MOLECULAR BIOLOGICAL ELUCIDATION OF LATE TROPANE ALKALOID BIOSYNTHESIS

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ABSTRACT

Tropane alkaloids (TA) are important and valuable secondary plant metabolites which occur in the families of Solanaceae, Erythroxylaceae, as well as Convolvulaceae, Moraceae, and Brassicaceae. These plant families produce the TAs hyoscyamine and scopolamine, cocaine, or calystegines, respectively. Due to the medicinal and pharmacological properties of hyoscyamine, scopolamine and cocaine, TA containing plants have been used for thousands of years. Even today, many drugs are derived from TAs

and are used in the treatment of various diseases. Of the TAs, scopolamine is the most in demand due to its pharmacological effects and legal status. The market supply of scopolamine is still met by conventional field cultivation of *Duboisia* hybrids. Climate change is resulting in less-stable growth conditions for traditional farming, which limits the reliability of agricultural yields and has warranted investigation of alternative TA production processes. One proposed approach is to transfer biosynthesis into biotechnological host which would enable TA production in containable and scalable fermentative infrastructure using low-cost compounds like tropine and phenyllactic acid.

All TA production approaches require profound knowledge of the biochemical pathways. To date, these pathways have not been fully elucidated, which limits their greater biotechnological application. This thesis seeks to increase the fundamental understanding of the molecular processes of the late stages of TA biosynthesis.

This thesis deals with the TA biosynthesis in *Duboisia* plants which is highly specialized and specifically localized. The spatial distribution of TA *in planta*, the expression of genes involved in the biosynthesis and the TA pattern during plant development are investigated and described. Moreover, it presents investigations towards identification of the littorine synthase, a currently unknown key enzyme in TA biosynthesis.

The results of this research add to our understanding of TA biosynthesis and provides important insights for the future development of alternative bio-production processes.

ZUSAMMENFASSUNG

Tropanalkaloide (TA) sind wichtige und wertvolle sekundäre Pflanzenmetabolite, die in den Familien der Solanaceae, Erythroxylaceae sowie in Convolvulaceae, Moraceae und Brassicaceae vorkommen. Diese Pflanzenfamilien produzieren die TA Hyoscyamin und Scopolamin, Cocain bzw. Calystegine. Aufgrund der medizinischen und pharmakologischen Eigenschaften von Hyoscyamin, Scopolamin sowie Cocain wurden TA enthaltende Pflanzen seit Jahrtausenden in der Ethnopharmakologie eingesetzt. Noch heute finden von den TA abgeleitete Arzneistoffe in vielen Indikationsgebieten ihren Einsatz. Von den TA ist die Nachfrage nach Scopolamin aufgrund seiner pharmakologischen Wirkungen und seines rechtlichen Status am größten. Die Marktversorgung von Hyoscyamin und Scopolamin wird bis heute durch den konventionellen Feldanbau von *Duboisia* Hybriden gesichert. Der Klimawandel allerdings führt dazu, dass aufgrund instabilerer Wachstumsbedingungen die Zuverlässigkeit landwirtschaftlicher Erträge einschränkt wird, was die Untersuchung alternativer TA-Produktionsprozesse notwendig macht. Ein Ansatz ist es, die Biosynthese in Mikroorganismen zu überführen, um aus günstigen Ausgangsstoffen wie Tropin und Phenylmilchsäure wertvolle TA wie Hyoscyamin oder Scopolamin herzustellen. Alle Ansätze zur TA-Produktion brauchen fundierte Grundlagen über die Biosynthese. Bis heute sind diese Biosynthesewege nicht vollständig aufgeklärt, was ihre stärkere biotechnologische Anwendung einschränkt. Diese Arbeit verbessert unser grundlegendes Verständnis molekularer Prozesse der späten Stadien der TA-Biosynthese.

Diese Arbeit befasst sich mit der Biosynthese der TA in *Duboisia* Pflanzen, die hochspezifiziert und lokalisiert ist. Die räumliche Verteilung der TA *in planta*, die Genexpression, der an der Biosynthese beteiligten Enzyme sowie das TA-Muster während des Pflanzenwachstums wurden untersucht und beschrieben. Des Weiteren werden Untersuchungen zur Identifizierung der Littorin Synthase, einem bislang unbekannten Schlüsselenzym der TA-Biosynthese, präsentiert.

