US Patent 5648376, 1997).
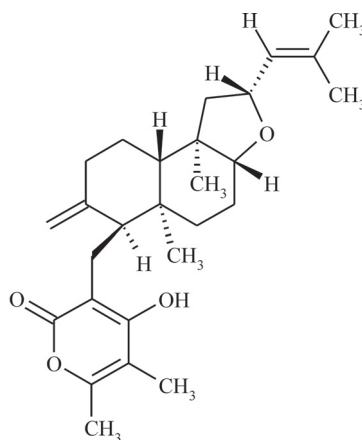
Figure 5. Subglutinol A

Amongst the bioactive natural products of endophytic origin described to date, the ones notable for their antitumour activity seem to have drawn quite an unequivocal attention, with paclitaxel (Taxol) as the most striking example (Figure 6).

Figure 6. Paclitaxel

Since its discovery in the 1960s, through structure elucidation completed in 1971 and FDA approval in 1992, this highly functionalized diterpenoid natural product has evolved to become a blockbuster drug with commercial sales of well over $3 billion in 2004 (Croteau, 2005). Since it was primarily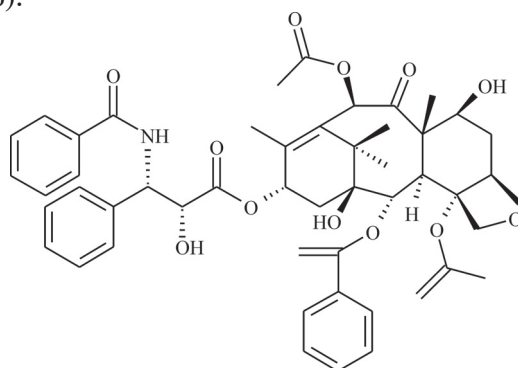 obtained from the inner bark of *Taxus brevifolia*, a relatively rare and slow-growing tree, the supply of this potent antineoplastic agent was soon to become the issue of scarcity, as even early estimations indicated that the demand for paclitaxel might exceed 300 kg, which would amount to 750 000 yew trees per year (Stierle *et al.*, 1995). The supply crisis, as well as the ecological implications resulting in the plant-endangered status, prompted the search for paclitaxel-producing microorganisms among the endophytic fungi of *Taxus*. While the 1990s brought quite an abundance of reports on paclitaxel-producing endophytes (e.g. Stierle *et al.*, 1993; Strobel *et al.*, 1996; Strobel *et al.*, 1997; Li *et al.*, 1998a&b; Noh *et al.*, 1999; Zhou & Ping, 2001), no conclusive follow-up data concerning fungal metabolite profile or genetic background of the biosynthetic pathway leading to paclitaxel in fungi is available at present. While the pursuit of the idea of a microbial paclitaxel source, providing for an inexhaustible supply of this antineoplastic blockbuster and novel taxanes, is being hampered by obtaining disappointingly low yields, the active ingredient is still mainly derived via chemical semisynthesis from the advanced taxoid, 10-deacetylbaccatin III, readily available from the needles of the European yew tree, *Taxus baccata*, being a renewable source and, to a lesser extent, by means of plant cell culture methods (Leistner, 2005; Frense, 2007). Nonetheless, the exciting progress that has been made in the elucidation of biosynthetic route leading to paclitaxel *in planta* due to fundamental works of Croteau and his co-workers (Walker & Croteau, 2001; Jennewein *et al.*, 2004a&b; Croteau, 2005; DeJong *et al.*, 2006; Nims *et al.*, 2006), as well as the recent advances in microbial genomics (Zazopoulos *et al.*, 2003; Stephanopoulos *et al.*, 2004; Keller *et al.*, 2005; van Lanen & Shen, 2006) and combinatorial biosynthesis (Nguyen, 2006; Floss, 2006; Klein-Marcuschamer *et al.*, 2007), might still revive and boost the interest in endophytic paclitaxel synthesizers.

Agricultural aspects: endophytic insecticides vs. tremorgenic mycotoxins

As the world becomes wary of ecological damage done by synthetic insecticides, endophytic research continues for the discovery of powerful, selective and safer alternatives (Strobel & Daisy, 2003).

From 1930 to 1970, millions of hectares in the temperate transition zone of the south central and southeastern USA were converted from revegetation shrubs and weeds to pastures of tall fescue (*Festuca arundinacea*). This grass was superior forage to native flora, but it was soon noticed that the expected livestock productiveness was not being met. An associated complex of symptoms episodically suffered by grazing livestock was termed 'tall fescue toxicosis' and consequently led to considerable economic losses to the US beef industry (Thompson & Stuedeman, 1993; Hoveland, 1993). Simultaneously, the livestock grazing on perennial ryegrass (*Lolium perenne*) pastures in New Zealand was afflicted by 'ryegrass staggers' (Gallagher *et al*., 1984; Bush *et al*., 1997). These problems spurred intense research that led to the isolation and identification of two respective fungal endophytes: *Neotyphodium coenophialum* and *Neotyphodium lolii*, both phylogenetically related to the ergot fungus *Claviceps purpurea* (Kuldau *et al*., 1997). Thus, the said endophytes were implicated in the associated livestock toxicoses, but both also greatly enhanced fitness of their plant hosts under biotic and abiotic stresses. Further investigation of the genus *Neotyphodium* (sexual state: *Epichloë*) proved its representatives capable of synthesizing four groups of alkaloids. Of these, the ergot alkaloids and tremorgenic lolitrems cause neurotoxic effects on grazing or granivorous vertebrates (Steyn & Vleggaar, 1985, Bacon *et al*., 1986). Peramine is an insect feeding deterrent and protects perennial ryegrass from the Argentine stem weevil, *Listronotus bonariensis*, a highly destructive insect pest in New Zealand (Rowan & Latch, 1994; Tanaka *et al*., 2005). Lolines are potent insecticidal and anti-aphid agents, not being implicated in any mammalian toxicoses (Jackson *et al*., 1996; Wilkinson *et al*., 2000). Obviously, the last of the aforementioned groups of alkaloids should provide for the most promising candidates to aid the agricultural arena.

As the forage grasses associated endophytes seem to constitute an extensively investigated and well documented microbial group (see further sections), they might prove to become a platform for the endophyte research to advance to the next level of modern microbial genomics and systems biology (Stephanopoulos *et al*., 2004; van Lanen & Shen, 2006).

Endophyte vs. host: the relationship

A conflict of interests?

Over the years, despite the controversy it seemed to have aroused in the early 1990s (Wennström, 1994), the term 'endophyte' has evolved from its original suggested definition, which described merely the location, to depict the nature of the association between the microbe and the host-plant (Wilson, 1995). To truly define the interaction, however, seems to be quite a task. In fact, it can vary in a seamless manner from mutualism to parasitism, based on a fine-tuned balance between the demands of the invader and the plant response (Kogel *et al.*, 2006).

A very elegant hypothesis has been proposed and extensively documented by Schulz and co-workers, postulating the relationship to be a 'balanced antagonism' (Figure 7) (Schulz & Boyle, 2005).

Figure 7. Balanced antagonism hypothesis (Schulz & Boyle, 2005)
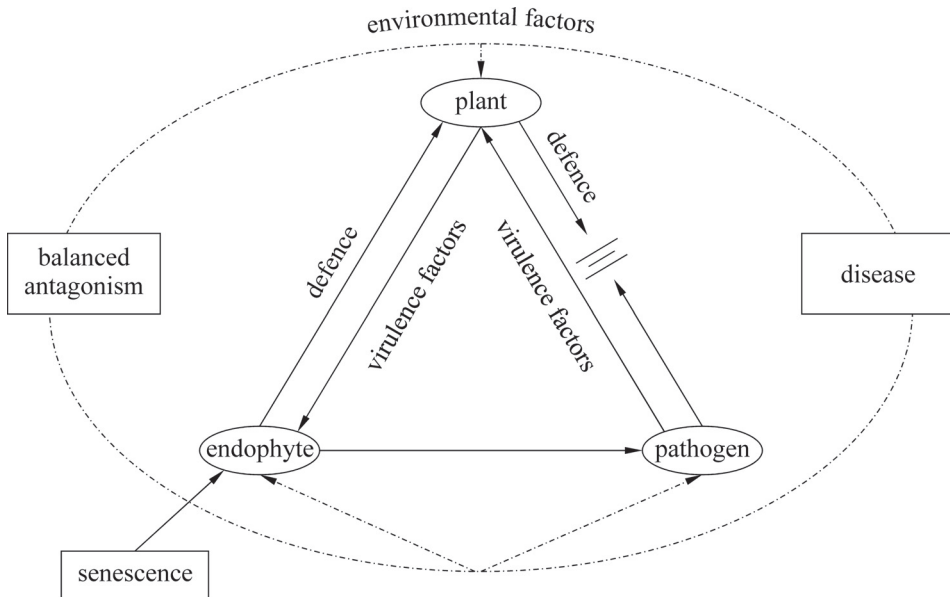
The said notion can be deciphered as equilibrium, under environmental, physiological and genetic control, that results in fitness benefits for both partners. On one hand, the theory depicts fungal endophytes as 'masters of phenotypic plasticity', able to infect as endosymbionts, to colonize cryptically, and finally to sporulate as pathogens or saprophytes. This creative

variability implies evolutionary potential. On the other hand, it does not ex-
clude the possibility of secondary metabolites being a contribution of the
endophytic partner to a mutualistic relationship. A very interesting example
of the protective power of an endophyte involves the aquatic plant *Rhyn-
cholacis penicillata* collected from a river system in Southwest Venezuela
where the harsh aquatic environment subjected the plant to constant beating
by virtue of rushing waters, debris, and tumbling rocks and pebbles. Such
circumstances should provide ample opportunity for pathogenic oomycetes
to invade the plant. Still, the plant population remained quite healthy. Upon
extraction and investigation of the endophytic flora of *R. penicillata*, a potent
anti-fungal bacterium, *Serratia marcescens*, was identified, which in turn
was shown to produce oocydin A (Figure 8), a novel anti-oomycetous com-
pound that obviously provided the plant with the requisite protection from
the water molds (Strobel *et al.*, 1999).
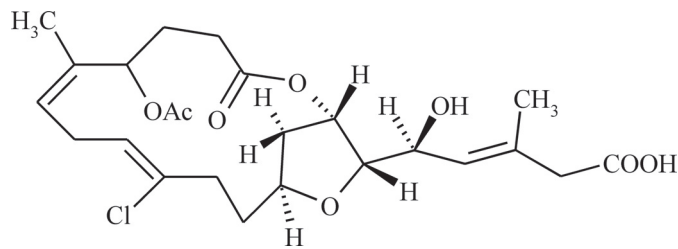
Figure 8. Oocydin A

Recently, it has been hypothesized that endophytes may protect their
host plants by scavenging the damaging reactive oxygen species (ROS)
generated by the plant defence mechanisms in response to environmental
stress (Rodriguez & Redman, 2005; Tanaka *et al.*, 2006). Thus, a strong acti-
vation of ROS due to biotic and abiotic stresses on part of the host followed
by an equally rapid free-radical-scavenging response of the microbe might
potentially be a prime mechanism in maintaining the delicate balance be-
tween the two.

To address the question asked in the title of this chapter: there is al-
ways a "conflict of interests" at all stages of relationships between endo-
phytic and plant partners (Smith & Read, 1997). However, the development
of tools for non-invasive observation of subcellular activities during the es-
tablishment of mutualistic interactions will provide a deeper understanding
of the mechanism that balances virulence against defence; hostility against
hospitality (Kogel *et al.*, 2006).

A relationship within a relationship

While fungal viruses have been shown capable of modulating plant-fungal symbioses by regulating the hypovirulence of pathogenic fungi (e.g. Dawe & Nuss, 2001; Ahn & Lee, 2001), the effect of mycoviruses on mutualistic fungal endophytes has not been described. But one report of a mycovirus from the well-known endophytic microbe, *Epichloë festucae*, was recorded (Zabalgogeazcoa *et al.*, 1998). Still, no decisive phenotypic traits have been associated with this virus since.

However, a very recent study reports a complex tripartite symbiosis involving a virus, a fungal endophyte and a plant (Márquez *et al.*, 2007). Thus far it was believed that the mutualistic relationship between a tropical panic grass from geothermal soils, *Dichanthelium lanuginosum*, and the fungus *Curvularia protuberata*, allowed both organisms to grow at high soil temperatures in Yellowstone National Park (Redman *et al.*, 2002). The in-depth investigation of this beneficial interaction showed, however, that the ability of the fungus to confer heat tolerance to its host plant is, in fact, related to a presence of yet another kind. To reflect its host of origin and its unique phenotype the responsible mycovirus was named *Curvularia* thermal tolerance virus (CThTV).

A physical interaction merely?
The plausibility of horizontal gene transfer (HGT) hypothesis

While enthusiasts call it 'the essence of the phylogenetic process and the driving force in a new paradigm for evolution' (Doolittle, 1999), sceptics describe it as no more than one of many phylogenetic anomalies (Kurland *et al.*, 2003). Though highly controversial, the hypothesis of horizontal gene transfer (HGT) seems quite seductive.

Hereby, two intriguing examples from the endophytic world are discussed. Both instances deal with the occurrence of identical natural products in unrelated taxa, namely: the host and the invader.

The presence of maytansinoids, potent cytotoxic agents, was first noticed in the Ethiopian shrub, *Maytenus serrata* (Kupchan *et al.*, 1972). Further investigation recorded their occurrence not only in higher plants (Wani *et al.* 1973; Ahmed *et al.*, 1981; Powel *et al.*, 1982), but also in mosses (Sakai *et al.*, 1988; Suwanborirux *et al.*, 1990) and, remarkably, in gram-positive *Actinomycetes* (Higashide *et al.*, 1977; Asai *et al.*, 1978). One could assume that the biosynthesis of these unique natural products has been repeatedly invented during evolution. However, the fact that approximately 48

genes are involved in the bacterial synthesis of maytansinoids (Yu *et al.*, 2002) makes it highly unlikely. Similarly, the aforementioned ubiquity of paclitaxel occurrence in yews as well as in taxonomically distant fungal microbes raises questions. Therefore, it seems possible that in the course of evolution a lateral (horizontal) gene transfer took place between different, taxonomically unrelated species, thus explaining the distant distribution of the antineoplastic secondary metabolites mentioned above between pro- and eukaryotes.

Nevertheless, before invoking HGT, alternative and often equally plausible, explanations ought to be thoroughly considered. In case of maytansinoids, all evidence seems to point to them being ultimately produced by plant associated microorganisms. Maytansine, the unique parent compound (Figure 9), was found neither in cell suspension cultures from *Maytenus buchananii* (Kutney *et al.*, 1981) nor in callus cultures raised from *Maytenus wallichiana* (Dymowski & Furmanowa, 1990) and *Putterlickia verrucosa* (Pullen *et al.*, 2003).
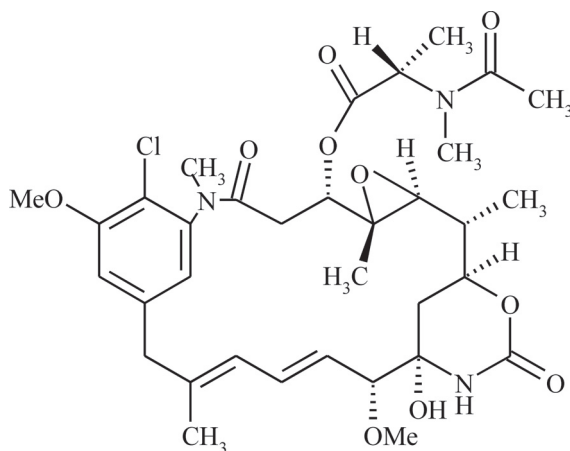
Figure 9. Maytansine

This is in line with the result of an in-depth search for the unique gene involved in maytansinoid biosynthesis, encoding for 3-amino-5-hydroxy-benzoic acid (AHBA) synthase, in *Putterlickia verrucosa* cell cultures. An extensive PCR based homology screen gave negative results only (Pullen *et al.*, 2003). These observations point to the conclusion that plants do not produce maytansinoids *ab initio*. However, an active role of the plant in an overall biosynthesis cannot be excluded, as it seems likely that the host converts a bacterially synthesized precursor into the final, biologically active

compound. Secondly, it is possible that maytansine is only produced as a consequence of a pathogen attack on the plant. The plants may contain a biologically inactive bacterially produced precursor, which is only converted into the potent final product in response to a signal resulting from the attack. Alternatively, and more plausibly, the bacterial production of the maytansinoid precursor could be triggered by a plant signal in response to the pathogen aggression (Cassady *et al*., 2004).

On the contrary, the bio-formation of paclitaxel seems to be a genuine feature of the yew host, as ample evidence supporting the production of the diterpenoid by sterile cell suspension cultures of *Taxus* has been provided (e.g. Ketchum & Gibson, 1996; Ketchum & Croteau; 1998; Yukimune *et al*., 2000; Wu & Lin, 2003; Naill & Roberts, 2005; Khosroushahi *et al*., 2006; Vongpaseuth & Roberts, 2007). This conclusion is further supported by the aforementioned work of Croteau and his associates who succeeded in the isolation of paclitaxel biosynthetic genes of plant origin. Interestingly, the taxadiene synthase gene has a long N-terminal targeting sequence for localization to and processing in the plastids, indicating that this gene is plant--derived rather then a fungal product (Koepp *et al*., 1995; Walker & Croteau, 2001). Accordingly, an extensive PCR based screen for taxadiene synthase gene in *Taxomyces andreanae*, the very first presumed endophytic taxane-producer (Stierle *et al*., 1993), failed to provide for any positive results (Staniek, unpublished data).

To sum up, the evidence for lateral gene transfer in eukaryotes remains largely anecdotal (Rosewich & Kistler, 2000). However tempting and attractive, the HGT hypothesis has to give way to a more plausible alternative postulating the endophyte-host co-evolution.

Investigating the genetic background of endophytic biosynthetic pathways: identification, molecular cloning, genetic analysis and manipulation of endophytic gene clusters

Clusters of functionally related genes are a general feature of prokaryotic gene organization, but were believed to be much less prevalent in eukaryotes. However, the advent of recombinant DNA methodologies in the 1980s enabled dramatic progress in the genetics and biochemistry of fungal secondary metabolism. This rapid progress was facilitated by what is now considered a hallmark characteristic of secondary metabolic biosynthetic pathways – the grouping of pathway genes in a contiguous cluster (Keller & Hohn, 1997; Keller *et al*., 2005).

The balansiaceous endophytes and their natural products

The balansiaceous endophytes form a unique group of closely related fungi with ecological requirements and adaptations distinct from those of other endophytes (Petrini, 1996). They belong to the clavicipitaceous genera *Epichloë* and *Balansia*, and their anamorphs *Neotyphodium* and *Ephelis* (Schardl *et al*., 2004).

As previously stated, due to their ecological and economic impact, theirs seems to be the best studied group of endophytes. The following section focuses on the recent advances in dissecting the molecular mechanisms driving the biosynthesis of their secondary metabolites.

Loline alkaloids (LA)

Unique not only in structure, comprising a saturated 1-aminopyrrolizidine-ring system, with a highly strained ether bridge between C-2 and C-7 (Figure 10) (Petroski *et al*., 1989), but also due to their potent, broad-spectrum insecticidal activities (Jackson *et al*., 1996; Wilkinson *et al*., 2000), loline alkaloids have remained a biosynthetic enigma till very recently (Blankenship *et al*., 2005).
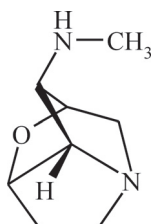
Figure 10. Loline

Establishing that the production of lolines in axenic cultures of *Neotyphodium uncinatum* can be regulated by culture conditions (Blankenship *et al*., 2001), which in turn suggests differential expression of genes involved in LA biosynthesis, prompted a notion that the isolation of the genes upregulated during loline production could be a first step in identifying possible enzymes catalyzing their biosynthesis. The recent development of novel highly effective methods for identifying differentially expressed transcripts, enabled the application of suppression subtractive hybridization technique (Diatchenko *et al*., 1996, 1999) and provided for a successful identification of two genes, *lolA* and *lolC*, associated with endophytic loline synthesis (Spiering *et al*., 2002).

These encouraging results spurred intense research on the molecular genetics of loline-alkaloid production, originating from a preliminary hypothesis of *lolA* and *lolC* being clustered (Spiering *et al*., 2005; US Patent 7183098, 2007). The question was tackled with an impressive arsenal of up-to-date techniques and ultimately resulted in the identification of double homologs of nine genes similarly arranged into two clusters, *LOL-1* and *LOL-2*. Long-range (LA: long and accurate) PCR and parallel screening of a partial genomic library of *N. uncinatum* to ascertain the existence of two highly similar, yet distinct gene clusters. Extensive genome walking into unknown regions yielding a total of 25 kb sequenced from *LOL-1* and 16 kb from *LOL-2*. Gene-prediction searches with the FGENESH programme (available from: http://www.softberry.com/berry.phtml) aided by RACE (rapid amplification of cDNA ends) and confirmed by reverse transcription-PCR based expression verification for identifying and mapping ORFs (open reading frames). Large-scale utilization of Basic Local Alignment Search Tool (NCBI) to ascribe the putative functions of the enzymatic entities encoded by the nine *lol* genes. And, last but definitely not least, the very first attempt of gene silencing by RNAi (RNA interference) performed on a mutualistic fungus, providing direct and indisputable evidence for involvement of *lolC* in loline biosynthesis.

All in all, an unprecedented endeavour leading to a final conclusion that the products of the *lol* genes thus identified may be sufficient for the biosynthesis of the entire loline-alkaloid three-ring structure in *Neotyphodium uncinatum*, from the primary precursor amino acids, L-proline and L-homoserine (Figure 11) (Blankenship *et al*., 2005; Spiering *et al*., 2005).
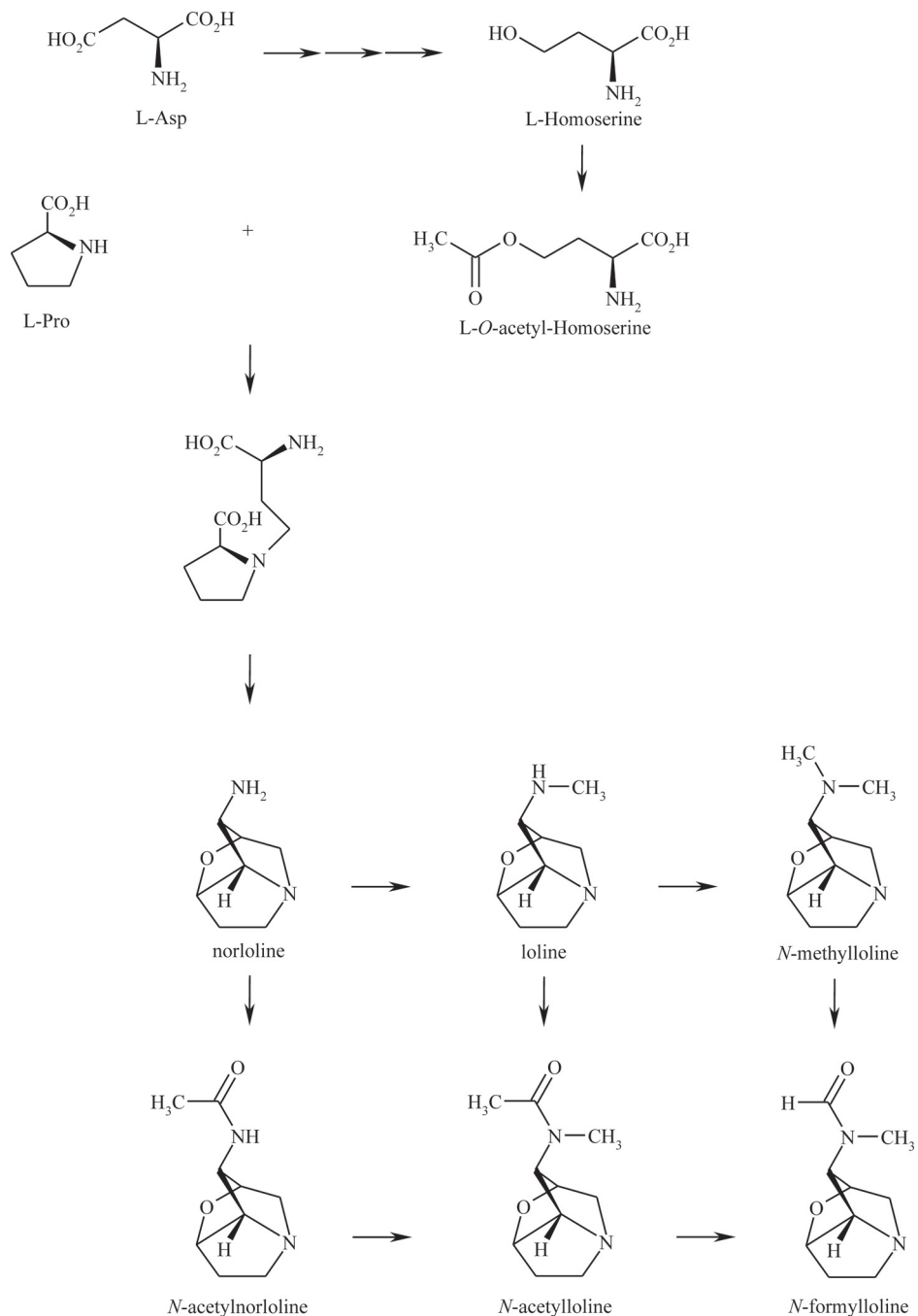
Figure 11. The loline alkaloid biosynthetic pathway (Blankenship *et al.*, 2005)

As spectacular as it may seem, the aforementioned accomplishment did not put a closure to solving the riddle of endophytic lolines. On the contrary, it contributed to further identification of single *LOL* gene clusters in *Epichloë festucae*, *Neotyphodium* sp. PauTG-1 and *Neotyphodium coenophialum* (Kutil *et al*., 2007).

To analyze the genetic architecture and to predict the evolutionary history of *LOL*, the five characterized clusters were compared by means of the coupled powers of phylogenetic approaches and other forms of sequence analysis. All *lol* genes anchored to the map for each species turned out to occur in strictly conserved order and orientation (Figure 12).
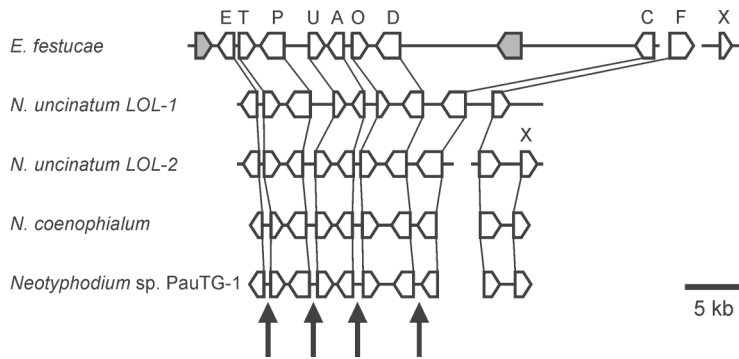
Figure 12. Comparative maps of *LOL* gene cluster(s) from four endophyte species; the shaded boxes in the *E. festucae* map indicate regions with homology to *pol* proteins; disconnected contig lines indicate genes that are sequenced, but not anchored to other *lol* genes; the contig of sequence known for this region in *E. festucae* continues beyond the boundaries shown; arrows along the bottom of the diagram indicate locations of putative promoter regions analyzed in the motif analysis (Kutil *et al*., 2007)

Furthermore, PhyloCon-based (Wang & Stormo, 2003) comparison of the putative *lol* gene promoter regions yielded the identification of four motifs conserved across the genes in all five clusters, with each motif having significant similarity to known fungal transcription factor binding sites in the TRANSFAC database (Wingender *et al*., 1996). Conservation of these motifs further supported the hypothesis of the *lol* genes being co-regulated (Spiering *et al*., 2002; Spiering *et al*., 2005).

Interestingly, the history of asexual *Neotyphodium* spp. includes multiple interspecific hybridization events (Tsai *et al*., 1994; Schardl, 2001; Clay & Schardl, 2002; Moon *et al*., 2004). Thus, comparing the clusters from

three seed-transmitted *Neotyphodium* anamorphs and their sexual counterpart, *E. festucae*, allowed determining which *Epichloë* ancestors were the most likely contributors of *LOL* in these asexual loline synthesizers. Perhaps the most striking outcome of this comparative analysis was that three *Neotyphodium* species with a history of *Epichloë typhina* as an ancestor possess nearly identical copies of *LOL* clusters (*N. uncinatum LOL-2*, *N. coenophialum*, *N.* sp. PauTG-1). This is most remarkable in light of the fact that there are no known isolates of the extant species *E. typhina* which express loline alkaloids (Leuchtmann *et al*., 2000). Thus, these data support a model of evolution in which the polymorphism in loline alkaloid production phenotypes among endophytic species is likely due to the loss of the trait over time (Kutil *et al*., 2007).

Ergopeptines

Ergot alkaloids are produced by ascomycetous fungi from discontinuous taxonomic groupings, including plant-associated fungal genera from the family *Clavicipitaceae* and some members of the order *Eurotiales*, including the human pathogen *Aspergillus fumigatus* (Panaccione, 2005). Due to their long biotechnological tradition, with manifold applications in therapy (Tudzynski *et al*., 2001), the physiology and biochemistry of ergopeptine formation have been studied in minute detail (Floss, 1976; Socic & Gaberc-Porekar, 1992; Gröger & Floss, 1998). Nevertheless, an uncomfortable premonition of scarcity in knowledge about the genetics of the biosynthetic route leading to these valuable alkaloids seemed to have arisen.

Yet again, the dynamic progress in the field of molecular genetics proved invaluable. The first step towards revealing the molecular background of ergopeptine biosynthesis was taken: the gene coding for dimethylallyl tryptophan synthase (DMATS) – the committed enzyme of the synthetic process in question (Figure 13a) – was cloned via reverse genetics (Tsai *et al*., 1995), identified by a differential cDNA screening approach (Arntz & Tudzynski, 1997) and characterized initially for *Claviceps fusiformis*, subsequently for *Claviceps purpurea* (Tudzynski *et al*., 1999), and finally for the phylogenetically distant *Aspergillus fumigatus* (Coyle & Panaccione, 2005; Unsöld & Li, 2005; Li & Unsöld, 2006).
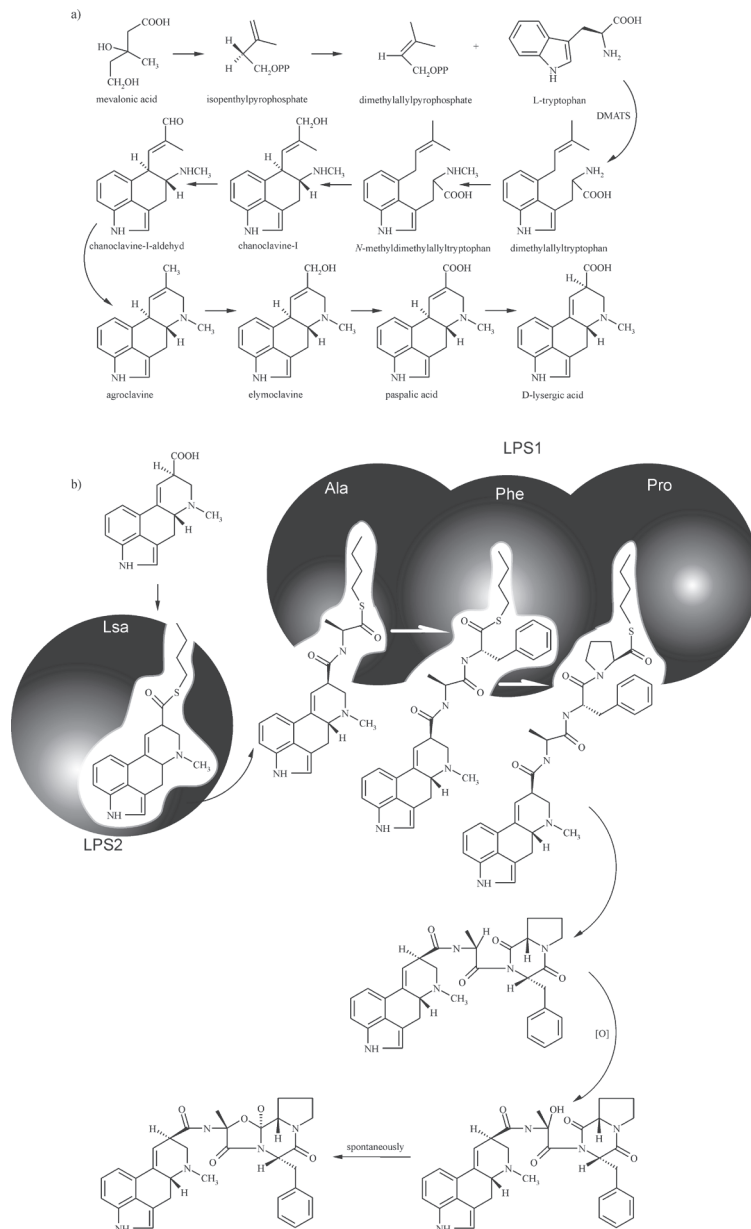
Figure 13. The ergot alkaloid biosynthetic pathway; biosynthesis of lysergic acid (a) & further ergopeptine formation, as exemplified by ergotamine (b) (Tudzynski *et al.*, 1999; Tudzynski *et al.*, 2001)

Fuelled by the conviction of secondary metabolite pathway genes clustering in fungal genomes (Keller & Hohn, 1997), large-scale studies have been taken up, resulting in the identification of gene clusters for ergot alkaloid biosynthesis in *C. purpurea* (Tudzynski *et al*., 1999; Correia *et al*., 2003; Haarmann *et al*., 2005; Haarmann *et al*., 2006) and *A. fumigatus* (Coyle & Panaccione, 2005; Unsöld & Li, 2005; Li & Unsöld, 2006); they contain 13 and 14 genes, respectively. It has been recently hypothesized that the fundamental ability to synthesize some type of ergot alkaloid was present in the most recent common ancestor of these *Ascomycetes*, accounting for the current phylogenetically discontinuous distribution of these secondary metabolites (Panaccione, 2005). Thus, genes shared between the two clusters are presumed to be responsible for the early steps common to the two organisms (Coyle & Panaccione, 2005).

The impressive achievements described hitherto, have been successfully conveyed into the realm of clavicipitaceous endophytic ergopeptine producers. *DMATS*, as a gene encoding for the enzymatic entity indispensable for ergot alkaloid production, was characterized for *Neotyphodium lolii* harboured by perennial ryegrass. Once the putative endophyte homolog was identified by degenerate PCR, its function was tested by gene knockout. Subsequent complementation experiments resulted in the ultimate confirmation of the postulated role of *DMATS* in the biosynthetic process in question (Wang *et al*., 2004). Analogous experimental procedure allowed for the characterization of two consecutive genes, displaying the capacity to encode nonribosomal peptide synthetases (NRPS), namely: *lpsA* (Pannacione *et al*., 2001; Panaccione *et al*., 2003) and *lpsB* (Tanaka *et al*., 2005; Fleetwood *et al*., 2007), playing a vital role in the ergopeptine formation (Figure 13b) (Riederer *et al*., 1996; Walzel *et al*., 1997; Correia *et al*., 2003). The ultimate objective: to determine whether ergot alkaloid biosynthetic genes were clustered in *Neotyphodium lolii* was pursued by using chromosome walking and Southern blot analysis and successfully attained (Fleetwood *et al*., 2007).

Interestingly, while gene sequence is relatively highly conserved between each of the three thus far identified ergot alkaloid gene clusters, there are several differences in gene order and the *N. lolii* cluster (*EAS*) seems to be more complex in structure and organization, as compared to its pathogenic counterparts (Figure 14). What is more, the BLASTN (NCBI) and MEME (Multiple EM for Motif Elicitation) (Bailey & Elkan, 1994) analyses of the *EAS* cluster revealed that its genes are closely associated with transpo-

son relics, including retrotransposons and autonomous as well as nonautonomous DNA transposons.
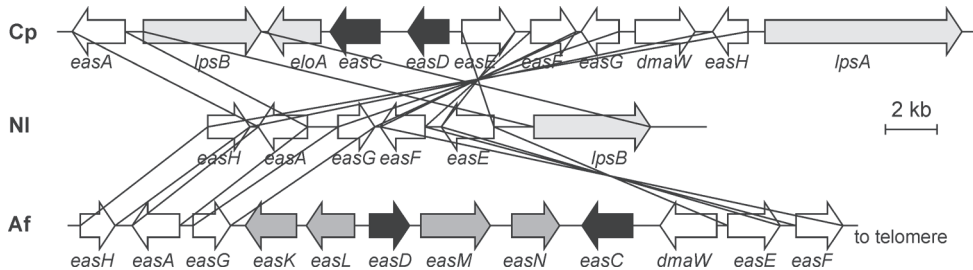
Figure 14. Comparative *eas* gene order among *C. purpurea* (Cp), *N. lolii* (Nl) and *A. fumigatus* (Af); black arrows, genes proposed to be required for ergot alkaloid synthesis, but not yet identified for *N. lolii*; light grey arrows, genes found in *N. lolii* and *C. purpurea*, but not in *A. fumigatus*; dark grey arrows, genes found only in *A. fumigatus*; *N. lolii dmaW, cloA* and *lpsA* are not shown, as their locations relative to *EAS* cluster are not known (Fleetwood *et al.*, 2007)

Finally, it has been shown that ergot alkaloids are specifically produced during biotrophic growth of *Epichloë* endophytes (Tanaka *et al.*, 2005). Attendant with this, the *eas* genes proved all to be expressed *in planta*, while giving no evidence for expression under axenic culture conditions. These results suggest that specific plant environment may be required for the induction of ergopeptine biosynthetic genes in the fungal endophyte (Fleetwood *et al.*, 2007).

Notably, ergot alkaloids have also been considered to constitute a chemotaxonomic signature of *Convolvulaceae* plants. However, recent studies on *Ipomoea asarifolia*, a representative of this dicotyledoneous plant family, indicated that the accumulation of the natural products in question depends, in fact, on the presence of a plant-associated clavicipitaceous fungus. Thus, it has been postulated that ergopeptines are not typical of the *Ascomycota*, the *Poaceae* or the *Convolvulaceae* alone, but, in contrast, they are very likely to prove a trait of a clavicipitaceous fungal taxon capable of colonizing certain higher plants in mutualistic symbiosis (Kucht *et al.*, 2004; Steiner *et al.*, 2006).

Lolitrems

Indole-diterpenes are a large, structurally diverse group of natural products commonly found in filamentous fungi of the genera *Penicillium*, *Aspergillus* and *Claviceps* (Mantle, 1987; Parker & Scott, 2004). Many of these compounds are potent mammalian tremorgenic mycotoxins (Steyn & Vleggaar, 1985) and fall into four major structural classes, namely the penitrems, janthitrems, paspalitrems and lolitrems (Figure 15) (Mantle & Weedon, 1994). They all share a common structural core comprised of a cyclic diterpene skeleton derived from geranylgeranyl diphosphate (GGPP) and an indole moiety derived from tryptophan, while different patterns of prenylation, hydroxylation, epoxydation and acetylation, and differences in ring stereochemistry around the basic indole-diterpene ring structure, are to be accountable for their structural diversity (Parker & Scott, 2004). The best known of these fungal tremorgens are the lolitrems produced by *Epichloë* endophytes in association with temperate forage grasses (Gallagher *et al.*, 1984).

The history of unravelling the molecular genetics underlying endophytic lolitrem production seems to encompass some of the elements of both the aforementioned biosynthetic puzzles of microbial alkaloids.