Die Ergebnisse dieser Forschung tragen zu einem verbesserten Verständnis der TA-Biosynthese bei und liefern wichtige Erkenntnisse für die zukünftige Entwicklung alternativer Bioproduktionsprozesse.

CHAPTER 1

INTRODUCTION

Parts of this chapter were published in

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1.1. PREFACE

"Alkaloids are naturally occurring compounds containing one or more nitrogen atoms. The name is derived from the basic nature of many members of this group, alkaloids from "alkaline-like". The definition of alkaloids is complex as many nitrogen-containing molecules do not necessarily belong to this group. Biogenic amines or amino sugars, for example, are natural plant products and *N*-containing but not defined as alkaloids. Tropane alkaloids (TAs) are a specific class of alkaloid and can be more specifically defined as all molecules that possess a tropane ring system (Grynkiewicz and Gadzikowska, 2008).

TAs are either esters of 3α-tropanole (tropine) or, to a lesser extent, 3β-tropanole (pseudotropine) and can be distinguished into three groups (Figure 1-1): TAs from Solanaceae plants like hyoscyamine and scopolamine, coca alkaloids like cocaine from *Erythoxylum coca*, and the recently discovered calystegines group, which are polyhydroxylated nortropane alkaloids (NTAs) mainly occurring in Convolvulaceae, Solanaceae, Moraceae, Erythrocylaceae and Brassicaceae (Dräger, 2003). In total, ~200 different TAs have been described (Gadzikowska and Grynkiewicz, 2002).

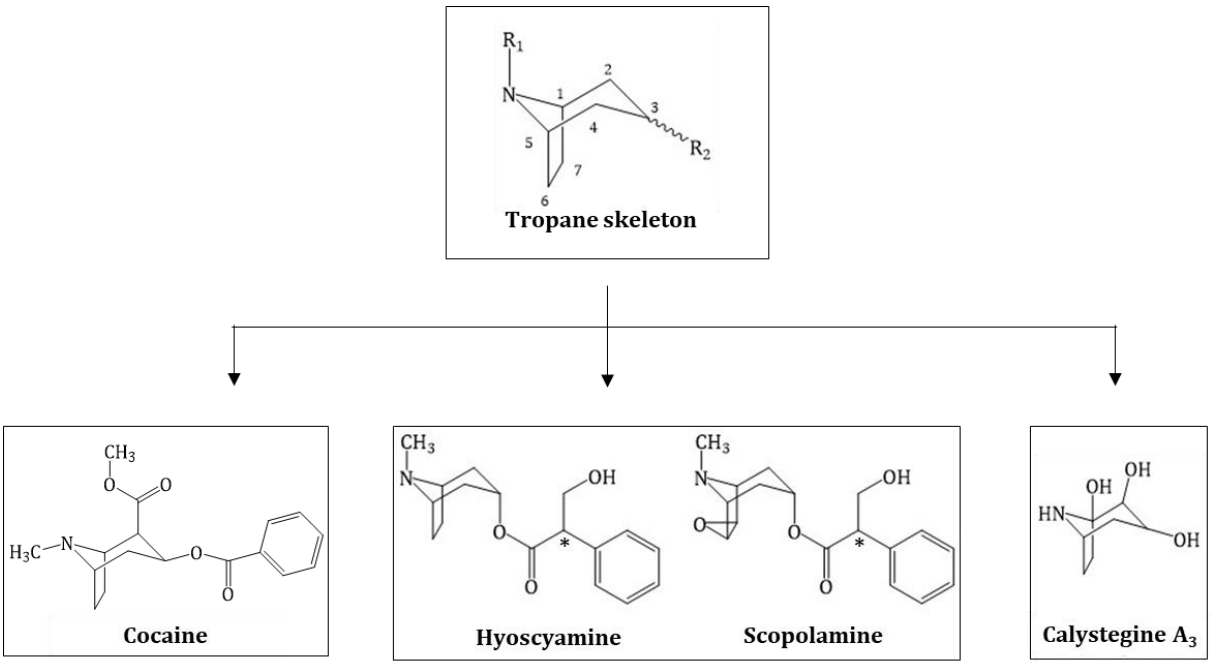

Figure 1-1: Structure of the tropane skeleton and the three major groups of TAs derived from this skeleton. * = stereocenters.