Initially, employing *Penicillium paxilli* as a model experimental system to dissect the biochemistry of indole-diterpene biosynthesis (Young *et al.*, 2001) (an obvious parallel can be drawn with the ergots and their model synthesizer: *Claviceps purpurea*) allowed for the isolation of three orthologous genes from the grass endophytes, *Neotyphodium lolii* and *Epichloë festucae*, and subsequent confirmation of their contiguous clustering (Young *et al.*, 2005). While further attempts to clone additional linked *ltm* genes in the endophytes by such approaches as inverse PCR were thwarted by the presence of large blocks of highly repetitive retrotransposon sequences flanking both sides of the cluster, an alternative strategy combining suppression subtractive hybrydization (a technique applied successfully in case of *LOL* characterization) with chromosome walking was taken up. As a result, two additional *LTM* gene clusters were identified and consequently proved to be linked with *LTM-1* (Young *et al.*, 2006).
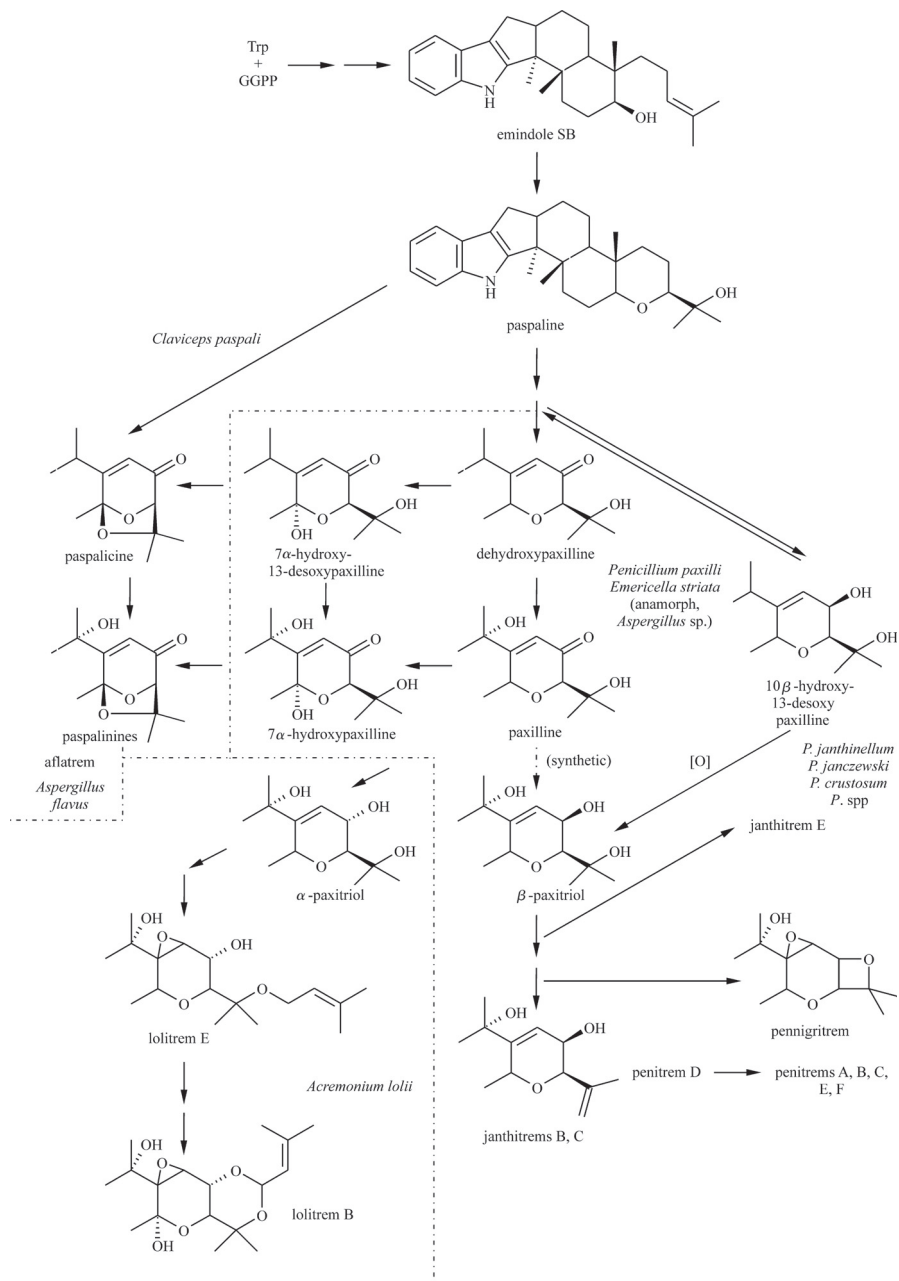
Figure 15. Proposed metabolic grid for indole-diterpenoid metabolites of particular species of *Acremonium*, *Aspergillus*, *Claviceps* and *Penicillium* linked according to sequential transformation in the diterpenoid moiety (Mantle & Weedon, 1994)

Ultimately, it has been shown that the genes for lolitrem biosynthesis in the endophytic anamorph *Neotyphodium lolii* form a complex genetic locus of at least three *LTM* gene clusters (Figure 16). What is more, the structure and organization of the said endophytic locus, as compared to the orthologous clusters from *Penicillium paxilli* (Young *et al*., 2001) and *Aspergillus flavus* (Zhang *et al*., 2004), has yet again proved to be of greater complexity (accordingly: *EAS* clusters of pathogenic vs. endophytic origin).

As sub-telomers are proposed to be 'a workshop of evolutionary genomic experimentation' (Wong & Wolfe, 2005), it has been suggested that the elaborate nature of the cluster in question may be accounted for by its location in such a rapidly evolving region of the endophytic genome (Gardner *et al*., 2002; Kellis *et al*., 2003; Machida *et al*., 2005; Nierman *et al*., 2005), and consequently driven by recombination and mutational processes associated with Type I transposon elements (Kempken & Kück, 1998) combined with the phenomenon of repeat induced point mutation (RIP) (Selker *et al*., 1987).
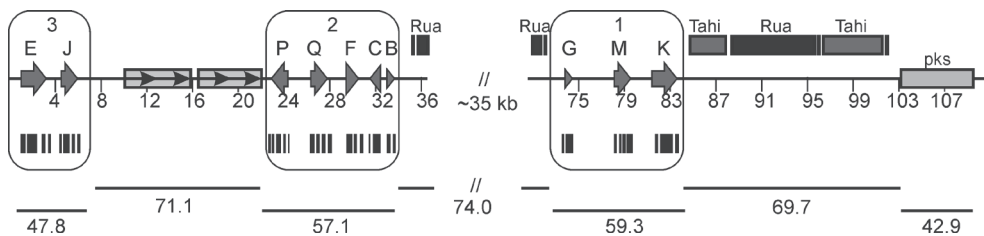
Figure 16. A physical map of the *N. lolii LTM* locus; the boundaries of the three *ltm* gene clusters are identified by boxes numbered 1 – 3; the genes, abbreviated to a single letter, are shown as arrows indicating the direction they are transcribed, with the exon structure shown as black blocks underneath each gene; the blocks between *ltm* clusters 2 and 3 show the positions of two imperfect direct repeats; the retrotransposon relics, Tahi and Rua, are identified by blocks above the sequence; the AT content of each region is indicated as a percentage at the bottom of the figure; the distance across the sequence, shown immediately below the genes, is represented in kb; the sequence containing *ltm* clusters 2 and 3 is separated from *ltm* cluster 1 by ~35kb (Young *et al*., 2006)

Thus, consecutive similarities between *LTM* and *EAS* loci seem to be brought to light. Firstly, the association of the genes composing both these endophytic clusters with the transposon relics appears to be of considerable importance (Young *et al*., 2006; Fleetwood *et al*., 2007). The abundance of

these relic sequences adds to the evolutionary potential of both clusters. It might also suggest that the regulation at the level of chromatin may be of consequence for *ltm* and *eas* genes, since chromatin remodelling as a method of coordinate gene regulation has been proposed as a possible factor causing selection pressure for secondary metabolite genes to be clustered (Martienssen & Colot, 2001; Volpe *et al*., 2002; Bok & Keller, 2004; Lee *et al*., 2005; Keller *et al*., 2005). Secondly, all ten genes identified at the *LTM* locus, as well as each gene in *EAS* cluster, have been shown to be highly expressed *in planta*, but only to a minimal extent or not at all – in axenic mycelial cultures, strongly suggesting that plant signalling is indispensable to induce their expression (Young *et al*., 2006; Fleetwood *et al*., 2007). However, the specific host stimuli required for the activation of the fungal genes remain to be identified.

Simultaneously, the aforementioned common traits underlying the ergopeptine and lolitrem biosynthetic processes seem to emphasize the ultimate uniqueness of lolines.

Maytansinoids from bacterial endophytes

As a matter of principle, hereby the elucidation of molecular background underlying the clustering of prokaryotic genes in the realm of endophytic secondary metabolism is shortly depicted. Thus, encompassing the diversity of microbial endophytic world, as well as acknowledging the early foundations set to boost the gene clustering investigation *per se* will be attempted.

The remarkable antitumor potency of maytansinoids, as well as the mystery surrounding their biosynthetic and evolutionary origins was reflected upon previously. It is noteworthy, however, that these 19-membered macrocyclic lactams are closely related to ansamycin antibiotics of microbial origin, such as rifamycin B and geldanamycin (Rinehart & Shield, 1976). In fact, the aforementioned similarity stimulated a search for maytansinoid-producing microorganisms, ultimately leading to the isolation of ansamitocins (Figure 17) from the *Actinomycetes*, *Actinosynnema pretiosum* ssp. *pretiosum* and a mutant strain *Actinosynnema pretiosum* ssp. *auranticum* (Higashide *et al*., 1977; Asai *et al*., 1978).
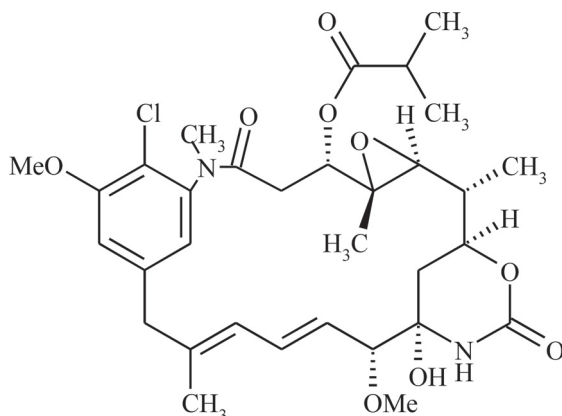
Figure 17. Ansamitocin P-3

The drive at the elucidation of microbial maytansinoids biosynthesis at the biochemical and genetic level was based upon the coupled forces of heterologous hybridization and PCR. The primary aim was to identify the gene encoding for the key enzyme in the assembly of 3-amino-5-hydroxybenzoic acid (AHBA) as the starter unit. Cloning by reverse genetics of the *AHBA synthase* from *Amycolatopsis mediterranei* (Kim *et al.*, 1998) provided the necessary probe and set the pace for identification, isolation and sequencing of the consecutive entities embedded in the biosynthetic gene cluster in question. A total of 250 kb of contiguous DNA was cloned and mapped. The appropriate gene inactivations were carried out to address the essential role of the AHBA gene homologues and to define the boundary of the biosynthetic locus (Yu *et al.*, 2002). Notably, the ansamitocin synthesis genes (*asm*) turned out to be contained not in one, but two clusters in the *Actinosynnema pretiosum* genome (Figure 18). This separation of genes encoding the biosynthesis of a given antibiotic into two essential clusters is unprecedented in *Procaryota*. Thus, it is proposed to be merely an inconsequential accident resulting from an evolutionary gene rearrangement which inserted 30 kb of non-essential DNA into the otherwise contiguous cluster (Yu *et al.*, 2002).
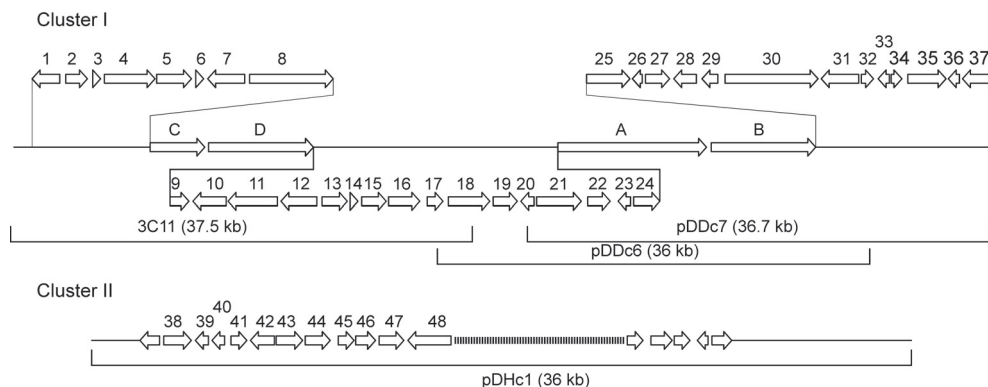
Figure 18. The ansamitocin biosynthetic gene cluster from *A. pretiosum;* asmABCD & asm19, polyketide synthase; asm22-24, asm43-45 & asm47, AHBA synthesis; asm13-17, methoxymalonate synthesis; asm7, asm10-12, asm19 & asm21, post-PKS modifications; asm2, asm8, asm18, asm29 & asm31, regulation (Floss, 2006)

Thus equipped with the essential genetic information and modern tools to manipulate the biosynthetic machinery, as well as urged by the extraordinary therapeutic potential, the research on microbial maytansinoids has recently entered a novel combinatorial stage. Two principal approaches are being explored. On one hand, the expression of all the *asm* genes, assembled into several cassettes in a heterologous host, such as *Streptomyces coelicolor* or *S. lividans* is being considered. This approach seems most promising in terms of elucidating the detailed biosynthetic process, but it has some major practical drawbacks, as the product titres are likely to be very low. On the other hand, it has been proposed to engineer mutations in the *asm* in the parent producer organism. This alternative approach has the advantage of all the regulatory elements and resistance mechanisms required for asnamytocin formation being present and functional (Cassady *et al*., 2004).

Undoubtedly challenging prospects, with substantial problems to be overcome and much more work needed, yet bearing a considerable potential for future drug discovery, optimization and production processes (Floss, 2006).

In summary, the accomplishments thus described might prove to become a vital platform and a blueprint for detailed studies of endophytic microbes *en masse* – their biosynthetic networks, evolutionary implications, host interdependencies, ecological, pharmaceutical and industrial significance – in the modern era of metabolic engineering and combinatorial biosynthesis (Stephanopoulos *et al*., 2004; Floss, 2006).

Concluding remarks and future perspectives

Biodiversity: a precious source of novelty. Not only in terms of unravelling numerous mysteries of nature – discovering a plethora of yet undescribed species, their evolutionary backgrounds, genetics and ecology, as well as the richness of thus implied new, potentially valuable molecules; but also a revolution of thought – an expended view promising to transform glimpses of reductionist research of the past years into snapshots of a dynamic world of systems biology, where cells grow, divide and produce, or organisms develop, differentiate and begin to deviate from the norm (Kate & Laird, 2000; Stephanopoulos *et al*, 2004; Kayser & Quax, 2007). Endophytic microbes seem to fit perfectly into this natural 'warehouse', only a small part of which we have been able to tap into so far.

The recent 'genomics revolution' has given momentum to considerable progress in the development of new technologies in bioscience, addressing specifically the arena of natural product biosynthesis. Whole-genome sequence mining (Lautru *et al*., 2005) and genome scanning as an alternative approach, providing an efficient way to discover natural product biosynthetic gene clusters without having the complete genome sequence (Zazopoulos *et al*., 2003); advances in microbial cell fermentation technology (Zengler *et al*., 2005; Weuster-Botzl *et al*., 2007) and metagenomics as a valuable alternative offering a cultivation-independent approach (Schloss & Handelsman, 2005); ample successes in heterologous expression and metabolic engineering (among many others: Alper *et al*., 2005; Schmidt *et al*., 2005; Wenzel *et al*., 2005; DeJong *et al*., 2006; Julsing *et al*., 2006; Li *et al*., 2006; Lindahl *et al*., 2006; Nims *et al*., 2006; Ro *et al*., 2006), the latter being in fact perceived as a progenitor of functional genomics and systems biology (Stephanopoulos *et al*., 2004; Tyo *et al*., 2007) – to name only a few highlights.

As hereby reviewed, some of the aforementioned novel strategies have enabled penetration into the previously inaccessible natural-product resources: microbial endophytes. While one has to be mindful that the problem we set out to address is several orders of magnitude larger than those with which we are familiar, no one can deny the opportunities that now present themselves.

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Chapter 3

Taxomyces andreanae
a presumed paclitaxel producer demystified?

Agata Staniek, Herman J. Woerdenbag, Oliver Kayser

Abstract

The 1990s brought an abundance of reports on paclitaxel-producing endo-phytes, initially heralded a discovery having tremendous implications for cancer therapy. As the vision of large-scale fermentation tanks producing vast quantities of relatively inexpensive paclitaxel and novel taxanes has faded and has been replaced by controversial silence, we carried out an in-depth investigation of *Taxomyces andreanae* – the very first presumed endo-phytic synthesizer of the diterpenoid. On one hand, metabolic profiling by means of chromatographic, spectroscopic and immunoenzymatic techniques predominant in literature was taken up. On the other, the experimental pro-cedure was brought to an alternative, previously unattempted level aiming at revealing the genetic background of paclitaxel biosynthesis in the endophyte.

The profound PCR-based screening for *taxadiene synthase* (*txs*) – a gene unique to the formation of the primary taxane-skeleton, as well as *phenylpropanoyl transferase* (*bapt*) encoding for the catalyst of the final acy-lation of the core structure rendering the ultimate efficacy of the drug, con-firmed the molecular blueprint for paclitaxel biosynthesis to be an inherent genetic trait of the endophyte. However, as the thorough metabolic analysis of *Taxomyces andreanae* commercial isolate brought no confirmation of endophytic paclitaxel production even after considerable up-scaling endeav-ours, we postulate that proclaiming the strain 'a fungus factory for Taxol' might have been premature.

Introduction

Since its discovery in the 1960s, through structure elucidation completed in 1971 and FDA approval in 1992, paclitaxel – a highly functionalized diterpenoid, has evolved to become a blockbuster drug with commercial sales of well over US $3 billion in 2004 (Croteau, 2005). Since it was primarily obtained from the inner bark of *Taxus brevifolia*, a relatively rare and slow-growing tree, the supply of this potent antineoplastic agent was soon to become the issue of scarcity. Even early estimations indicated that the demand for paclitaxel might exceed 300 kg per year, which would amount to 750 000 yew trees (Stierle *et al.*, 1995). The supply crisis, as well as the ecological implications resulting in the plant-endangered status, prompted the search for paclitaxel-producing microorganisms among the endophytic fungi of *Taxus*. This approach yielded the discovery of *Taxomyces andreanae* reported in 1993 (Stierle *et al.*, 1993).

Initially dubbed 'a hot commodity' (Stone, 1993), the fungus was to become an inexhaustible supplier of the valuable diterpene. After nearly two decades, however, no conclusive follow-up data on the endophytic metabolite profile or the genetic background of the paclitaxel biosynthetic pathway is available.

While the original study (Stierle *et al.*, 1993) relied solely on metabolic means to confirm the endophytic taxane production, applying chromatographic, spectroscopic and immunoenzymatic detection methods shortly after removing the fungal symbiont from its plant host, our research concerned a commercially available pure *Taxomyces andreanae* strain (CBS 27992) and was brought to an alternative, more advanced level. Namely, in parallel to utilizing the aforementioned techniques to confirm taxane presence in fungal cultures, we also addressed the question of molecular blueprint for paclitaxel production being an inherent genetic trait of the endophyte.

The presumed route leading to paclitaxel *in planta*, encompassing genes encoding for the consecutive biosynthetic enzymes, has been extensively investigated and well documented by Croteau and co-workers (Walker & Croteau, 2001). The paclitaxel pathway in *Taxus* is considered to require 19 enzymatic steps from the ubiquitous diterpenoid precursor geranylgeranyl diphosphate (GGPP) which is subsequently cyclized to taxa-4(5),11(12)-diene. Following stages involve a series of cytochrome P450-mediated oxygenations, an array of acylations, oxetane ring formation and several

steps of side chain assembly, resulting ultimately in the synthesis of pacli-
taxel (Figure 1).

geranylgeranyl $\xrightarrow{\textit{txs}}$ taxadiene
diphosphate

baccatin III + β-phenylalanoyl
CoA

$\textit{bapt}$

3'-N-debenzoyl-
-2'-deoxytaxol
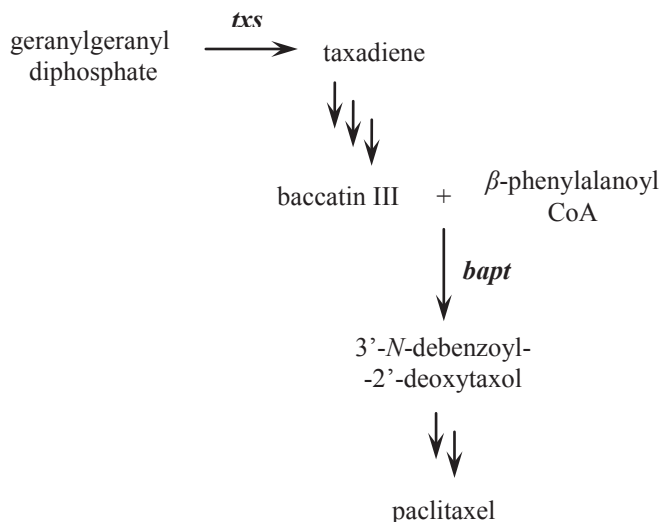
paclitaxel

Figure 1. A simplified outline of paclitaxel biosynthesis indicating steps crucial
for our investigation

Taxadiene synthase (TXS, EC 4.2.3.17) is considered to be an enzyme
catalyzing the committed step of paclitaxel biosynthesis (Wildung & Cro-
teau, 1996), i.e. the slow bioconversion of the parent olefin (GGPP) to
taxadiene, whereas phenylpropanoyl transferase (BAPT, EC 2.3.1.-) pro-
vides for the final attachment of the side chain – a structural requirement for
anticancer efficacy (Walker *et al*., 2002). For this reason, the in-depth study
of *txs* and *bapt* as target genes, bearing clear implications for paclitaxel bio-
synthetic pathway elucidation in *Taxomyces andreanae*, was taken up. It was
further anticipated that determining the intraspecific homology by alignment
studies might shed some light on the widely debated hypothesis of horizontal
gene transfer (Rosewich & Kistler, 2000; Kurland *et al*., 2003), thus explain-
ing the distant distribution of the secondary metabolites in question between
taxonomically unrelated species.

Materials & methods

Chemicals

Paclitaxel ($\geq$ 95%, HPLC) and baccatin III ($\geq$ 95%, HPLC) reference compounds as well as benzoic acid, thiamine, biotin and pyridoxal were purchased from Sigma-Aldrich (St. Louis, USA). Glucose, fructose, sucrose, peptone and yeast extract were supplied by Duchefa (Haarlem, the Netherlands). All other chemicals and organic solvents, including the deuterated methanol and chloroform, were delivered by Merck (Darmstadt, Germany).

Plant and fungal material

Taxus baccata L. was purchased at a local market in Groningen and authenticated at the Botanical Garden 'De Kruidhof', Buitenpost, the Netherlands. A voucher specimen (gro-ASOK-01) is deposited in our department.
Taxomyces andreanae (CBS 27992) was obtained from Centraalbureau voor Schimmelcultures (CBS), Institute of the Royal Netherlands Academy of Arts and Sciences, Utrecht, the Netherlands.

Fungal cultivation

Initial cultivation was performed in Sabouraud-Dextrose medium (peptone 10 g/L, glucose 20 g/L, pH 5.6). A slightly modified S7 medium (Stierle *et al.*, 1993) comprising: glucose 1 g/L, fructose 3 g/L, sucrose 6 g/L, peptone 1 g/L, sodium acetate 1 g/L, yeast extract 250 mg/L, thiamine 1 mg/L, biotin 1 mg/L, pyridoxal 1 mg/L, $Ca(NO_3)_2$ 6.5 mg/L, phenylalanine 5 mg/L, $MgSO_4$ 3.6 mg/L, $CuSO_4$ 1 mg/L, $ZnSO_4$ 2.5 mg/L, $MnCl_2$ 5 mg/L, $FeCl_3$ 2 mg/L, benzoic acid 100 mg/L, 1M KH_2PO_4 buffer (pH 6.8) 1 mL/L was the experimental medium.
Growth incubation was conducted at room temperature with moderate rotation speed (100 rpm) under a day/night regime (16/8 h; 1200 lux). 1, 5 and 10 L cultivations were attempted. Subculturing was performed by medium refreshment of a one-week-old fungal suspension in a 1:100 ratio.

Extract preparation

Crude and purified extracts of dried *Taxus baccata* needles were prepared as described previously (Grothaus *et al.*, 1995). After Büchner-filtration, the fungal mycelium was macerated and extracted with CH_2Cl_2:MeOH (1:1), while the filtrate was extracted with CH_2Cl_2. The solvent was removed from

the organic extracts by rotary evaporation under reduced pressure at ~ 40ºC (Strobel *et al*., 1994).

TLC

TLC analysis was conducted on silica gel plates Merck F_{254} (5×10 cm) with CH_2Cl_2:ACN (7:3, v/v) as the solvent system. Taxane detection was carried out by UV light absorption (254 nm) and spraying with vanillin/H_2SO_4 (Strobel *et al*., 1994).

MPLC

The Büchi MPLC system comprised the following units: C-615 pump manager, two C-605 pump modules, C-660 fraction collector, C-630 UV monitor and a PC computer. A Büchi C-690 series borosilicate glass column (230 × 15 mm), packed with reversed phase silica gel RP_{18} was applied to perform the separation. Water (solvent A) and methanol (solvent B) were the mobile phase. The solvent flow was set at 10 mL/min and the UV detection wavelength at 220 nm. Dry fungal extracts were dissolved in 2 mL of methanol and injected to the system. The fractions were collected by signal or by volume (max. 15 mL/tube). The analysis was commenced at 20 % B, followed by linear gradient with an endpoint at 40 min, 100 % B and finalized by 15 min of isocratic 100 % B elution. The separation procedure was monitored by TLC.

HPLC

The HPLC system comprised an LC-10AT pump, a SCL-10Avp controller, a FCV-10AL low pressure gradient mixer and an SPD-M10A diode array detector (Shimadzu CLASS-VP), operated with CLASS-VP software version 6.12SP4. Low pressure gradient chromatography was performed using an Eclipse XDB C_{18} column (250 × 4.6 mm with 5 μm packing). The injection volume was 50 μL and the flow rate was set at 1.5 mL/min at room temperature. The mobile phase system consisted of A: H_2O:ACN (80:15.44, w/w) with 10 mL 0.1 M phosphoric acid/L, and B: H_2O:ACN (20:62.56, w/w) with 10 mL 0.1 M phosphoric acid/L. The separation was commenced at 45 % solvent B for 5 min, followed by a linear gradient with given time endpoints: 24 min, 100 % B; 26 min, 100 % B; 28 min, 45 % B; 30 min, 45 % B (adopted from Ballero *et al*., 2003). The detection limit for paclitaxel

reference was established as the amount of analyte that provides a signal-to-noise ratio of 3, and amounted to 400 ng/mL.

HPLC-MS

A Perkin-Elmer SCIEX API-3000 apparatus (Toronto, Canada) with atmospheric pressure electrospray ionisation and triple-quadrupole mass spectrometer operating in the positive mode was used for the HPLC-MS analysis. The instrument was equipped with a Perkin-Elmer series 200 HPLC micro-gradient pump and an autosampler. The mass spectrometer was interfaced to an Alltima C_{18} column (150 × 2.1 mm, 5 μm particle size). The mobile phase comprised: 100 % H_2O (solvent A) and 100 % ACN (solvent B) both containing 0.1 % formic acid. The separation was commenced at 45 % solvent B for 1 min, followed by a linear gradient with given time endpoints: 20 min, 80 % B; 22 min, 80 % B; 24 min, 45 % B; 25 min, 45 % B. The injection volume was 30 μL and the flow rate 1 mL/min at room temperature. The positive ion mode was employed and spectra were obtained with a spray voltage of 5.2 kV. The source temperature was 450°C. The scan rate was 2 sec/scan, a full ion scan was applied in the range of 100-1100 amu with a step size of 1 amu and 10 V entrance potential. Nitrogen was used both as the nebulizer and curtain gas at a pressure of 0.7 Torr and a flow rate of 13 mL/min. Data processing was performed using Analyst version 1.4 software (MDS Sciex, Concord, Canada).

NMR

^{1}H (500 MHz) and ^{13}C (125.69 MHz) one- and two-dimensional NMR spectra were recorded on a Varian INOVA-500 spectrometer.

Immunodetection

CIEIA – a competitive inhibition enzyme immunoassay system for the quantitative detection of taxanes in biological matrices sensitive to paclitaxel at a concentration of 0.5 ng/mL (Grothaus *et al*., 1993) was performed using a commercially available kit (Hawaii Biotech, Inc., Aeia, Hawaii).

DNA isolation

Efficient extraction of plant and fungal DNA was accomplished using DNeasy® Plant Mini Kit (Qiagen Inc., USA) according to the manufacturer's protocol. Plasmid DNA purifications were performed using QIAprep® Spin

Miniprep Kit (Qiagen Inc., USA). PCR products were purified with QIAquick® PCR Purification Kit & QIAquick® Gel Extraction Kit (Qiagen Inc., USA).

PCR

Temperature gradient PCR reactions were conducted with 2 alternative enzymes: *Taq* DNA Polymerase (5U/μl) (Fermentas GmbH, Germany) & *Phusion*™ DNA Polymerase (2U/μl) (Finnzyme OY, Finland), in the Eppendorf Mastercycler gradient thermocycler.

Thermal conditions

Taq: 95°C for 3 min (initial denaturation), 30 × [95°C for 30 sec (denaturation), 40 sec (annealing) in temperature gradient, 72°C (extension) for the period of time dependent on the length of the amplified gene fragment and enzyme efficiency], 72°C for 5 min (final extension). *Phusion*™: 98°C for 1 min (initial denaturation), 30 or 35 × [98°C for 10 sec (denaturation), 20 sec (annealing) in temperature gradient, 72°C (extension) for the period of time dependent on the length of the amplified gene fragment and enzyme efficiency], 72°C for 10 min (final extension).

Primers

taxadiene synthase gene:
  a specific primer set for the amplification of *txs* sequence encoding the full-length protein (Besumbes *et al.*, 2004)
- txs_fwd (5'-atggctcagctctcatttaatg-3')
- txs_rev (5'-tgccaatacaataataagtc-3')

alternative specific forward starter for the amplification of a truncated *txs* sequence encoding the mature enzymatic entity (without the plastid targeting signal peptide)
- txs_fwd_noPTS (5'-atgagcagtagcactggcactagca-3')

specific primer set for the amplification of highly conserved core DNA fragment of *txs* (Zhou *et al.*, 2007)
- ctxs_fwd (5'-caaacccatgtcgaattgagaag-3')
- ctxs_rev (5'-caagtttgcatacactctggaatct-3')

degenerate PCR primers
- LHQ_fwd (5'-ytrcaycargargarytraargarytr-3')
- LHQ_rev (5'-yarytcyttyarytcytcytgrtgyar-3')

- DDXXD_fwd (5'-gtnathgaygayacntaygaygaytay-3')
- DDXXD_rev (5'-rtartcrtcrtangtrtcrtcdatnac-3')
- DDXXDspec_rev (5'-aaagatgtcagccatatcatcaaaaag-3')

phenylpropanoyl transferase gene (Zhang *et al.*, 2008)

- bapt_fwd (5'-cctctctccgccattgacaa-3')
- bapt_rev (5'tcgccatctctgccatactt-3')

The primers were purchased from Invitrogen[TM]/ Illumina[®] laboratory and Operon Biotechnologies, Germany.

Molecular cloning & sequence analysis

Taq and A-tailed *Phusion*[TM] amplification products were ligated into pGEM[®]-T & pGEM[®]-T Easy Vector Systems (Promega Corporation, USA), whereas cloning experiments on the blunt-ended PCR products synthesized with *Phusion* polymerase were conducted by means of Zero Blunt[®] TOPO[®] PCR Cloning Kit (Invitrogen, UK). The obtained plasmid vectors were transformed into NovaBlue Singles[TM] chemically competent *E. coli* cells (Novagen, Inc., USA), or XL1-blue competent *E. coli* strain (Stratagene, USA). Endonuclease digestions of DNA inserts were performed using restriction enzymes from New England BioLabs Inc., USA. Sequence analysis was performed by ServiceXS B.V., Leiden, the Netherlands and Macrogen Ltd., Seoul, Korea.

Results & discussion

In order to gain the first insight into the presence of taxane compounds in the extracts of *Taxomyces andreanae* prepared from 1, 5 and 10 L cultures, thin layer chromatography was employed. As R_f values of analyzed mixtures' components corresponded to the one of paclitaxel reference (0.30 – 0.34) yielding the same intense blue colour reaction with the vanillin – sulphuric acid spray as the authentic compound, the initial indication of endophytic paclitaxel production seemed to have been provided for the organic extracts of fungal culture medium filtrates. High performance liquid chromatography was applied subsequently. The chromatograms of culture broth filtrate extracts revealed a peak eluting around the retention time of 10.96 min corresponding, more or less, to that of the reference compound (10.91 min).

While both baccatin III and paclitaxel were successfully detected in the purified extract of *Taxus baccata* needles, providing for a positive control of

taxane presence verification by means of HPLC-MS, no such reaffirmation concerning endophytic taxane production was obtained.

As current chromatographic methods for the determination of taxanes in biological matrices are considered to be relatively insensitive, an immunoenzymatic system (CIEIA), with the reactivity towards paclitaxel at concentrations 800 times lower than those detectable by HPLC, was employed. The average taxane concentration values in crude and purified extracts of *Taxus baccata* needles amounted to 270 µg/mL and 20 µg/mL respectively, which constitutes 0.05% of dry weight and corresponds to taxane concentration in *Taxus* needles as previously reported (Grothaus *et al.*, 1995). Despite numerous trials, results obtained for the samples of fungal origin remained inconsistent and led to the conclusion that the diterpenoids of interest were absent from the endophytic extracts.

Medium pressure liquid chromatography was applied as a preparative method for the isolation of individual natural products from the complex fungal extracts, with a major effort put on the identification of the intriguing paclitaxel-mimicking constituent. The separation procedure was monitored by TLC and reaction with vanillin/H_2SO_4 spraying reagent. The signal representing the compound in question was registered at a retention time of 14.10 min while 6 consecutive fractions of the eluate showed intense blue staining on the silica gel plates. The pure fungal metabolite was further subjected to HPLC analysis and eluted at 5.66 min. While present in the original chromatograms of endophytic extracts, this peak did not correspond to the one of paclitaxel registered at 10.91 min.

The endophytic compound (**1**) obtained was an amorphous white solid (7 mg). The TLC and HPLC pure metabolite was subjected to mass spectrometry as well as ^{1}H and ^{13}C 1D/2D NMR. The spectral data was in good agreement with an unusual tripeptide 3-amino-2-hydroxy-4-phenylbutanoic acid – Val – Phe (Figure 2).
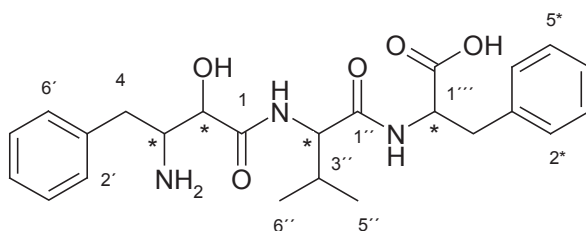
Figure 2. 3-amino-2-hydroxy-4-phenylbutanoic acid – Val – Phe

According to LC-MS/MS the molecular mass was determined at 441.53 (calcd for $C_{24}H_{31}N_3O_5$). The fragmentation pattern showed three major fragments of (*m/z/%*) 257.2 (100), 239.2 (23), 216.2 (20), and 198.2 (48). The assignment of proton resonances gave following correlation: PBA (3-amino-2-hydroxy-4-phenylbutanoic acid)2 (3.83 ppm, d, 10 Hz), PBA3 (3.5 ppm, dd, 10 Hz, 8 Hz), PBA4 (2.72 and 2.85 ppm, dd, 8 Hz); PBA2´-6´ (7.25-7.37ppm, m), Val2´´ (6.42 ppm, d, 15 Hz), Val3´´(2.18 ppm, m), Val5´´(0.96 ppm, t, 19 Hz), Val6´´(0.89 ppm, t, 19 Hz), Phe2´´´(3.98 ppm, d, 21 Hz), Phe3´´´(3.16 ppm, dd, 3.32, dd, 21 Hz), Phe2*-6*(7.25-7.37 ppm, m). A 13C NMR showed 24 resonances as documented in Table 1.

Table 1. 13C NMR data of compound **1**

C	1
PBA1	171.3
PBA2	77.2
PBA3	54.8
PBA4	39.9
PBA1´	140.8
PBA2´	131.5
PBA3´	127.8
PBA4´	126.3
PBA5´	127.8
PBA6´	131.5
Val1´´	168.3
Val2´´	61.8
Val3´´	30.1
Val4´´	20.0
Val5´´	18.1
Val6´´	19.7
Phe1´´´	54.6
Phe2´´´	38.2
Phe1*	134.9
Phe2*	127.9
Phe3*	128.4
Phe4*	127.4
Phe5*	128.4
Phe6*	127.8

Values in ppm were recorded at 125.69 MHz (in CDCl$_3$)

The basic tool of research on the genomic level was the polymerase chain reaction. The initial PCR trials were carried out using specific primers designed after very highly conserved sequences in the *txs* cDNAs from *Taxus brevifolia* and *Taxus chinensis*, as reported in literature (Besumbes *et al.*, 2004), and allowing the amplification of the full-length *Taxus baccata* gene. As this original transcript encompasses a long *N*-terminal targeting sequence for localization to and processing in the plastids, consecutive reactions were performed using an alternative forward oligonucleotide for the amplification of a truncated *txs* sequence encoding the mature enzymatic entity.

Further investigation required designing suitable degenerate primer pairs providing for amplification of the target gene. The ultimate design was preceded by an extensive study concerning the nature of prenyltransferases as a group of enzymes encompassing taxadiene synthase, e.g. (Lesburg *et al.*, 1997; Bohlman *et al.*, 1998; Trapp & Croteau, 2001), and completed by means of the NCBI Conserved Domain Search for *txs* revealing the presence of highly conserved amino acid sequences characteristic of terpene cyclases of plant as well as microbial origin (NCBI. Available at http://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=cd00868& version=v2.06. Accessed May 17, 2007). Although the evolutionary relationship of plant and microbial prenyltransferases is unclear, all of these enzymes contain the aspartate-rich DDXXD motif involved in coordination of divalent metal ions for substrate binding (Tarshis *et al.*, 1994). This region was, therefore, an obvious choice for primer design basis. An alternative primer set (LHQ) was constructed with reference to an up-stream highly conserved amino acid sequence of unknown function, as identified by the NCBI Search tool.

All the aforementioned oligonucleotides were employed in various combinations, in a wide range annealing temperature gradient, with and without DMSO included in the reaction mixture, and with two alternative DNA polymerases as reaction catalysts. This extensive PCR-based screen yielded a number of potentially interesting amplification products. These were either directly sequenced or, if applicable, further purified and employed as templates for nested PCR experiments.

Primary alignment studies at the nucleotide and amino acid level did not reveal any significant similarities between the sequences of the analysed DNA fragments and those of *txs*, nor any other known gene of prenyltransferase family, be it of plant or microbial origin.

Final experimental steps addressing the genetic capacity of *Taxomyces andreanae* to synthesize the tricyclic taxane core were prompted by a recent publication postulating successful PCR-based screening for *txs* presence in several endophytic entities. Thus, the genomic DNA of fungal origin served as a template yet again, in hope for efficient annealing with the specific starters proposed by Zhou *et al*. (2007). As no amplification products could be observed while applying a standard quantity of 30 PCR cycles, the cycle-number was increased to 35 finally yielding the desired amplicon (Figure 3a). Further successful semi-degenerate/nested (ctxs_fwd & DDXXD_rev and ctxs_fwd & DDXXDspec_rev) chain reactions, sequencing and alignment studies followed, ultimately confirming *txs* presence in the genome of *Taxomyces andreanae* (97% sequence similarity with *txs* of *Taxus* sp. within the conserved region amplified). Interestingly, the desired sequence could not be retrieved while utilizing the forward starter annealing to the plant *txs* fragment immediately following the plastid targeting sequence (txs_fwd_noPTS) in combination with the core reverse oligonucleotide (ctxs_rev), suggesting that the functional fungal gene is further cropped on its *N*-terminus.

While crucial for the taxane biosynthetic pathway elucidation, the detection of *taxadiene synthase* affords no definitive proof for endophytic paclitaxel production. Thus, in order to gain one, *phenylpropanoyl transferase* was selected as an alternative molecular marker (Zhang *et al*., 2008). The core fragment of the gene diagnostic for one of the very last steps of paclitaxel biosynthesis was successfully amplified (Figure 3b), cloned and sequenced, showing high homology with its plant counterparts (96% sequence identity). *Bapt* detection, in turn, allows speculating about the origins of the *β*-phenylalanoyl-paclitaxel side chain. Namely, the fact that phenylalanine constitutes one of the building blocks of the tripeptide identified in course of our research deems it plausible that, what we initially considered to be merely a paclitaxel counterfeit, is actually its crucial progenitor.

a)

b)
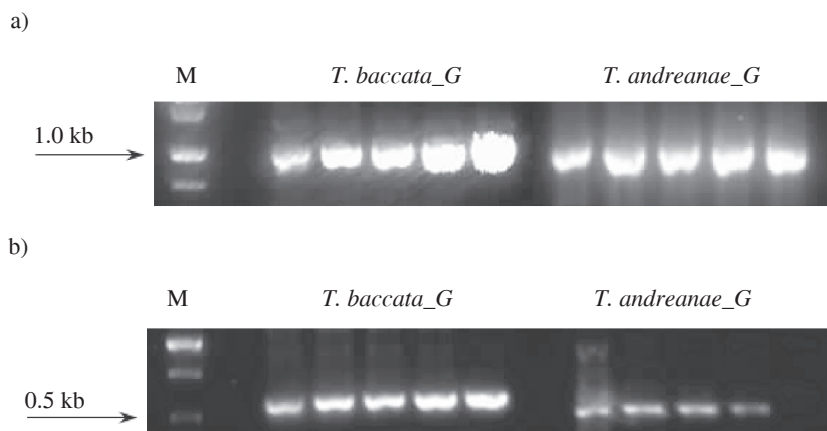
Figure 3. PCR analysis for the presence of a) *taxadiene synthase* (~ 1.0 kb) and b) *phenylpropanoyl transferase* (~ 0.5 kb) in *Taxomyces andreanae; Taxus baccata* needles were a source of plant DNA samples, providing for a positive control of *txs* & *bapt* presence verification (M – GeneRuler^{TM} 1 kb DNA Ladder, Fermentas; G – annealing temperature gradient: 45 - 65°C)

To sum up, the absence of taxane metabolites from endophytic extracts derived from 1, 5 and 10 L cultures proves *Taxomyces andreanae* incapable of independent (*ex planta*) paclitaxel production under axenic culture conditions. Thus, while initially unable to detect the fungal *taxadiene synthase*, we primarily ascribed the biosynthesis of paclitaxel *ab initio* to be a genuine feature of the yew host rather than its microbial inhabitant in question (Staniek *et al*., 2008). Interestingly, the presence of the *N*-terminal plastid targeting sequence within *taxadiene synthase* transcript of plant origin, further supported by ample evidence of the antineoplastic diterpene production by sterile cell suspension cultures of *Taxus* (Ketchum & Gibson, 1996) and the successful isolation of paclitaxel biosynthetic genes therefrom (Walker & Croteau, 2001), seemed indeed to indicate that this gene was plant-derived rather than a fungal product.