Biosynthesis of the tropane ring system is homologous in organisms which produce these three TA classes. TA biosynthesis begins with the amino acids ornithine or arginine and their intermediate putrescine, continuing to the common *N*-methyl-Δ^1-pyrrolinium cation precursor of all TAs. This is the branch point of cocaine, hyoscyamine/scopolamine and calystegine as well as nicotine biosynthesis (Ziegler and Facchini, 2008).

Although all TAs have a high degree of structural similarity due to their tropane ring, the pharmacological effects of these compounds differ significantly. Cocaine and hyoscyamine/scopolamine are able to pass the blood-brain barrier and commit dose-dependent hallucination and psychoactive effects. Calystegines do not cause these effects due to their polarity as well as hydrophilicity and consequent inability to pass this barrier.

The cultivation of coca plants, the extraction of cocaine and production of other cocaine-containing drugs as well as their trade**,** with a few exceptions, is illegal and cocaine is the 2nd most frequently consumed illicit drug globally (Schultze-Kraft, 2016). Due its legal designation, research has only been conducted on pathway elucidation in order to understand cocaine biosynthesis, however, research of large-scale commercial production has not been conducted (legally). As the calystegines are a newly discovered group of TAs without any pharmaceutical, medicinal or economic interest, little research has thus far been performed on this group of TAs. In contrast, the cultivation and production of scopolamine is of major economic interest due to its miscellaneous pharmaceutical applications. Indeed, global demand for this compound is increasing. Moreover, scopolamine is one of the Essential Medicines of the World Health Organization (WHO) (WHO, 2015). Hyoscyamine and scopolamine are extracted from the *Duboisia* plants being cultivated on large plantations in Queensland, Australia (Grynkiewicz and Gadzikowska, 2008). Climate change and resulting new biotic and abiotic factors challenge the pharmaceutical industry to produce consistently high volumes of scopolamine. To overcome this issue, alternative production methods have been also tested. (…)

1.2. HISTORY AND CHEMICAL ELUCIDATION OF TROPANE ALKALOIDS

The TAs cocaine, scopolamine, and calystegines share a common tropane moiety. Nevertheless, these compounds cause very different physiological effects in humans. Cocaine manifests its effects in the synaptic cleft by inhibiting the dopamine, noradrenaline, and serotonin reuptake while scopolamine acts as a competitive muscarinic receptor antagonist. The ingestion of both substances may lead to hallucinations and psychoactive effects or death (Langmead et al., 2008; Rothman et al., 2001). Calystegines, on the other hand, are not absorbed into the central nervous system (CNS) due to their hydrophilicity and consequently, exhibit no psychoactive effects in humans (Dräger, 1994).

1.2.1. Hyoscyamine and scopolamine

TA producing Solanaceae plants are distributed globally. *Duboisia* plants are found in Australia and New Caledonia, while *Datura* plants, which had their origin in Asia and America, grow worldwide except in polar und subpolar climate zones. Members of the genus *Atropa* and *Hyoscyamus* have origins in Europe, Asia, as well as North Africa and were introduced to the USA, Canada and Australia (Griffin and Lin, 2000). All plants are simple to cultivate and readily found in nature, highly potent, and, consequently, have a long history in traditional medicines from different cultures. Until single compounds were isolated, whole plant or herbal preparations of these plants including extracts, ointments, or teas were used for medicinal applications. Earliest reports of hyoscyamine or scopolamine-induced states of perception reach back into antiquity. Over 3,000 years ago, *Mandragora* extracts were added to beer in Egypt to lower amounts of alcohol in these beverages. In Russia and China, *Datura* extracts, and in Europe *Hyoscyamus* was added to enhance the thrilling effect of beer. A physician in Babylonia documented the analgesic property of the nightshades to reduce toothache (Müller, 1998). In addition to hallucinogenic and analgesic effects, nightshades have a history of being used as poisons, for example, a wave of unexplainable mortality in the French high-society during was attributed to these plants (Ulbricht et al., 2005). In Australia, indigenous people exploited the TA-containing *Duboisia* plants for centuries for their cholinergic activity (Heinrich and