However, the ultimate success of our profound screen for the microbial *taxadiene synthase*, accompanied by the detection of *bapt*, encoding for the enzyme affording the efficacious antineoplastic natural product, provides crucial evidence for the molecular blueprint of paclitaxel production being an inherent genetic trait of *Taxomyces andreanae*. Consequently, the results thus obtained seem to give a new insight to the controversial hypothesis of horizontal gene transfer (HGT). One might assume that the biosynthesis of

paclitaxel has been repeatedly invented during evolution. However, the fact that approximately 20 genes are involved in the formation of this highly functionalized and unique diterpenoid (Walker & Croteau, 2001) makes it rather unlikely. Therefore, it seems possible that a lateral transfer of genetic information shaped the evolutionary trajectory of taxonomically unrelated, yet co-existing, species. Moreover, postulating HGT to be a driving force in the evolution of fungal gene clusters – a phenomenon now considered a hallmark characteristic of secondary metabolic biosynthetic pathways (Lawrence & Roth, 1996; Rosewich & Kistler, 2000), rises an intriguing question as to whether the genes responsible for paclitaxel formation in *Taxomyces andreanae* are indeed grouped in a contiguous cluster. Therefore, although deemed largely anecdotal thus far (Rosewich & Kistler, 2000; Kurland *et al*., 2003), the ultimate plausibility of the horizontal gene transfer hypothesis should be revisited and further investigated.

All in all, our results suggest that specific plant environment may be required for the induction of paclitaxel biosynthetic genes in the fungal symbiont. Identifying the regulatory mechanisms will be of considerable future interest and will provide further insight into the true nature of the fine-tuned equilibrium of plant-microbe interactions (Staniek *et al*., 2008; Hines & Zahn, 2009).

Regardless of the aforementioned fundamental speculations, practical aspects should be decisively invoked. As the highly desirable search for sustainable and economically feasible paclitaxel sources has tempted various authors to draw premature conclusions proclaiming endophytes to be true taxane bio-factories, e.g. (Stierle *et al*., 1993; Strobel *et al*., 1996; Chakravarthi *et al*., 2008), and reviewers still seem to dwell on the potential of the microbial taxane synthesizers to revolutionize the pharmaceutical arena (Miller *et al*., 2008), we contend that such reports should be viewed as extraordinary claims that demand ultimate justification, namely: unambiguous evidence for molecular blueprint underlying the postulated microbial paclitaxel biosynthesis, suggesting a means for its activation and manipulation.

Acknowledgements

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Chapter 4

Wollemia nobilis
in vitro cultivation attempts of the living fossil

Agata Staniek, Mateusz Szamałek, Herman J. Woerdenbag, Oliver Kayser

Introduction

In August of 1994 David Noble made a tremendous discovery of the oldest living conifer species in the world. It was found in a deep narrow sandstone canyon in Wollemi National Park, a 496 000 ha forest reserve located in New South Wales, merely 150 km from Sydney, and called *Wollemia nobilis*. Quite ironically, the aboriginal meaning of the term 'wollemi' translates to 'watch out, look around you' (Hill, 1996). Yet, a rugged Australian wilderness managed to keep its secret for millions of years.

   Wollemia nobilis represents a whole new monotypic genus (Jones *et al.*, 1995) belonging to the ancient Araucariaceae family, which reached maximum diversity during the Jurassic and Cretaceous periods between 200 and 65 million years ago. The worldwide range of the 'monkey puzzle tree' family was greatly reduced at the time of the extinction of the dinosaurs, although its representatives were an important component of the forests of southern supercontinent Gondwana in the early Tertiary period. Replaced by flowering plants, Araucariaceae include now but three genera – *Araucaria*, *Agathis* and *Wollemia* (Atmajda, 2004). The Wollemi pollen is indistinguishable from the fossilized pollen *Dilwynites*, abundant in the Australian record from mid-Cretaceous to mid-Tertiary (94-30 million years ago). The foliage of *W. nobilis* is virtually identical to that of one of its supposed fossil ancestors, the late Jurassic (150 million years ago) *Agathis jurrasica*. According to available data, the estimated age of *Wollemia nobilis* is 200-90 million years (Figure 1). Its origin however still remains an enigma (Gilmore & Hill, 1997; Setoguchi *et al.*, 1998; Stefanovic *et al.*, 1998).

Figure 1. Fossil of Wollemi pine (http://www.wollemipine.com)

Wollemi pine is a monoecious tree growing up to 40 m. Only three Wollemi groves, of 40, 17 and 3 trees respectively, are known today (Snelling, 2006). The trunks range up to 1.2 m in diameter. The mature trees possess the capacity to develop new shoots at or below the soil level – the phenomenon known as 'coppicing'. This results in forming multiple trunks of different ages by the large and old plants (Figure 2). The trunks of Wollemi pine have a highly unusual brown, knobbly cork-like bark. The crown is slender and columnar. Instead of having dark-green leaves, like the conifers of today, the Wollemi leaves are of bright-green colour (Hill, 1996). Striking differences between juvenile and adult leaves are seen (Chambers, *et al*. 1998). The reproductive organs are borne on sporophylls arranged in cones or cone-like structures called strobili, located at the end of lateral growing shoots (Offord *et al.*, 1999). Annually produced seeds are light and winged, and most probably dispersed by wind. Only the largest oldest trunks reproduce sexually, however vegetative reproduction is also possible, and occurs through rudimentary buds, carried in the axis of leading vertical shoots (Burrows *et al*., 2003). Forming epicormic shoots, as well as coppicing are adaptive features increasing the tree's chance of survival. However, those habits indicate that Wollemi species may be extensively clonal. Indeed, it has been shown that the population in question is highly inbred, having no detectable genetic variation (Peakall *et al*., 2003). Thus, each genetic individual seems to play a key role in the future evolution of the monotypic conifer, as the genetic diversity within the species constitutes the full genetic range of the genus.

Figure 2. 'Multi-stemming' habit of *Wollemia nobilis* growing in natural conditions (http://www.wollemipine.com)

The aforementioned considerations, combined with the immense popularity of the 'green dinosaur' keenly sought after as a horticultural plant, reinforce the necessity of reducing the threat to the population in the wild from illegal collectors. For this reason, the Australian government has undertaken an unprecedented conservation programme, making *Wollemia nobilis* commercially available as a potted plant (NSW Department of Environment and Conservation, 2006). Thus, in early 2006 first Wollemi specimens left 'the land down under', opening a window into an unimaginably ancient past – a great opportunity of studying this extraordinary conifer worldwide.

Materials & methods

Plant material

A juvenile (1-year-old, 30 cm in length) *Wollemia nobilis* W. G. Jones, K. D. Hill & J. M. Allan, bearing the registered Wollemi pine[TM] logo was purchased in 2008 from Arboretum Kalmthout, Belgium – a representative of Wollemi Australia exclusively licensed by the Royal Botanic Gardens Sydney (RBGS) through NSW National Parks and Wildlife Service (NPWS) to propagate and market the Wollemi pine in Australia and internationally. The conifer was additionally authenticated at the Botanical Garden 'De Kruidhof', Buitenpost, the Netherlands. A voucher specimen (gro-ASOK-02) is deposited in our department.

Callus generation attempts

Numerous attempts to obtain the callus and liquid tissue cultures of *Wollemia nobilis* were made. To this end, two sterilization methods (Table 1), a variety of media (Tables 2-6), with and without activated charcoal (0.5 %), as well as alternative light conditions (1 200 vs. 2 500 lux) were employed. Freshly budded Wollemi shoots were used and the explants were cultivated at room temperature under a day/night regime of 16/8 h.

Table 1. Sterilization methods

	method A	method B
70 % ethanol	10 sec.	10 sec.
15 % NaOCl : H_2O (1:1)	5 min.	20 min. (stirring)
3 × H_2O	2 min.	20 min. (stirring)

Table 2. B5 medium (Gamborg *et al.*, 1968) and its modifications

ingredient	concentration [g/L]		
agar or agarose	8.0		
sucrose	40.0		
$CoCl_2$ x $6H_2O$	2.5×10^{-5}		
$CuSO_4$ x $5H_2O$	2.5×10^{-5}		
Na_2MoO_4 x $2H_2O$	2.5×10^{-4}		
KI	7.5×10^{-4}		
$ZnSO_4$ x $7H_2O$	0.002		
H_3BO_3	0.003		
$MnSO_4$ x H_2O	0.01		
FeNaEDTA	0.0367		
$CaCl_2$	0.11323		
$MgSO_4$	0.12156		
NaH_2PO_4	0.13044		
$(NH_4)_2SO_4$	0.134		
KNO_3	2.5		
nicotinic acid	0.001		
pyridoxine HCl	0.001		
thiamine HCl	0.01		
myo-inositol	0.1		
	B5	*B5var*	*B5wol*
kinetin	2.0×10^{-4}	2.0×10^{-4}	-
NAA	5.0×10^{-4}	5.0×10^{-4}	-
IAA	5.0×10^{-4}	5.0×10^{-4}	-
2,4-D	0.002	0.002	5.0×10^{-4}
casein enzymatic hydrolysate	-	2.0	-

Table 3. MS medium (Murashige & Skoog, 1962) and its modifications

ingredient	concentration [g/L]		
agar or agarose	8.0		
$CoCl_2$ x $6H_2O$	2.5×10^{-5}		
$CuSO_4$ x $5H_2O$	2.5×10^{-5}		
Na_2MoO_4 x $2H_2O$	2.5×10^{-4}		
KI	8.3×10^{-4}		
$ZnSO_4$ x $7H_2O$	0.0086		
H_3BO_3	0.0062		
$MnSO_4$ x H_2O	0.0169		
FeNaEDTA	0.0367		
$CaCl_2$	0.33202		
$MgSO_4$	0.18054		
KH_2PO_4	0.17		
NH_4NO_3	1.65		
KNO_3	1.9		
nicotinic acid	5.0×10^{-4}		
pyridoxine HCl	5.0×10^{-4}		
thiamine HCl	1.0×10^{-4}		
glycine	0.002		
myo-inositol	0.1		
	MS	*vdSalm*	*MSwol*
BAP	0.001	0.002	-
IAA	0.001	0.002	-
2,4-D	-	-	5.0×10^{-4}
sucrose	40	30	40

Table 4. Murashige & Miller medium (Miller & Murashige, 1976)

ingredient	concentration [g/L]
agar or agarose	8.0
sucrose	30.0
$CoCl_2$ x $6H_2O$	2.5×10^{-5}
$CuSO_4$ x $5H_2O$	2.5×10^{-5}
Na_2MoO_4 x $2H_2O$	2.5×10^{-4}
KI	8.3×10^{-4}
$ZnSO_4$ x $7H_2O$	0.0086
H_3BO_3	0.0062
$MnSO_4$ x H_2O	0.0169
FeNaEDTA	0.0367
$CaCl_2$	0.33202
$MgSO_4$	0.18054
KH_2PO_4	0.17
NH_4NO_3	1.65
KNO_3	1.9
thiamine HCl	4.0×10^{-4}
myo-inositol	0.1
IAA	0.002
kinetin	0.002

Table 5. Quoirin & Lepoivre medium (Quoirin & Lepoivre, 1977)

ingredient	concentration [g/L]
agar or agarose	8.0
sucrose	30.0
$CoCl_2$ x $6H_2O$	2.5×10^{-5}
$CuSO_4$ x $5H_2O$	2.5×10^{-5}
Na_2MoO_4 x $2H_2O$	2.5×10^{-4}
KI	8.3×10^{-4}
$ZnSO_4$ x $7H_2O$	0.0086
H_3BO_3	0.0062
$MnSO_4$ x H_2O	7.6×10^{-4}
FeNaEDTA	0.0367
$Ca(NO_3)_2$	0.57892
$MgSO_4$	0.17579
KH_2PO_4	0.27
NH_4NO_3	0.4
KNO_3	1.8
thiamine HCl	4.0×10^{-4}
myo-inositol	0.1
GA	0.002
BAP	0.002

Table 6. Westvaco WV3 medium (Coke, 1996)

ingredient	concentration [g/L]
agar or agarose	8.0
sucrose	30.0
$CoCl_2$ x $6H_2O$	2.5×10^{-5}
$CuSO_4$ x $5H_2O$	2.5×10^{-5}
Na_2MoO_4 x $2H_2O$	2.5×10^{-4}
KI	8.3×10^{-4}
$ZnSO_4$ x $7H_2O$	0.0086
H_3BO_3	0.031
$MnSO_4$ x H_2O	0.01516
FeNaEDTA	0.0367
$CaCl_2$	0.42588
$MgSO_4$	0.90379
KH_2PO_4	0.27
KCl	0.65679
KNO_3	0.91006
thiamine HCl	4.0×10^{-4}
myo-inositol	0.1
BAP	5.0×10^{-4}
2,4-D	0.003
L-glutamine	2.29

All the media components were purchased from Duchefa Biochemie, the Netherlands.

Results & discussion

Callus cultures of *Wollemia nobilis* turned out to be extremely difficult to initiate and maintain. Numerous attempts to improve the cultivation conditions were made by employing a variety of media supplemented with a myriad of growth regulators in different combinations and concentrations (Tables 2-6). As activated charcoal is believed to play a significant role in the improvement of cell growth and development (Thomas, 2008), it was also included in the media composition. However, the pronounced adsorbing properties of charcoal said to decrease the detrimental effects of toxic metabolites, phenolic exudation and brown exudates accumulation had no impact on the initiation or growth of Wollemia callus. As observed by van Uden *et al.* (1990), the contact between plant tissue and agar seemed to be disadvantageous for the formation and development of callus derived from a conifer *Callitris drummondi*. Therefore we also substituted agar with agarose as a solidifying agent for our test plates. Unfortunately, to no avail.

All in all, our strive for obtaining callus and cell suspension cultures of *Wollemia nobilis* resulted merely in observing a slight callus formation on the shoot explants sterilized in less stringent conditions (method A), placed on Westvaco medium and cultured under 1 200 lux (Figure 3). However, we did not succeed in maintaining a well growing Wollemia callus and thus were not able to initiate cell suspension cultures of the ancient conifer.

8 mm

Figure 3. Indications of Wollemi callus formation on Westvaco medium

Nonetheless, the aforementioned endeavours brought about the isolation of an interesting endophytic strain, *Cladosporium langeronii* bearing indications of paclitaxel production (Staniek *et al.*, 2010).

To our knowledge, this is the first description of procedure optimization for the cultivation of callus-tissue of the oldest living tree species in the world. While ultimately unattained, the aim should not be abandoned. Indeed, as the aforementioned formulation was relatively recently proposed in

response to limited success of the efforts to develop tissue culture methods for utilization with Loblolly pine (*Pinus taeda*) (Coke, 1996), it does not come as a great surprise that it showed some efficiency while employed in the venture for *in vitro* cultivation of a fellow conifer. Thus, the Westvaco medium formulation seems to provide a platform for further optimization for an effective initiation and sustained growth of callus and cell suspension cultures of *Wollemia nobilis* that could prove a milestone in further investigation of the botanical enigma of the 'green dinosaur'.

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Essential oil constituents derived from different organs of a relictual conifer *Wollemia nobilis*

Agata Staniek, Remco Muntendam, Herman J. Woerdenbag, Oliver Kayser

Abstract

The chemical composition of the essential oil of leaves (0.9%, w/v) and twigs (0.3%, w/v) of *Wollemia nobilis* (Araucariaceae) – a remnant species thought to have been extinct for 65 million years – was investigated by GC/MS. The main constituents of both leaf- and twig-derived oil samples were: 16-kaurene (61.8% and 38.2% respectively) and germacrene D (9.9% and 22%). The principal difference was a considerably more pronounced sesquiterpene presence in the twig-oil, amounting to 33.5%, than in its folial counterpart (23.4%). On the contrary, while remaining the dominant group in both oil samples under investigation, diterpenoids were relatively more abundant in leaf-derived oil constituting 65.3%, versus 41.7% detected in twigs. To our knowledge, this is the first report dealing with the essential oil composition of Wollemi pine twigs, as opposed to the leaf-derived volatiles.

Introduction

The discovery of Wollemi pine in 1994 made international headlines, hailed as the 'botanical find of the century'. The unique conifer was found in a deep Triassic sandstone gorge in Wollemi National Park within 150 km of Sydney. Interestingly, the aboriginal meaning of the word 'wollemi' translates to 'watch out, look around you' (Hill, 1996). Yet, a rugged Australian wilderness managed to keep its secret for millions of years.

Subsumed, on a morphological basis, to the 200 million year old conifer family Araucariaceae, *Wollemia* constitutes a whole new monotypic genus (Jones *et al.*, 1995). While the evolutionary relationships within the 'monkey puzzle tree' family are poorly known, *Wollemia* was confirmed to be distinct from the related genera *Araucaria* and *Agathis* by Gilmore and Hill (1997) through DNA sequencing of the plastid gene *rbc*L. The sequence data, combined with different ranges of other conifer taxa, imply that *Wollemia* derived prior to its taxonomic counterparts and may be the earliest derived genus in Araucariaceae (Setoguchi *et al.*, 1998). An alternative hypothesis suggests that *Wollemia* be a sister group to *Agathis* with these two forming a clade that is sister to *Araucaria* (Gilmore & Hill, 1997; Stefanovic *et al.*, 1998). Despite the origin of the relictual pine remaining an evolutionary enigma, the preliminary investigation of the chemistry of *W. nobilis* is consistent with that of other araucaroids, with the abundance of diterpenoids in their leaf oils being a seemingly characteristic trait (Brophy *et al.*, 2000).

As surveyed at allozyme, amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR) loci, the genetic diversity of Wollemi pine proved to be exceptionally low. It is considered, in fact, the most extreme case known in planta accounted for by the combination of such probable contributing factors as small population effects (less than a hundred trees occurring in the wild, in an inaccessible canyon), clonality and below-average genetic variation within the botanical family (Peakall *et al.*, 2003). Thus, each genetic individual seems to play a key role in the future evolution of the monotypic conifer, as the genetic diversity within this species constitutes the full genetic range of the genus. Moreover, microflora and other species associated with the microhabitat provided by the relictual pine are vital and unique elements contributing to the biodiversity of Wollemi National Park, New South Wales and, indeed, the Australian continent. It is noteworthy that *Pestalotiopsis guepini* – a paclitaxel producing fungus, was found to be a representative of the endophytic flora harboured by *W. nobilis* (Strobel *et al.*, 1997).

The aforementioned considerations, combined with the immense popularity of the 'green dinosaur' keenly sought after as a horticultural plant, reinforce the necessity of reducing the threat to the population in the wild from illegal collectors. For this reason, the Australian government has undertaken an unprecedented conservation programme, making *Wollemia nobilis* commercially available as a potted plant (NSW Department of Environment and Conservation, 2006). Thus, in early 2006 first Wollemi specimens left 'the land down under', opening a window into an unimaginably ancient past – a great opportunity of studying this extraordinary conifer worldwide.

The present study aims at a detailed analysis of the chemical composition of essential oil samples derived from different plant organs: leaves and twigs. Since the only report on the chemistry of Wollemi pine available to date (Brophy *et al*., 2000) deals with the analysis of leaves obtained from mature trees indigenous to Australia, we also hope to draw parallels and point out possible discrepancies, as our samples originate from a juvenile Wollemi specimen cultivated *ex situ*.