Jäger, 2015). *Hopwoodii*, also called pituri, produces the alkaloid nicotine, which is arguably more widely popular for common use than the *Duboisia* plants *leichhardtii* and *myoporoides* (Endo and Yamada, 1985).

Solanaceae plants have been given several names due to their historical and widespread use. These names often reflect the type of application and respective pharmacological action. *Datura stramonium* L., which was introduced to European medicine by Romani immigrants (Griffin and Lin, 2000), is called asthma herb due to its application in mitigating the symptoms of asthma. If *Datura* herb is smoked, a bronchorelaxation effect has been documented. Further names are "thornapple" after the hooked capsule or "horse poison" due to the toxic effects after ingestion on equines that are particularly sensitive to TAs (Naudé, 2007). The common *Datura* name jimsonweed is derived from Jamestown (USA) and it was reported that in 1676, settlers ingested this weed with fatal results. The intoxications were described and documented vividly leading to this additional name

The isolation and structural elucidation of TAs from Solanaceae plants began with the discovery of atropine. In 1832, this alkaloid was isolated by the H. F. G. Mein, however, he did not publish his results (Döbereiner, 1842). One year later, P. L. Geiger and O. Hesse (1833) published the isolation of atropine, a nitrogen containing, alkaline substance, from *Atropa belladonna* L. and *Hyoscyamus niger* L.. They described early investigations regarding the medicinal use, different isolation methods and chemical properties (Geiger and Hesse, 1833). The stereochemical relation between atropine and hyoscyamine was elucidated by K. Kraut and W. Lossen almost fifty years later (Lossen, 1869; Wolffenstein, 1922). They were able to elucidate the reaction mechanism of the alkaline hydrolyzation of hyoscyamine and detected that the cleavage products of both, hyoscyamine and atropine, are tropic acid and tropine. From this it was concluded that atropine is the racemate of hyoscyamine (Wolffenstein, 1922). A. Ladenburg (1879) discovered that the reverse reaction of the hydrolysis is possible by boiling the educts in hydrochloric acid and established a frequently used method of esterifying tropine with numerous organic acids (Ladenburg, 1879).

1.2.2. Cocaine

The first reports of the use of cocaine date back to 3,000 B.C. in Ecuador (Grinspoon and Bakalar, 1981). The cultivation and chewing of coca plant leaves is assumed to have originated on the Eastern slope of the Ecuadorian or Peruvian Andes by the Inca peoples. Tribesmen traditionally chewed the leaves of the coca plant together with lime to release the alkaloids, both for spiritual purposes such as burial ceremonies or to give strength and energy, and also to tolerate thin air high at high altitudes in the mountains. The coca plant and its invigorating effect was believed to be a mysterious gift of the gods. Before the Spanish soldiers entered South America, chewing coca leaves was reserved only for the tribal leaders. After the Spanish conquest of South America, its use was spread of over the continent and no longer socially limited. Cocaine was isolated for the first time in 1855 by F. Gaedecke. He published his results in the journal Archiv der Pharmacie and called the substance, isolated from coca leaves, erythroxyline (Gaedecke, 1855). Working on cocaine was an interesting field, but due to the limited access to plant samples available in Europe, little research could be conducted. Albert Niemann, who received enough supply of coca leaves for research, was able to proceed his study and improved the isolation process as well as the general knowledge of cocaine and its mode of action (Niemann, 1860). The chemical formula of cocaine was determined in 1862 by W. Lossen, who also dealt with the analysis of atropine. Subsequently, the first chemical synthesis and the elucidation of its chemical structure was achieved by R. Willstätter in 1898. About fifty years later, the stereochemistry was elucidated by the Swiss chemists E. Hardegger and H. Ott (Hardegger and Ott, 1954). However, not all published reports on cocaine and its chemistry were scientifically or ethically correct. In 1885, S. Freud published his work “Über Coca” and recommended cocaine as an almost miracle medicine, with local anesthetic properties, which is best for the treatment for postnatal depression and morphine addiction – a dangerous application for a substance with such high addictive potential (Freud, 1885).

1.2.3. Calystegines

Polyhydroxylated NTAs like calystegine do not show any psychoactive effects due to their inability to pass the blood-brain barrier based on their hydrophilicity. In addition, they exhibit minimal pharmacological activity. As a result, this class of NTAs have not found use in ancient medicines. Recently, researchers proposed that these compounds inhibit mammalian and plant glucosidases, although until now they do not have any pharmacological application and have received little research attention (Jocković et al., 2013). The first structures of polyhydroxylated NTAs were published in 1990 (Dräger, 2003).

1.3. PHARMACOLOGY OF TROPANE ALKALOIDS AND THEIR ROLE AS DRUG LEAD SUBSTANCES

1.3.1. Scopolamine, hyoscyamine and anisodamine and their derived drugs

Hyoscyamine and scopolamine are widely used as anticholinergic drugs. They affect the central and peripheral nervous system as competitive, non-selective muscarinic acetylcholine receptor (mAChR) antagonists, that prevent binding of the physiological neurotransmitter acetylcholine. In humans, two acetylcholine receptor types are known: Muscarinic and nicotinic receptors, which are named after their agonists, muscarine (Figure 1-2) and nicotine. Muscarine is a poison of the toadstool mushroom *Amanita muscaria* and acts on the mAChR of the synapses like acetylcholine, with the difference that the acetylcholinesterase does not metabolize it.

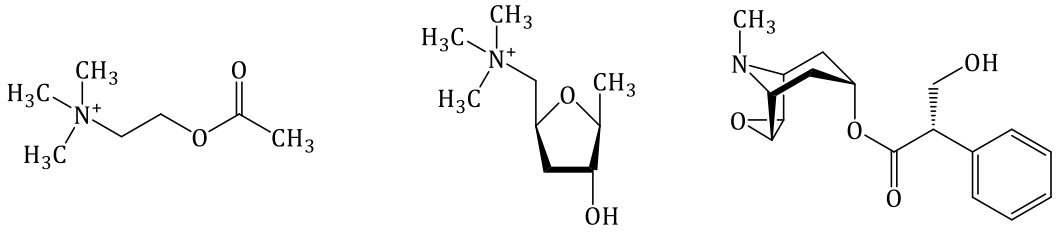

Figure 1-2: Comparison of the chemical structures of acetylcholine, muscarine, and scopolamine. Scopolamine is protonated in the body due to the physiological pH and is present as a quaternary ammonium salt.

The mAChRs are a subclass of the G-protein-coupled receptors (GPCRs) family, containing five subtypes (M1-M5). M1, M3, and M5 that are coupled with the stimulating Gq receptors and generate cytosolic calcium transients via the phospholipase C signaling pathway. M2 and M4, on the other hand, couple with the Gi protein and inhibit the adenylyl cyclase (Langmead et al., 2008). In particular, M1 receptors occur in the central nervous system and ganglia where they are involved in memory and learning processes. M2 receptors are found in the heart and are lower in abundance than M1 receptors. M3 receptors are involved in the contraction of the smooth muscles. M4 receptors were detected in the forebrain, hippocampus, and striatum, they are likely involved in pain processes (Wess et al., 2003). The physiological action of M5 receptors is not yet elucidated, however, it is assumed that these receptors are involved in brain microcirculation and mediate vasoconstriction, vasodilatation, and activation of nitric oxide synthase (Watts et al., 2008).