Materials & methods

Plant material

A juvenile (1-year-old, 30 cm in length) Wollemi pine bearing the registered Wollemi pine[TM] logo was purchased in 2008 from Arboretum Kalmthout, Belgium – a representative of Wollemi Australia exclusively licensed by the Royal Botanic Gardens Sydney (RBGS) through NSW National Parks and Wildlife Service (NPWS) to propagate and market the Wollemi pine in Australia and internationally. The conifer was additionally authenticated at the Botanical Garden 'De Kruidhof', Buitenpost, the Netherlands. A voucher specimen (gro-ASOK-02) is deposited in our department.

Isolation procedure

The oil samples were separately isolated from 14.2 g of air-dried and freshly ground (1 mm) leaves as well as 6.3 g of air-dried and coarsely ground twigs by hydrodistillation for 3 h in 300 mL water, according to the determination of the essential oil content in vegetable drugs, using the apparatus described in the *Nederlandse Farmacopee* (1966). Xylene (100 µL) was used as the collection liquid, and the oil was stored at -20°C until analyzed. The oil was diluted 50 times with cyclohexane prior to GC/MS analysis.

Gas Chromatography-Mass Spectrometry

A Shimadzu GCMS QP5000 system was used (Shimadzu Corporation, Japan) equipped with a GC-17A gas chromatograph, an AOC-20i auto-injector, and GCMS Solution version 1.10 software. The GC conditions were: column, Zebron Capillary GC Column, ZB-5 MS (15 m x 0.25 mm; film thickness 0.1 μm); oven temperature programme, 50-310°C at 5°C/min; injector temperature, 260°C; carrier gas, He; total flow, 59.3 mL/min; split ratio, 21:1; injected volume, 2.0 μL. MS conditions: ionization energy, 70 eV; ion source temperature, 250°C; interface temperature, 300°C; scan speed, 4 000 scans/s; mass range, 34-600 u.

The identity of the components was assigned by comparison of their retention indices, relative to C_9-C_{29} *n*-alkanes, with mass spectral databases and from literature (Adams, 2001; Joulain & König, 1998; Flavor & Fragrance Library Shimadzu Benelux, 's-Hertogenbosch, the Netherlands, 2003). The percentages of the components were calculated from the GC peak areas, using the normalization method.

Results & discussion

Hydrodistillation of the leaves and twigs of *Wollemia nobilis* yielded 0.9%, w/v and 0.3%, w/v oil, respectively. In total 42 components could be identified in the leaves and 40 in twigs, corresponding to 95.9% and 77.8% of the oil derived from the respective organs. To our knowledge, this is the first report dealing with the essential oil composition of Wollemi pine twigs, as opposed to the leaf-derived volatiles.

Table 1. Composition of the leaf and twig oils of *Wollemia nobilis*

compound	RI[a]	leaves [%]	twigs [%]
β-pinene	975	0.8	0.5
β-myrcene	995	0.4	0.3
3-methylene-1,7-octadiene	1033	2.2	1.5
4-carene	1058	nd[b]	tr[c]
octen-1-ol acetate	1119	tr[c]	0.2
6-camphenol	1122	0.4	0.2
(*E*)-3(10)-caren-4-ol	1135	0.9	0.3
verbenol	1143	0.2	0.1
6,6-dimethyl-2-methylene-bicyclo[2.2.1]-heptan-3-one	1154	0.1	nd[b]
Z-*β*-terpineol	1170	0.2	0.2
8-oxo-*cis*-ocimene	1184	nd[b]	0.1
2-acetyl-2-carene	1185	0.4	nd[b]
myrtenol	1190	0.1	tr[c]
verbenone	1198	0.2	nd[b]
(*E*)-3(10)-caren-2-ol	1214	0.1	nd[b]
carvone	1232	tr[c]	nd[b]
2,2-dimethylvaleroyl chloride	1238	tr[c]	tr[c]
bergamol	1249	tr[c]	tr[c]
(*Z*)-2-decenal	1253	tr[c]	nd[b]
bornyl acetate	1267	0.2	0.3
p-mentha-6,8-dien-2-ol acetate	1317	0.1	nd[b]
p-menth-8-en-2-ol acetate	1325	0.4	0.5
cyclobuta[1,2:3,4]dicyclopentene, decahydro-3a-methyl-6-methylene-1-(1-methylethyl)-[1S-(1.*α*,3a.*α*,3b.*β*,6a.*β*,6b.*α*)]	1345	tr[c]	0.1
sativene	1348	nd[b]	tr[c]
1-ethenyl-1-methyl-2,4-bis(1-methylethenyl) cyclohexane	1356	0.2	0.2
7,11-dimethyl-3-methylene-1,6,10-dodecatriene	1373	0.4	0.5
unknown, possible sesquiterpene[d]	1388	0.1	tr[c]
β-*cis*-ocimene	1399	0.2	0.2
germacrene D	1450	9.9	22.0
germacrene B	1460	0.8	1.2

α-muurolene	1493	nd[b]	tr[c]
spathulenol	1539	2.5	2.2
filipendulal	1546	0.4	0.2
(*iso*)-aromadendrene epoxide	1555	2.1	1.7
[1ar-(1a.*α*,4.*β*,4a.*β*,7.*α*,7a.*β*,7b.*α*]-decahydro-1,1,4,7-tetramethyl-1*H*-cycloprop[e]azulen-4-ol	1560	0.4	0.5
aromadendrene (2)-oxide	1605	1.4	1.9
α-cadinol	1613	0.4	0.5
(*iso*)-geraniol	1637	0.5	nd[b]
trans-longipinocarveol	1639	0.7	0.8
caryophyllene oxide	1650	nd[b]	tr[c]
trans-Z-α-bisabolene epoxide	1662	0.1	tr[c]
tetrahydrogeranyl acetate	1816	0.7	0.2
phtalic acid, butyl-2-ethylhexyl ester	1833	tr[c]	tr[c]
unknown, possible sesquiterpene[e]	1857	3.2	1.7
sandaracopimar-15-en-8.*β*.-yl acetate	1875	0.8	0.6
phylocald-15-ene	1920	1.0	1.6
kaur-16-ene	2023	61.8	38.2
4*β*,17-(acetoxy)-kauran-18-al	2052	1.7	0.4
kaur-16-en-18-oic acid, methyl ester	2123	nd[b]	1.0
total amount identified [%]		95.9	77.8
essential oil v/w [%]		0.9	0.3
grouped components			
monoterpene hydrocarbons		1.3	1.0
oxygenated monoterpenes		5.9	3.7
sesquiterpene hydrocarbons		14.6	25.7
oxygenated sesquiterpenes		8.8	7.8
diterpene hydrocarbons		62.8	39.8
oxygenated diterpenes		2.5	2.0

[a] retention index relative to C_9-C_{29} *n*-alkanes on a ZB-5 MS column
[b] not detected
[c] trace (<0.1%)
[d] MS, 70 eV, *m/z* (rel. int.): 204 [M]$^+$ (6.5), 189(6.8), 161(23.6), 147(8.4), 121(32.8), 105(28.9), 91(40.9), 41 (100)
[e] MS, 70 eV, *m/z* (rel. int.): 272[M]$^+$ (6.7), 257(22,0), 230(3.7), 202(2.5), 187(5.1), 175(5.7), 161(5.7), 148(12.7), 137(35.6), 119(20.4), 105(30.4), 91(40.6), 81(45.8), 41(100)

Comparative analysis of the investigated oil samples, as presented in Table 1, indicates a more pronounced monoterpene content in Wollemi foliage, reaching 7.3%, while the twig-derived oil contains 4.5%. The principal monoterpenoid constituents of both oils are β-pinene (0.8% and 0.5%, for leaves and twigs, respectively) and β-myrcene (0.4% and 0.3%). Sesquiterpene presence is considerably more pronounced in the twig-derived oil sample, amounting to 33.5%, than in its folial counterpart containing 23.4% of the C_{15} terpenes. On the contrary, while remaining the dominant group in both oil samples under investigation, diterpenoids are relatively more abundant in leaf-derived oil constituting 65.3%, as opposed to 41.7% detected in twigs. One might speculate that this observation could be accounted for by the formation of 'active isoprene units' – basic C_5 terpene building blocks, isopenthenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), proceeding via two alternative pathways. While the long known mevalonate (MVA) pathway (McGarvey & Croteau, 1995; Ruzicka, 1953), localized in the cytosol, provides the isoprene units for sesquiterpene biosynthesis, the plastid-localized methylerythritol phosphate (MEP) pathway, discovered and investigated only in recent years by Rohmer *et al.* (known, therefore, also as the *Rohmer pathway*) (Flesch & Rohmer, 1988; Rohmer *et al.*, 1993; Rohmer *et al.*, 1996; Rohmer, 2008) is thought to feed the biosynthesis of mono- and diterpenoids. The aforementioned cell-compartmentation (Trapp & Croteau, 2001) seems to be in accord with the postulated direct light interference with the non-mevalonate pathway (Seemann *et al.*, 2006), and consequently with the obviously prevalent cross-talk between the cytoplasmic and plastidial biosynthetic routes (Hemmerlin *et al.*, 2003; Schuhr *et al.*, 2003). Hence the significantly higher amounts of C_{15} terpenoids observed in the plant organs less photosynthetically active and less exposed to the light than the foliage. Germacrene D is the most prevalent sesquiterpenoid identified in both analyzed samples (9.9% in the leaf- and 22% in the twig-oil), accompanied by such hydrocarbons as germacrene B (0.8% and 1.2%), as well as an oxygenated C_{15} representative – spathulenol (2.5% and 2.2%). But one twig-specific sesquiterpenoid component – α-muurolene could be detected. As previously noted, diterpenoids constitute the most quantitatively significant fraction of both oil samples, yet qualitatively – the least versatile. Next to the most abundant 16-kaurene (61.8% and 38.2%, for leaves and twigs, respectively), only three C_{20} representatives were present in both samples, namely: phyllocald-15-ene (1% and 1.6%), an oxygenated derivative of sandaracopimarene (0.8% and 0.6%) and 4β,17-(acetoxy)-kauran-18-al (1.7% and

0.4%), while an ester of kaur-16-en-18-oic acid could additionally be detected in the twig-derived oil.

An alternative goal of the hereby presented study was to draw conclusions as to the presumed disparities between our observations and previously published data concerning the chemistry of *W. nobilis* foliage (Brophy *et al.*, 2000). The first notable difference seems to be the overall yield of the discussed experimental procedures. While Brophy *et al.* report 29 structures, accounting for 88.2% of the leaf oil sample recovered in 0.5%, our study detects 42 terpenoids corresponding to 95.9% of the hydrodistillate retrieved in 0.2% from the raw plant material. Thus the sensitivity of the gas-chromatographic separation technique coupled to the mass spectrometry based detection method utilized herein seems to exceed that of the previously reported methodology. As far as the particular terpene fractions, discrepancies of both quantitative and qualitative nature could be observed. Monoterpene content proposed in the previous study is significantly higher than the amount of C_{10} terpenes found in our sample (12% vs. 7.6%, relative to the entire amount of chemical structures identified in respective experiments). Moreover, while *α*-pinene was the most prevalent representative reported before (8.8%), our investigation showed no presence of this monoterpene, with its *β* isomer detected in a significantly lower amount (0.8%). Both studies suggest that germacrene D be the most abundant amongst leaf sesquiterpenoids. They differ, however, on the total percentage of sesquiterpenes present in folial oils – 15.4% proposed by Brophy *et al.*, as opposed to 24.4% detected herein. The most consistent results were obtained while investigating the C_{20} terpene fractions, with the total amount of diterpenes reaching around 70% of the structures identified in both samples, and 16-kaurene being the most prominent representative.

Several reasons accounting for the aforementioned disparities could be put forth. First of all, the plant material put to analysis herein was dried prior to distillation, which in turn could explain the overall higher content of oxygenated terpenes in our oil sample, as opposed to fresh foliage studied in Australia – significantly more abundant in isoprenoid hydrocarbons rather than their oxidized metabolites. Indeed, while revealing no other differences, be it of qualitative or quantitative nature, the analysis of oil derived from fresh leaves of our Wollemia specimen confirmed the decreased influence of oxygenation processes (data not shown). What is more, while the group of Brophy investigated leaves of wild mature Wollemi trees obtained from their natural habitat – Wollemi National Park, our sample was derived from a ju-

venile pot-cultivated specimen propagated in Arboretum Kalmthout, Belgium. Thus environmental, ecological and developmental considerations could be postulated.

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Screening the endophytic flora of *Wollemia nobilis* for alternative paclitaxel sources

Agata Staniek, Herman J. Woerdenbag, Oliver Kayser

Abstract

The endophytic flora of *Wollemia nobilis* was investigated in search for alternative paclitaxel producers. On one hand, metabolic profiling of the obtained specimens using an immunoenzymatic technique was done. On the other, we aimed at revealing the genetic background of presumed paclitaxel biosynthesis in the isolates.

We found an indication of endophytic taxane production in the extracts of two strains, *Phomopsis* sp. and *Cladosporium langeronii*. A PCR-based screening for *taxadiene synthase*, a gene unique to the formation of the taxane skeleton, confirmed the molecular blueprint for paclitaxel biosynthesis to be an inherent characteristic of the latter. Although this result makes *C. langeronii* an interesting candidate for further study, we postulate that proclaiming it 'a fungus factory for paclitaxel', as has been done for several other endophytes in the past, might be yet premature.

Introduction

Paclitaxel – the first billion dollar blockbuster drug of plant origin (Croteau, 2005), is a highly functionalized diterpenoid, primarily obtained from the inner bark of *Taxus brevifolia* (Wani *et al*., 1971). While the search for alternative sources of this compound brought quite an abundance of reports on paclitaxel producing endophytes in the 1990s (e.g. Stierle *et al*., 1993; Strobel *et al*., 1996; Li *et al*., 1998a&b; Noh *et al*., 1999), no conclusive follow-up data concerning fungal metabolite profile or genetic background of the biosynthetic pathway leading to paclitaxel is available, and the world's market still relies on yew-derived supply of the diterpene.

    Wollemia nobilis, an ancient conifer thought to have been extinct for millions of years was discovered only in 1994 in a remote Triassic sandstone gorge of the Wollemi National Park in the Blue Mountains north-west of Sydney (Hill, 1996). This relictual pine was found to harbour a paclitaxel producing endophyte *Pestalotiopsis guepini* (Strobel *et al*., 1997). Thanks to the unprecedented conservation programme undertaken by the Australian government (NSW Department of Environment and Conservation, 2006), making Wollemi pine commercially available as a potted plant, the great opportunity of studying this extraordinary conifer worldwide is now at hand.

    In the present study we investigate the endophytic flora of *Wollemia nobilis* in search for new paclitaxel synthesizing specimens. In parallel to utilising an immunoenzymatic detection technique to confirm taxane presence in fungal cultures, as well as investigating the presumed cytotoxic activity of the extracts therefrom, we also addressed the question of molecular blueprint for paclitaxel production being an inherent genetic trait of the endophytic isolates. The route leading to paclitaxel *in planta* requires 19 enzymatic steps (Walker & Croteau, 2001) and taxadiene synthase (TXS, EC 4.2.3.17) is considered to be a catalyst of the committed one (Wildung & Croteau, 1996), i.e. the slow bioconversion of the parent olefin (geranylgeranyl pyrophosphate, GGPP) to taxadiene – the tricyclic taxane core. Thus, our PCR-based screen for *txs* as a target gene not only bears clear implications for paclitaxel biosynthetic pathway elucidation in the endophytes, but might also shed some light on the controversial hypothesis of horizontal gene transfer (Rosewich & Kistler, 2000; Kurland *et al*., 2003). Namely, if one accepts the notion that the origin for fungal paclitaxel production arose through lateral transfer of genetic information from *Taxus* spp., and accounts for the wide means of dispersal possessed by fungi, it is conceivable that fungal paclitaxel synthesizers may be found in many plants worldwide, in-

cluding the relictual Wollemi pine. Furthermore, we hope to gain more insight into the fine-tuned equilibrium of plant-microbe interactions (Staniek *et al.*, 2008; Hines & Zahn, 2009) under evolutionary, environmental, physiological and genetic control.

Materials & methods

Chemicals

Paclitaxel ($\geq$ 95%, HPLC) reference compound as well as Potato Dextrose Agar medium were purchased from Sigma-Aldrich (St. Louis, USA). Glucose, peptone and Gamborg B5 medium were supplied by Duchefa (Haarlem, the Netherlands). All other chemicals and organic solvents were delivered by Merck (Darmstadt, Germany).

Plant material

Taxus baccata L. was purchased at a local market in Groningen and authenticated at the Botanical Garden 'De Kruidhof', Buitenpost, the Netherlands. A voucher specimen (gro-ASOK-01) is deposited in our department.
A juvenile (1-year-old, 30 cm in length) *Wollemia nobilis* W. G. Jones, K. D. Hill & J. M. Allan, bearing the registered Wollemi pine[TM] logo was purchased in 2008 from Arboretum Kalmthout, Belgium – a representative of Wollemi Australia exclusively licensed by the Royal Botanic Gardens Sydney (RBGS) through NSW National Parks and Wildlife Service (NPWS) to propagate and market the Wollemi pine in Australia and internationally. The conifer was additionally authenticated at the Botanical Garden 'De Kruidhof', Buitenpost, the Netherlands. A voucher specimen (gro-ASOK-02) is deposited in our department.

Isolation, cultivation & identification of fungal endophytes

Strains # 1, 3 & 4 were isolated from the surface-treated (70% ethanol for 10 min) Wollemi pine needles by hyphal tip transfer method (Strobel *et al.*, 1997). Strain # 2 was isolated from the surface-treated (70% ethanol for 10 sec, 15 % NaOCl:H_2O, 1:1 for 3 min, 3 × sterile water for 2 min) needles while attempting to generate plant callus cultures on B5 medium (Gamborg *et al.*, 1968) containing 0.5 mg/L 2,4-dichlorophenoxyacetic acid under a day/night regime (16/8 h; 2 500 lux) (van Uden *et al.*, 1990).

All strains were reintroduced onto Potato Dextrose Agar (PDA), and subsequently transferred into Sabouraud-Dextrose broth (peptone 10 g/L, glucose 20 g/L, pH 5.6). A slightly modified S7 medium (Stierle *et al.*, 1993) comprising: glucose 1 g/L, fructose 3 g/L, sucrose 6 g/L, peptone 1 g/L, sodium acetate 1 g/L, yeast extract 250 mg/L, thiamine 1 mg/L, biotin 1 mg/L, pyridoxal 1 mg/L, $Ca(NO_3)_2$ 6.5 mg/L, phenylalanine 5 mg/L, $MgSO_4$ 3.6 mg/L, $CuSO_4$ 1 mg/L, $ZnSO_4$ 2.5 mg/L, $MnCl_2$ 5 mg/L, $FeCl_3$ 2 mg/L, benzoic acid 100 mg/L, 1M KH_2PO_4 buffer (pH 6.8) 1 mL/L was the experimental medium. Growth incubation was conducted at room temperature with moderate rotation speed (100 rpm) under a day/night regime (16/8 h; 1 200 lux). Subculturing was performed by medium refreshment of a one-week-old fungal suspension in a 1:100 ratio. The volume of experimental cultures was 1 L.
Strain identification was performed by Centraalbureau voor Schimmelcultures (CBS), Institute of the Royal Netherlands Academy of Arts and Sciences, Utrecht, the Netherlands, according to standard procedures:

1: *Phomopsis* sp. (Uecker, 1988)

Genus identification was based on the sequence of the ITS (internal transcribed spacer) of the nuclear ribosomal DNA. The gene sequence was obtained by PCR using primers V9G (de Hoog & van den Ende, 1998) + LS266 (Masclaux *et al.*, 1995) and ITS1 + ITS4 (White *et al.*, 1990). The same primers were used in the sequencing reactions. A contig was formed using the sequenced DNA fragments. The cultures remained sterile and species identification based on DNA sequences was not possible by lack of appropriate reference sequences.

2: *Cladosporium langeronii* (Fonseca, Leão & Nogueira) Vuill. (Zalar *et al.*, 2007)

Identification was based on the sequence of the ITS of the nuclear ribosomal DNA. The sequence was determined using the primers V9G and LS266 for both PCR and sequencing reactions.

3: *Acremonium*-like anamorph

The anamorph produced in our subcultures was structurally very simple, and similar forms are known to occur in various species of *Acremonium*, but also in *Verticillium* and related Hypocrealean fungi.

The sequence of the ITS of the nuclear ribosomal DNA was determined using the primers V9G and LS266 for both PCR and sequencing reactions. The (partial) sequence of the β-tubulin was determined using the primers Bt10 (O'Donnell & Cigelnik, 1997) and BT2b (Glass & Donaldson, 1995) for both PCR and sequencing reactions. This fungus belongs to the Sordariomycetes, but no sufficiently close matches were found for the ITS rDNA and β-tubulin genes with reference strains to more precisely assess its phylogenetic position.