TAs are absorbed from the gastrointestinal tract, rapidly distributed into the tissues, and excreted predominantly through the renal system (EFSA, 2013). The short half-life in plasma and dose-dependent adverse effects limit the administration of scopolamine to transdermal application (Renner et al., 2005). After absorption, scopolamine experiences a significant first-pass effect, because only a minor amount (2.6 %) is excreted in the urine in the pharmacologically active form (Kanto et al., 1989). Cytochrome P450 enzymes seem to be especially involved in the metabolism of scopolamine by oxidative demethylation. Inhibition of CYP3A4 by ingestion of grapefruit juice prolonged the t_{max} and increased the AUC_{0-24h} value of scopolamine metabolization (Renner et al., 2005). Additionally, it has been observed that scopolamine and its apo- and nor-metabolites are conjugated to glucuronide (glucuronidation) or sulfate during phase II metabolism for excretion into urine. Scopolamine and hyoscyamine do not accumulate in the human body, nor exhibit genotoxic or chronic toxicity, an adverse effects due to long-term exposure have not been reported (EFSA, 2013).

Occurring side effects of anticholinergic drug substances occur from inhibition of the parasympathetic nervous system. Symptoms include decelerated heart rate, dry mouth, and reduced perspiration. At higher therapeutic oral doses, increased heart rate, inhibition of the respiratory tract secretory activity as well as bronchodilation,

and mydriasis have been observed. Sweating is also inhibited which is accompanied by a consequent rise in body temperature.

Scopolamine

Scopolamine causes mydriatic, spasmolytic and local anesthetics effects yet exhibits side effects which can be hallucinogenic and even lethal. The most important mode of application for scopolamine is transdermal, a technology which was developed as transdermal therapeutic systems (TTS) in 1981. Scopoderm TTS® is the trade name for a scopolamine delivery system used in the treatment of motion sickness. During the Second World War, scopolamine was used to treat shell shock, psychoactive side effects, and also motion sickness (Heinrich and Jäger, 2015). The drawbacks of scopolamine lay in the manifold peripheral and central nervous system side effects. To overcome these issues, scopolamine derivates have been developed, leading to its classification as a drug lead substance.

Hyoscyamine and atropine

Hyoscyamine and atropine have similar modes of action and effects as scopolamine. The pharmacological action of TAs is stereoselective, due to the difference of the stereoisomers concerning affinity and binding to muscarinic receptors. This results in different potency between *S*-(–)- and *R*-(+)- isomers of hyoscyamine: The *S*-(–)-isomer is estimated to be 30–300 fold more potent than the *R*-(+)- isomer (Gyermek, 2002). The *S*-(-)-isomer of hyoscyamine is not stable and is racemized rapidly to atropine, which is a 1:1 mixture of the two forms. Atropine is very stable over time and hence, it used for medicinal applications instead of hyoscyamine. Both, atropine and scopolamine have a characteristic, dose dependent action on the cardiovascular system, which is clinically useful for resuscitation.

Anisodamine

Anisodamine, which is isolated from *Anisodus tanguticus*, a Tibetan regional plant, is less toxic than atropine and scopolamine. It has a long tradition in folkloric Asian medicine especially in the treatment of septic shock by improvement of blood flow in microcirculation, and also in various circulatory as well as gastric disorders with similar effects to atropine and scopolamine.

Homatropine, cyclopentolate and tropicamide

Homatropine, the mandelic acid ester of tropine, is used in ophthalmology to evoke a more rapid and less paralytically effect than atropine. This is a major advantage over atropine and, consequently, homatropine was launched as a new mydriatic by Merck Darmstadt in 1883 as one of the first synthetic drugs (Sneader, 2005). Other modified mydriatic agents are cyclopentolate, which is used especially for pediatric eye examinations, and tropicamide, which has been approved in ophthalmology since 2005.

Trospium chloride

Trospium chloride is a quaternary ammonium 3α-nortropane derivate esterified with benzylic acid. This synthetic anticholinergic is not able to cross blood-brain barrier and relaxes the smooth muscle in the bladder. Its main application is to treat urgency and reflex incontinence (Rudy et al., 2006).