4: *Lecythophora* anamorph of *Coniochaeta velutina* (Fuckel) Cooke (Weber, 2002)

Identification was based on the sequence of the ITS of the nuclear ribosomal DNA. The sequence was determined using the primers ITS1 and ITS4 for both PCR and sequencing reactions. This sequence matched with the ITS sequence of *Coniochaeta velutina*, however, the cultures only developed the anamorphic state.

Extract preparation

Taxus, the original paclitaxel producer, was employed as a reference organism providing for positive controls of the experimental procedures. Crude and purified extracts of dried *Taxus baccata* needles were prepared as described previously (Grothaus *et al*., 1995).
After Büchner-filtration, the fungal mycelia were macerated and extracted with CH_2Cl_2:MeOH (1:1), while the filtrates were extracted with CH_2Cl_2. As the separation proved difficult in case of specimen # 3, the entire culture broth was extracted with CH_2Cl_2. The solvent was removed from the organic extracts by rotary evaporation under reduced pressure at ~ 40ºC (Strobel *et al*., 1994).

Immunodetection

CIEIA – a competitive inhibition enzyme immunoassay system for the quantitative detection of taxanes in biological matrices (Grothaus *et al*., 1993) was performed using a commercially available kit (Hawaii Biotech, Inc., Aeia, Hawaii). The extracts (10 mg dry residue) were dissolved in a CIEIA compatible solvent containing 20% methanol.

DNA isolation

Efficient extraction of plant and fungal DNA was accomplished using DNeasy® Plant Mini Kit (Qiagen Inc., USA) according to the manufacturer's protocol.

Plasmid DNA purifications were performed using QIAprep® Spin Miniprep Kit (Qiagen Inc., USA).

PCR products were purified with QIAquick® PCR Purification Kit & QIAquick® Gel Extraction Kit (Qiagen Inc., USA).

PCR

Temperature gradient PCR reactions were conducted with *Phusion*™ DNA Polymerase (2U/µl) (Finnzyme OY, Finland), in the Eppendorf Mastercycler gradient thermocycler.

Thermal conditions

98°C for 1 min (initial denaturation), 35 × [98°C for 10 sec (denaturation), 20 sec (annealing) in temperature gradient, 72°C (extension) for the period of time dependent on the length of the amplified gene fragment and enzyme efficiency], 72°C for 10 min (final extension).

Primers

taxadiene synthase gene (Zhou *et al.*, 2007)
- ctxs_fwd (5'-caaacccatgtcgaattgagaag-3')
- ctxs_rev (5'-caagtttgcatacactctggaatct-3')

The primers were purchased from Operon Biotechnologies, Germany.

Molecular cloning and sequence analysis

A-tailed *Phusion*™ amplification products were ligated into pGEM®-T & pGEM®-T Easy Vector Systems (Promega Corporation, USA). The obtained plasmid vectors were transformed into XL1-blue competent *E. coli* strain (Stratagene, USA). Endonuclease digestions of DNA inserts were performed using restriction enzymes from New England BioLabs Inc., USA. Sequence analysis was performed by Macrogen Ltd., Seoul, Korea.

Cytotoxic activity tests

Cytotoxic activity of the extracts was investigated against a human ovarian cancer cell-line OVCAR-3 (ATTC[®]) by means of MTS assay (CellTiter 96[®] AQ$_{ueous}$ Non-Radioactive Cell Proliferation Assay, Promega Corporation, USA).

The cancer cells were routinely cultured in DMEM (Invitrogen[®]), supplemented with 10% of heat inactivated fetal bovine serum (Invitrogen[®]) and suitable antibiotics: 1% penicillin/streptomycin (Invitrogen[®]), at 37ºC in the presence of 5% CO_2. The addition of paclitaxel reference, plant and fungal extracts, dissolved in 1% DMSO sterilized by filtration (0.2 μm) and diluted with cell culture medium, in consecutive 2-fold dilutions was preceded by seeding the test cells into 96-well plates (5000 cells/well) and 24 h incubation. Cell viability was assessed 72 h later by means of the MTS assay, following the instructions of the manufacturer. All assays were performed in triplicate.

Microscopy

Microscopic observations and imaging were performed by means of the Olympus CKX41 inverted microscope coupled with the Altra 20 Soft Imaging System.

Results & discussion

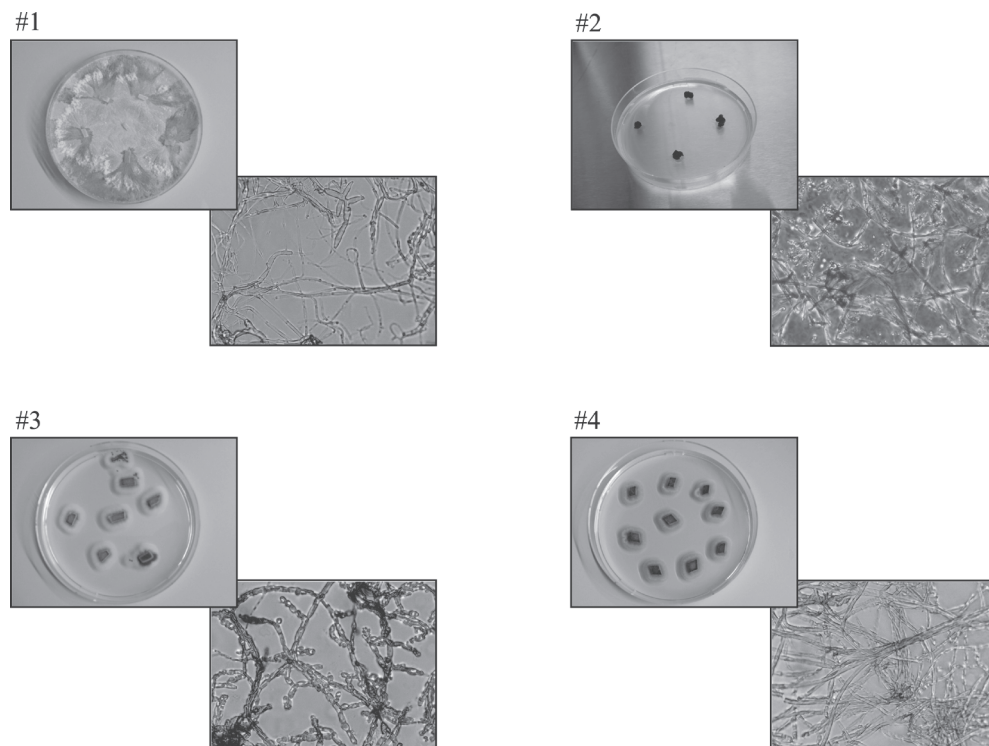Four distinct isolates of endophytic fungi were recovered from the sampled *Wollemia nobilis* needles (Figure 1).

Figure 1. Macro- and micromorphological (Olympus CKX41SV, ×400) characteristics of endophytic isolates

Each fungus was grown in the experimental medium, extracted as described, and assayed for paclitaxel. As current chromatographic methods for the determination of taxanes in biological matrices are considered to be relatively insensitive, an immunoenzymatic system (CIEIA) was employed in order to gain insight into the presence of taxane compounds in the endophytic extracts. The reactivity of this assay towards paclitaxel was 0.5 ng/mL. The average taxane concentration values in crude and purified extracts of *Taxus baccata* needles, providing for a positive control of taxoid presence verification, amounted to 270 µg/mL and 20 µg/mL respectively, which constitutes 0.05% of dry weight and corresponds to taxane concentrations in *Taxus* needles as previously reported (Grothaus *et al*., 1995). Of the fungi isolated, specimens # 1 and # 2 were the most interesting. Organic

extracts of their culture medium filtrates provided evidence of paclitaxel at a concentration of 10.1 (± 2.7) ng/mL and 19.4 (± 0.4) ng/mL respectively and of 13.4 (± 1.0) ng/mL in the mycelial extract for the isolate #2 alone.

The presumed cytotoxic effect of the fungal extracts was further investigated. To ensure for the positive control of proliferation arrest, the ovarian cancer cells were treated with paclitaxel, as well as with the purified extract of *Taxus baccata* needles. The IC_{50} of paclitaxel in the cell line tested was approximately 50 nM as found previously (Kolfschoten *et al*., 2002) and corresponded to the amount of the drug in the reference plant material. We found a clear cytotoxic effect of the fungal extracts (Table 1), but it is uncertain whether these effects are to be ascribed to paclitaxel due to poor solubility and minute amounts of the drug detected.

Table 1. Cytotoxic effect of endophytic extracts

	IC_{50} [mg/mL]
# 1	0.16 (±0.04)[a] 0.10 (±0.01)[b]
# 2	0.30 (±0.05)[a] 0.65 (±0.07)[b]
# 3	- [c]
# 4	0.40 (±0.06)[a] - [b]

[a] medium filtrate extract
[b] mycelial extract
[c] culture broth extract

To further address the question of paclitaxel biosynthesis being an inherent characteristic of the fungal isolates, our research continued at a genomic level, with polymerase chain reaction being the basic tool of investigation. Thus, the genomic DNA of all fungal specimens served as a template in hope for efficient annealing with the specific starters proposed by Zhou *et al*. (2007). While the PCR-based screen yielded a number of potentially interesting amplification products, the genetic capacity to synthesize the tricyclic taxane core was ultimately confirmed only for the isolate # 2 (Figure 2). High similarity of the amplicon obtained with its plant counterparts (96% sequence identity within the conserved region) (Figure 3) gives unequivocal

evidence for the presence of *taxadiene synthase* in the fungal genome and allows to ascribe the biosynthesis of paclitaxel *ab initio* to be a genuine feature of *Cladosporium langeronii*. This makes the isolate an interesting candidate for further in-depth study involving the confirmation of txs enzyme activity followed by fermentation up-scaling and optimization as well as genetic engineering.
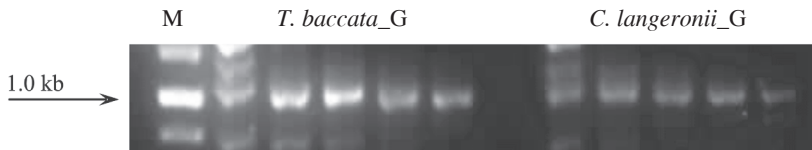

Figure 2. PCR analysis of the presence of *taxadiene synthase* (~ 1.0 kb) in *Cladosporium langeronii*; *Taxus baccata* needles were a source of plant DNA samples, providing for a positive control of *txs* presence verification (M – GeneRuler[TM] 1 kb DNA Ladder, Fermentas; G – annealing temperature gradient: 45 - 65°C)

While our study failed to re-establish the previously reported endophytic paclitaxel producer, *Pestalotiopsis guepini* harboured by *Wollemia nobilis* in its natural habitat (Strobel *et al.*, 1997), it recovered interesting isolates bearing indications of presumed taxane biosynthesis, be it on a metabolic (*Phomopsis* sp. & *Cladosporium langeronii*) or on a genomic level (*Cladosporium langeronii*). It also allows to speculate on the origins of fungal paclitaxel and seems to emphasize the impact of environmental factors on its prevalence. On one hand, fungi may be an independently evolved system for paclitaxel production. However, the fact that the biosynthesis of this highly functionalized and unique diterpenoid requires approximately 20 genes (Walker & Croteau, 2001) makes this rather unlikely. Therefore, it seems plausible that a horizontal transfer of genetic information shaped the evolutionary trajectory of taxonomically unrelated, yet co-existing, species and further influenced the dispersal of endophytic taxane synthesizers in a given ecosystem. Recent reports on paclitaxel producing fungi harboured not only by *Taxus*, but also by other species (Kumaran *et al.*, 2009; Kumaran & Hur, 2009), seem to be in accord with that notion thus suggesting that alternative taxane synthesizers may be found in many plants worldwide, including a *Wollemia nobilis* specimen cultivated *ex situ*.

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Score = 1463 bits (792),  Expect = 0.0
Identities = 860/891 (96%), Gaps = 12/891 (1%)
Strand=Plus/Plus

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Query  2140  TGCAGAACCATATCTTAGAGACGCACTTGCAACGAAAATCTCAACCAATACAAAACTATA  2199
             ||||||||||||||||||||||||||||||||||  |||||||||||||||||||||| ||
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             | ||  |||||||||||||||||||||||||||||||| |||||||||||||||||||||
Sbjct  309   GTCT-AGAAGCCGGAAGTTATATTGATTCATATGACGATGATTATGTATGGCAGAGGAAG  367

Query  2439  ACTCTATACAGGTGAGTTCAAATATA-TCCCCATTACCCTTTAATTTTATATAGAATTTA  2497
             |||  |||||||||||||||||||||| ||  ||||||| ||||||||||||||||||||
Sbjct  368   ACTTTATACAGGTGAGTTCAAATATAATCAC-ATTACCCTTCAATTTTATATAGAATTTA  426

Query  2498  TGACCCGTTGTTGTATCGATTGCTATCTTTTCTCAGTACTGAGGTTATAATTCGTGCAAC  2557
             ||| |||||||||||||||||||||||||||||||  ||||||||||||||||  ||||||
Sbjct  427   TGAGCCGTTGTTGTATCGATTGCTATCTTTTCTCAATACTGAGGTTATAATCCGTGCAAC  486

Query  2558  AGAATGCCATCTTTGAGTAATTCAAAATGTTTAGAATTGGCAAAATTGGACTTCAATATC  2617
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  487   AGAATGCCATCTTTGAGTAATTCAAAATGTTTAGAATTGGCAAAATTGGACTTCAATATC  546

Query  2618  GTACAATCTTTGCATCAAGAGGAGTTGAAGCTTCTAACAAGGTGGGTGATAAATTGTCAT  2677
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  547   GTACAATCTTTGCATCAAGAGGAGTTGAAGCTTCTAACAAGGTGGGTGATAAATTGTCAT  606

Query  2678  ACCTTCTCAATTTATTCAAAACCTAATTTTTTGGGGAGGAGAACATGAAGTAATAATCTA  2737
             |||||||||||||||||||||||||||||||||||  ||||||||||||||||||||||||
Sbjct  607   ACCTTCTCAATTTATTCAAAACCTAATTTTTTGGGAAAGAGAACATGAAGTAATAATCTA  666

Query  2738  CTAACATTATGGTTTTTTGGTCATAAGATGGTGGAAGGAATCCGGCATGGCAGATATAAA  2797
             |||||||||||||||||||||||||||||||||||||||||||| |||||||||||||||
Sbjct  667   CTAACATTATGGTTTTTTGGTCATAAGATGGTGGAAGGAATCTGGCATGGCAGATATAAA  726

Query  2798  TTTCACTCGACACCGAGTGGCGGAGGTTTATTTTTCATCAGCTACATTTGAACCCGAATA  2857
             |||||||||||||||||||||||||| ||||||||||||||||||||||||||||||||||
Sbjct  727   TTTCACTCGACACCGAGTGGCGGAAGTTTATTTTTCATCAGCTACATTTGAACCCGAATA  786

Query  2858  TTCTGCCACTAGAATTGCCTTCACAAAAAATTGGTTGTTTACAAGTCCTTTTTGATGATAT  2917
             |||||||||| ||||||||||||||| ||| |||| |||||| |||||||| ||||||||
Sbjct  787   TTCTGCCACTCGAATTGCCTTCACAAAA-TTGGT-GTTTACA-GTCCTTTT-GATGATAT  842

Query  2918  GGCTGACATCTTTGCAACACTAGATGAATTGAAAAGTTTCACTGAGGGAGT    2968
             |||||||||||||||| |||||||||||||||| ||| ||||||| |||||
Sbjct  843   GGCTGACATCTTTGCA-CACTAGATGAATTGAAA-GTT-CACTGAAGGAGT    890
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Figure 3. *Taxadiene synthase* sequence alignment
(http://blast.ncbi.nlm.nih.gov)
Query: *T. baccata txs* gene, accession no. AJ320538,
Sbjct: *C. langeronii txs* sequence obtained in course of research

Regardless of the aforementioned fundamental speculations, practical aspects should be decisively invoked yet again. As we discussed elsewhere (Staniek *et al*., 2009), specific plant environment might be required for the sustainable synthesis of the drug. Indeed, while a recent publication (Kumaran & Hur, 2009) reports three strains of *Phomopsis* isolated from three different gymnosperms to be paclitaxel producers, the highest yields are ascribed to the one harboured by *Taxus cuspidata* – the original yew synthesizer of the valuable antineoplastic agent. It is also noteworthy that the overwhelming majority of reports dealing with taxane producing endophytes describe the fungal symbionts shortly after removal from their plant hosts, not taking full account of the possible impact and nature of the interdependencies between the naturally co-existing species (Staniek *et al*., 2008; Hines & Zahn, 2009). But one, very recent paper communicates an endeavour to re-establish the co-habitat by proposing a promising co-culture system for *Taxus chinensis* var. *mairei* and its endophyte *Fusarium mairei* (Li *et al*., 2009).

As the highly desirable search for reliable and economically feasible paclitaxel sources seems to have revived the interest in endophytes as potential taxane bio-factories, we still contend that such reports should be viewed as extraordinary claims that demand ultimate justification, namely: unambiguous evidence for molecular blueprint underlying the postulated microbial paclitaxel biosynthesis, suggesting a means for its induction and manipulation, as well as taking heed of the interactions between plant hosts and their fungal inhabitants.

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Chapter 7

Summary

Concluding remarks & future perspectives

Whether suffering a pathogenic attack, basking in symbiotic comfort, or seemingly symptomless, plants constantly participate in molecular interplay with various classes of microbial organisms. One of the means of interorganismal communication in this dynamic continuum are secondary metabolites. The chemical diversity bearing pharmaceutical potential thus implied reaches beyond the plant kingdom and offers an expended view promising to transform glimpses of reductionist research of the past years to snapshots of an exuberant world of systems biology. Endophytes seem to fit perfectly into this natural 'warehouse', only a small part of which we have been able to tap into so far.

The introductory section of the hereby presented thesis (**chapter 2**) provides an elaborate overview on the current state of knowledge about endophytic organisms – microbes colonizing internal tissues of all plant species, creating a huge biodiversity with yet unknown novel natural products presumed to push forward the frontiers of drug discovery (Staniek *et al*., 2008).

Paclitaxel, the world's first billion dollar anticancer blockbuster, was primarily obtained from *Taxus brevifolia*. While the search for alternative sources of the powerful antineoplastic agent brought an array of reports on paclitaxel producing endophytes, causing quite a controversy over the past two decades, the world's market still relies on yew-derived supply of the valuable diterpene.

The primary objective of the research presented in this thesis is to investigate the potential of endophytes as alternative paclitaxel sources in all its intriguing aspects. On one hand, we strive to dissipate the ambiguity surrounding the very first presumed endophytic paclitaxel producer, *Taxomyces andreanae*, harboured by the original yew synthesizer of the drug (Stierle *et al*., 1993) (**chapter 3**). On the other, prompted by the extraordinary findings of recent years (Hill, 1996; Strobel *et al*., 1997), we describe the isolation and screening of the endophytic flora of an ancient conifer *Wollemia nobilis* in search for other paclitaxel producing specimens (**chapter 6**). In parallel to utilizing chromatographic, spectroscopic and immunoenzymatic detection methods prevalent in literature to confirm taxane presence in endophytic cultures, our research was brought to an alternative level addressing the question of molecular blueprint for paclitaxel production being an inherent genetic trait of the fungal isolates. Our PCR-based, in-depth study of *taxadiene synthase* and *baccatin III phenylpropanoyl transferase* as target genes not only bears clear implications for paclitaxel biosynthetic pathway elucidation

in the endophytes, but also allows to speculate on the origins of fungal taxanes. On one hand, fungi might be an independently evolved system for paclitaxel production. However, the fact that the biosynthesis of this highly functionalized and unique diterpenoid *in planta* involves approximately 20 genes (Walker & Croteau, 2001) makes this rather unlikely. Therefore, it seems plausible that a horizontal transfer of genetic information shaped the evolutionary trajectories of taxonomically unrelated, yet co-existing, species and further influenced the dispersal of endophytic taxane synthesizers in a given ecosystem and, indeed, worldwide (Staniek *et al.*, 2009; Staniek *et al.*, 2010b).

Moreover, postulating horizontal gene transfer to be a driving force in the evolution of fungal gene clusters – a phenomenon now considered a hallmark characteristic of secondary metabolic biosynthetic pathways (Keller & Hohn, 1997; Keller *et al.*, 2005), rises an intriguing question as to whether the genes responsible for paclitaxel formation in *Taxomyces andreanae* and other presumed endophytic taxane producers are indeed grouped in a contiguous cluster. Equipped with this essential genetic information and modern tools to manipulate the biosynthetic machinery, the research on microbial paclitaxel synthesizers could enter a novel combinatorial stage. While several heterologous systems including *Escherichia coli* (Huang *et al.*, 2001), *Saccharomyces cerevisiae* (DeJong *et al.*, 2006; Engels *et al.*, 2008) and *Pichia pastoris* (Schmeer & Jennewein, 2009) were already exploited for expression of plant-derived genes encoding early paclitaxel biosynthetic enzymes, to engineer and co-mobilise a functional gene cluster in a parent producer microorganism affords the advantage of all the regulatory elements being present and functional. This undoubtedly promising approach seems all the more challenging in view of our further findings concerning pure *Taxomyces andreanae* strain. Namely, despite considerable up-scaling endeavours, metabolic profiling of the commercial isolate brought no confirmation of endophytic paclitaxel production in axenic culture conditions. This result suggests that specific plant environment may be required for the induction of paclitaxel biosynthetic genes in the fungal symbiont. Identifying these triggering mechanisms will be of considerable future interest, not only providing further insight into the true nature of the fine-tuned equilibrium of plant-microbe interactions, but also revealing their tremendous therapeutical potential. In fact, a recent report communicates an unprecedented endeavour to re-establish the intriguing co-habitat by proposing a promising co-culture system for *Taxus chinensis* var. *mairei* and its endophyte *Fusarium mairei*

(Li *et al*., 2009). The next challenge lies in the further integration of these approaches to develop a comprehensive picture of how life history traits of both 'players' interact with the environment to shape evolutionary trajectories (Burdon & Thrall, 2009).

Wollemia nobilis, the aforementioned host of endophytic paclitaxel synthesizers, is an exciting object of investigation *per se*. Its discovery in 1994, hailed as the 'botanical find of the century', opened a window into an unimaginably ancient past – a great opportunity of studying the conifer thought to have been extinct for millions of years. **Chapter 4** describes our efforts to obtain callus and cell suspension cultures of Wollemi pine. While ultimately unattained, despite extensive optimization attempts, the aim should not be abandoned as it could prove a milestone in further unravelling of the botanical enigma of 'the living fossil'. In **chapter 5** we turn our attention to the chemistry of *Wollemia nobilis*, proposing a comparative analysis of the essential oil constituents derived form its different organs, namely leaves and twigs. The results obtained allow speculation on the fundamentals of the formation of 'active isoprene units' – basic C_5 terpene building blocks, proceeding via two alternative pathways. While the long known mevalonate (MVA) pathway, localized in the cytosol, provides the isoprene units for sesquiterpene biosynthesis, the plastid-localized methylerythritol phosphate (MEP) pathway, discovered and investigated only in recent years by Rohmer *et al*. (known, therefore, also as the *Rohmer pathway*) is thought to feed the biosynthesis of mono- and diterpenoids. The aforementioned cell-compartmentation seems to be in accord with the postulated direct light interference with the non-mevalonate pathway, and consequently with the obviously prevalent cross-talk between the cytoplasmic and plastidial biosynthetic routes. Hence the significantly higher amounts of sesquiterpenoids observed in the plant organs less photosynthetically active and less exposed to the light than the foliage boasting the abundance of mono- and diterpenes (Staniek *et al*., 2010a).

In summary, the research presented in this thesis investigates the potential of endophytes as yet untapped, prolific source of pharmaceutically relevant natural products, with an unequivocal attention focused on paclitaxel. As the highly desirable search for sustainable and economically feasible sources of this excellent antitumour agent has tempted various authors to draw premature conclusions proclaiming endophytes to be independent taxane bio-factories, we contend that the answer to the issue of paclitaxel supply crisis might lie within the interplay between the plant hosts (often them-

selves unique, like the relictual Wollemi pine) and their microbial inhabitants, under evolutionary and environmental control. While one has to be mindful that the problem we set out to address is several orders of magnitude larger than those with which we are familiar, no one can deny the opportunities that present themselves in the era of modern functional genomics and systems biology.

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Atakowane przez patogeny, korzystające z harmonii symbiozy czy też nie zdradzając żadnych objawów, rośliny nieustannie uczestniczą w molekularnej współgrze z różnorodnymi klasami mikroorganizmów. Jednym ze sposobów komunikacji w owym wzajemnym oddziaływaniu są metabolity wtórne. Tak implikowana chemiczna różnorodność niosąca farmaceutyczny potencjał wykracza więc poza królestwo roślin i oferuje poszerzony pogląd obiecujący zamienić migawki redukcjonistycznego myślenia dominującego naukę minionych lat na pełen obraz biologii systemowej. Endofity zdają się idealnie pasować do tego naturalnego 'magazynu', z którego bogactwa zdołaliśmy do tej pory uszczknąć jedynie odrobinę.

Wstęp niniejszej dysertacji (**rozdział 2**) dostarcza gruntownej oceny obecnego stanu wiedzy na temat organizmów endofitycznych – mikrobów (głównie grzybów) kolonizujących wewnętrzne tkanki wszystkich gatunków roślin i tym samym tworzących ogromną bioróżnorodność dotąd nieznanych oryginalnych produktów naturalnych mogących odegrać znaczącą rolę na arenie farmaceutycznej (Staniek *et al*., 2008).

Paklitaksel, pierwszy na świecie lek przeciwnowotworowy przynoszący bilionowe zyski, wyizolowano pierwotnie z kory cisu zachodniego (*Taxus brevifolia*). Podczas gdy poszukiwanie alternatywnych źródeł tego niezwykle skutecznego cytostatyku przyniosło szereg publikacji o endofitach zdolnych do jego produkcji wywołując sporo kontrowersji w ciągu dwóch ostatnich dekad, światowy rynek nadal polega na zasobach paklitakselu pozyskiwanego z surowców roślinnych.

Głównym celem badań przedstawionych w dysertacji jest ocena wszystkich intrygujących aspektów potencjału endofitów jako alternatywnych źródeł paklitakselu. Z jednej strony usiłuje ona rozwikłać tajemniczość otaczającą pierwszego z sugerowanych endofitycznych producentów cytostatyku, *Taxomyces andreanae*, wyizolowanego z cisu, a więc pierwotnego, roślinnego 'dostawcy' leku (Stierle *et al*., 1993) (**rozdział 3**). Z drugiej zaś, w odpowiedzi na niezwykłe odkrycia minionych lat (Hill, 1996; Strobel *et al*., 1997), opisuje izolację oraz badanie endofitycznej flory kolonizującej prehistoryczne drzewo iglaste, wolemię szlachetną (*Wollemia nobilis*) w poszukiwaniu kolejnych okazów syntetyzujących paklitaksel (**rozdział 6**). Równolegle do stosowania dominujących w literaturze metod chromatograficznych, spektroskopowych i immunoenzymatycznych służących detekcji taksanów w kulturach endofitów, usiłowano nadać procedurze badawczej alternatywny, bezprecedensowy przebieg i odpowiedzieć na pytanie czy molekularny wzór biosyntezy paklitakselu jest nieodłączną, genetyczną cechą

badanych mikroorganizmów. Dogłębna, oparta o jedną z podstawowych technik genomiki funkcjonalnej – łańcuchową reakcję polimerazy (PCR), analiza *syntazy taksadienu* oraz *transferazy fenylowopropanoylowej bakkatyny III* jako docelowych genów nie tylko jasno sugeruje możliwość wyjaśnienia endofitycznej ścieżki metabolizmu wtórnego, prowadzącej do biosyntezy paklitakselu, lecz pozwala także spekulować na temat genezy taksanów w królestwie grzybów. Z jednej strony, grzyby mogą stanowić system o niezależnej ewolucji produkcji paklitakselu. Jednakże fakt, iż biosynteza tego niezwykle rozbudowanego, unikatowego diterpenoidu *in planta* wymaga około 20 genów (Walker & Croteau, 2001) czyni powyższą tezę mało prawdopodobną. Dlatego wydaje się wiarygodne, że horyzontalny transfer informacji genetycznej mógł ukształtować trajektorie ewolucji taksonomicznie niespokrewnionych, lecz koegzystujących gatunków oraz wpłynąć na rozprzestrzenienie endofitycznych producentów taksanów w danym ekosystemie oraz, idąc dalej, na całym świecie (Staniek *et al*., 2009; Staniek *et al*., 2010b).

Ponadto, postulat utożsamiający horyzontalny transfer genów (HGT) z siłą wiodącą w ewolucji klasterów genów u grzybów – zjawisko uważane obecnie za nieodłączną cechę grzybowych genomów (Keller & Hohn, 1997; Keller *et al*., 2005), stwarza intrygujące pytanie czy geny odpowiedzialne za formację paklitakselu u *Taxomyces andreanae* i innych endofitycznych producentów taksanów są rzeczywiście zgrupowane w klaster. Posiadanie tej istotnej wiedzy genetycznej połączone z wykorzystaniem nowoczesnych narzędzi pozwalających na manipulację w biosyntetycznej maszynerii mogłoby spowodować wejście badań nad alternatywnymi producentami paklitakselu w nowy kombinatoryczny etap. Podczas gdy kilka heterologicznych systemów, jak *Eschgerichia coli* (Huang *et al*., 2001), *Saccharomyces cerevisiae* (DeJong *et al*., 2006; Engels *et al*., 2008) i *Pichia pastoris* (Schmeer & Jennewein, 2009) zostało już wykorzystanych do ekspresji genów cisu kodujących enzymy wczesnych kroków w biosyntezie paklitakselu, inżynieria i komobilizacja funkcjonalnego kalsteru genów u oryginalnego producenta posiada bezsporną zaletę obecności i funkcjonalności wszelkich elementów regulujących. W obliczu wniosków płynących z dogłębnej analizy czystego szczepu *Taxomyces andreanae*, to niewątpliwie obiecujące podejście zdaje się stanowić tym większe wyzwanie. Mianowicie, pomimo kultywacji na szeroką skalę, analiza metabolitów komercyjnego izolatu nie przyniosła potwierdzenia produkcji paklitakselu przez endofit w aksenicznych warunkach hodowli. Wynik ten sugeruje, że specyficzne środowisko roślinne może być

niezbędne do indukcji genów biosyntezy paklitakselu grzybowego symbionta. Identyfikacja specyficznych mechanizmów indukujących otworzy perspektywę nie tylko poznania prawdziwej natury wyrafinowanego ekwilibrium interakcji pomiędzy roślinami a mikrobami, lecz także odkrycia pełni ich ogromnego potencjału terapeutycznego. Jedyne dotychczas, niedawno opublikowane doniesienie komunikuje bezprecedensową próbę przywrócenia intrygującej współzależności proponując obiecujący system ko-hodowli cisu chińskiego, *Taxus chinensis* var. *mairei* i jego endofitu, *Fusarium mairei* (Li *et al*., 2009). Dalszym wyzwaniem jest integracja wyżej wymienionych nowatorskich założeń i ukształtowanie wszechstronnego poglądu na sposób, w jaki historyczne rysy obu 'graczy' oddziaływując z otaczającym środowiskiem, kształtują trajektorie ewolucji (Burdon & Thrall, 2009).

Wolemia szlachetna (*Wollemia nobilis*), wspomniany wcześniej gospodarz endofitów syntetyzujących paklitaksel, stanowi eksytujący przedmiot badań *per se*. Jej rozpoznanie w roku 1994, okrzyknięte mianem 'botanicznego odkrycia XX wieku', otworzyło drzwi do niewyobrażalnie odległej przeszłości, umożliwiając studia nad gatunkiem uważanym za wymarły przez miliony lat. **Rozdział 4** opisuje starania pozyskania kallusa i zawiesiny komórek wolemii szlachetnej. Choć celu ostatecznie nie osiągnięto, pomimo zakrojonej na znaczną skalę optymalizacji warunków hodowli *in vitro*, nie należy zaprzestawać dążeń, które mogą okazać się kamieniem milowym na drodze do rozwiązania botanicznej enigmy tej 'żyjącej skamieliny'. W **rozdziale 5** szczególną uwagę zwrócono na chemię wolemii szlachetnej, proponując analizę porównawczą składu olejków eterycznych pozyskanych z różnych organów rośliny, mianowicie liści i gałązek. Otrzymane wyniki pozwalają na spekulację dotyczącą istoty formacji 'aktywnych jednostek izoprenowych' – podstawowego budulca związków terpenoidowych, powstających na drodze alternatywnych ścieżek metabolicznych. Podczas gdy znana od dawna, zlokalizowana w cytozolu, ścieżka mewalonianowa (MVA) dostarcza jednostek izoprenowych do biosyntezy seskwiterpenów, specyficzna dla plastydów ścieżka fosforanu metylerytritolu (MEP), odkryta i badana stosunkowo niedawno przez grupę Rohmera (znana także jako *ścieżka Rohmera*) zasila formację mono- i diterpenów. Wspomniana kompartmentacja zdaje się odzwierciedlać postulowany bezpośredni wpływ światła na ścieżkę nie-mewalonianową oraz wewnątrzkomórkową kros-komunikację pomiędzy cytoplazmatycznym i plastydowym szlakiem biosyntezy. Stąd też znacznie wyższe ilości seskwiterpenoidów odnotowane w organach o mniej

nasilonej aktywności fotosyntetycznej i w mniejszym stopniu wystawionych na dziłanie promieniowania świetlnego niż liście obfitujące w związki mono- i diterpenowe (Staniek *et al.*, 2010a).

Podsumowując, dociekania przedstawione w niniejszej dysertacji badają potencjał endofitów jako jeszcze niepoznanego obfitego źródła produktów naturalnych o znaczeniu farmaceutycznym, skupiając uwagę jednoznacznie na paklitakselu. Podczas gdy wysoce pożądane poszukiwania niewyczerpalnych i ekonomicznie opłacalnych źródeł tego doskonałego cytostatyku kuszą do wyciągania przedwczesnych wniosków proklamujących endofity jako niezależne 'bio-fabryki taksanów', niniejsza rozprawa argumentuje, iż odpowiedź na wyczerpanie dostępnych zasobów paklitakselu może kryć się we wzajemnej interakcji roślin (często niezwykle wyjątkowych, jak prechistoryczna *Wollemia nobilis*) z zasiedlającymi je mikroorganizmami pod ewolucyjną i ekologiczną kontrolą. Mimo świadomości ogromu wyzwania, bogactwo innowacyjnych możliwości wydaje się niezaprzeczalne w rodzącej się dobie genomiki funkcjonalnej i biologii systemowej.

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Planten produceren stoffen waarmee ze zich ondermeer beschermen tegen bacteriën en schimmels en tegengaan dat ze worden aangevreten door dieren. Deze stoffen noemen we secundaire metabolieten. Secundaire metabolieten zijn chemisch gezien heel divers en vaak biologisch actief. Hierdoor vormt het plantenrijk een bron voor potentiële nieuwe geneesmiddelen. Ook vindt er via secundaire metabolieten communicatie plaats tussen planten en micro-organismen die met elkaar samenleven, waardoor specifieke en farmaceutisch interessante verbindingen kunnen ontstaan. Endofyten zijn schimmels die van nature in planten voorkomen en met hen een symbiose vormen. Zij zijn in dit opzicht heel interessant en tot nu toe nog weinig onderzocht.

Het onderzoek in dit proefschrift richt zich op de mogelijke vorming van paclitaxel in een aantal endofyten in samenspel met hun gastheerplanten.

Hoofdstuk 2 is een introductie op het onderwerp en geeft een uitgebreid overzicht over onze huidige kennis van endofyten. Hun grote biodiversiteit met tot nu toe onbekende secundaire metabolieten kan uiterst waardevol zijn voor het ontwikkelen van nieuwe geneesmiddelen.

Paclitaxel is een diterpeen dat oorspronkelijk afkomstig is uit de bast van *Taxus brevifolia*, en als cytostaticum wordt gebruikt bij bepaalde vormen van kanker. Het is een 'blockbuster' in de farmaceutische industrie. De hoeveelheden paclitaxel die in de taxusboom voorkomen zijn echter laag, en de isolatie en zuivering tijdrovend. Onderzoek naar alternatieve bronnen voor de productie van paclitaxel leidde in de jaren negentig van de vorige eeuw tot publicaties over endofyten die paclitaxel konden produceren. Dit is ronduit controversieel gebleken, ondanks de aanvankelijk positieve berichten in de (wetenschappelijke) media. Momenteel is de taxusboom nog steeds de enige bruikbare bron voor paclitaxel.

Wij onderzochten de endofyt *Taxomyces andreanae* die van nature voorkomt in *Taxus brevifolia*, met het doel de heersende onduidelijkheid weg te nemen (**hoofdstuk 3**). Verder beschrijven we de isolatie en screening van endofyten uit de oeroude conifeer *Wollemia nobilis* en hun vermogen om paclitaxel te synthetiseren (**hoofdstuk 6**).

We gebruikten standaard chromatografische, spectroscopische en enzymimmunologische technieken om de aanwezigheid van paclitaxel in cultures (kweken op laboratoriumschaal) van *Taxomyces andreanae* aan te tonen. We vonden echter geen aanwijzingen dat paclitaxel in de cultures werd gevormd. Daarnaast onderzochten we of de endofyten genen bevatten die betrokken zijn bij de vorming (biosynthese) van paclitaxel. Met behulp van PCR (polymerase chain reaction) richtten we ons op de genen *taxadiene*

synthase en *baccatin III phenylpropanoyl transferase*. Wij konden de aanwezigheid van deze genen in *Taxomyces andreanae* aantonen.

Het is theoretisch mogelijk dat geïsoleerde endofyten paclitaxel maken. Maar toch is het onwaarschijnlijk, aangezien er in de plant ongeveer twintig verschillende genen bij de biosynthese betrokken zijn. Een meer realistische mogelijkheid is het optreden van 'horizontal gene transfer'. Hierbij wordt gedurende de evolutie genetisch materiaal uitgewisseld tussen organismen die met elkaar samenleven. In zo'n ecosysteem zijn de organismen samen verantwoordelijk voor het eindproduct. De specifieke omgeving van de plant is nodig om de genen in de endofyt, die een rol spelen in de biosynthese van paclitaxel, 'aan' te zetten.

Wollemia nobilis, de eerder genoemde conifeer en gastheer van paclitaxel-producerende endofyten, is een interessant object voor wetenschappelijk onderzoek. De plant werd in 1994 ontdekt, nadat men ervan was uitgegaan dat zij al miljoenen jaren geleden was uitgestorven. Zij wordt beschouwd als een levend fossiel. In **hoofdstuk 4** beschrijven we onze pogingen om van de plant callus- en suspensiecultures te maken. Helaas is het ons niet gelukt om celcultures te maken van *Wollemia nobilis* die goed in het laboratorium kunnen groeien. In **hoofdstuk 5** kijken we naar de chemische verbindingen die de plant produceert. We vergeleken de samenstelling van de vluchtige olie uit verschillende organen van de plant, bladeren en twijgjes. De vluchtige olie bestond voornamelijk uit mono-, sesqui- en diterpenen, met de sesquiterpenen 16-kaureen en germacreen D als belangrijkste componenten. Net als paclitaxel zijn deze vluchtige oliebestanddelen gevormd uit isopreen als bouwsteen. De biosyntheseroutes houden dus verband met elkaar.

Uit *Wollemia nobilis* isoleerden wij de endofyten *Phomopsis* sp. and *Cladosporium langeronii*. Met behulp van PCR konden we de aanwezigheid van het gen *taxadiene synthase* in deze endofyten aantonen. Net als in *Taxomyces andreanae* uit *Taxus brevifolia* is de blauwdruk voor de paclitaxel biosynthese ook aanwezig in deze endofyten uit *Wollemia nobilis*.

Het is interessant om na te gaan of verschillende endofyten die paclitaxel kunnen maken ook overeenkomende genclusters bezitten. Zulke kennis vergroot de mogelijkheden om paclitaxel onder gecontroleerde omstandigheden te produceren via zogenaamde combinatoriële biosynthese. Bij combinatoriële biosynthese worden genen van het ene organisme in een ander overgezet, met als doel bepaalde cruciale stappen in de biosynthese gemakkelijker (en rendabeler) te laten verlopen.

Op basis van de resultaten in dit proefschrift concluderen we dat eerdere publicaties die claimen dat endofyten kunnen dienen als alternatief voor de productie van paclitaxel prematuur zijn geweest. Om een toekomstig tekort aan paclitaxel als geneesmiddel te voorkomen en om de oorspronkelijke leverancier *Taxus brevifolia* voor uitsterven te behoeden, zijn absoluut andere en meer rendabele bronnen nodig. De huidige ontwikkelingen op het gebied van genomics en systeembiologie, in combinatie met de biodiversiteit die de natuur biedt, zullen hiertoe zeker bijdragen.

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