Tropisetron

Tropisetron possesses a tropane skeleton, but due to its mechanism of action it belongs to the serotonin receptor antagonists. It is applied to antiemetic therapy in cases of nausea and vomiting during chemotherapy and additionally as analgesic in fibromyalgia (Sorbe et al., 1994).

N-butylscopolamine

To minimize adverse effects on the central nervous system, scopolamine has been modified by *N*-butylation and, in this form, it cannot longer pass the blood-brain barrier. *N*-butylscopolamine is used to treat abdominal pain from cramping, renal colic, and bladder spasms (Mutschler et al., 2008). Available dosage forms are as tablets or film-coated tablets (also available in combination with paracetamol), rectal suppositories (also available in combination with paracetamol) or solutions for injection and the according drug products are Buscopan® or Buscofem®.

Tiotropium bromide, ipratropium bromide and oxitropium bromide

In traditional medicine, smoking of *Datura* leaves have been frequently used to treat asthmatic symptoms. Bronchodilation is caused by blocking of M3 receptors located on smooth muscle cells in the bronchi. Scopolamine and hyoscyamine are the TAs responsible for this effect. To reduce the adverse and intoxicating effects of this treatment, tiotropium bromide and ipratropium bromide were developed and are also administered by inhalation. Tiotropium bromide (Spiriva®; released on the market in 2005) is dominantly used in the treatment of chronic obstructive pulmonary disease (COPD) while ipratropium bromide (Atrovent®; released 1975) is used in the treatment of COPD (in combination with salbutamol, a β2-adrenergic receptor agonist) and asthma (Barnes, 2000). Oxitropium bromide is less known and less used than the previous ones, but also acts as an anticholinergic bronchodilator for the treatment of asthma and COPD.

Benzatropine

Benzatropine is a selective M1 muscarinic acetylcholine receptor antagonist with central nervous effects. Chemically, it is a combination of the tropine skeleton of atropine and the benzohydryl skeleton of diphenhydramine. It partially blocks cholinergic activity in the basal ganglia and increases the availability of dopamine by blocking its reuptake. This increases dopaminergic activity, therefore, it has found use

in the treatment of early stages of Parkinson's disease (Schlagmann and Remien, 1986).

Scopolamine and its use as in antidepressant therapy

Scopolamine may also be suitable for the application in CNS diseases. It is known that scopolamine and other muscarinic receptor antagonists have an effect on the cognition processes, sensory functions (for example pain perception), and stress responses. As there is considerable evidence supporting the cholinergic-adrenergic hypothesis of mania and depression, the clinical effects of scopolamine as a central acting inhibitor of the muscarinic receptor has been tested. Several randomized double-blind studies have been performed and demonstrated contrasting outcomes. Some studies found scopolamine to have a rapid and prominent effect (Yu et al., 2019) while others found no benefit from scopolamine over placebo (Park et al., 2018) for the treatment of these conditions. The contrasting findings indicate that more extensive studies are needed to verify the use of scopolamine for treatment of CNS diseases.

1.3.2. Cocaine derived drugs

Although cocaine has been used for a long time and by many people, little is known about it is use in treatment of neurobiology and pharmacology. The application of cocaine is legally restricted and consequently, the research is limited. It is known that cocaine exhibits different pharmaceutical modes of action like local anesthetic properties, CNS stimulating actions, and cardiovascular effects. However, these modes of action may alter according to the way of application - oral, nasal, by chewing, inhalation or by injection (Williams, 1977).

The central nervous effects such as euphoria, relief of fatigue and boredom as well as psychic stimulation are mainly explained by the resulting excess of dopamine after cocaine consumption. Cocaine inhibits the reuptake of dopamine, noradrenaline, and serotonin, thus increasing their concentration in the synaptic cleft of the limbic system (Rothman et al., 2001). The intake of cocaine has an influence on the brain which is

detectible in an electroencephalogram (EEG). However, the effects are inconsistent and may appear as increased or lowered signals in EEGs (Berger, 1931). The local anesthetic properties of cocaine by topical application are achieved by blocking the ion channels in neural membranes. Cocaine is absorbed by the mucosa after application and paralysis rapidly occurs in the peripheral ends of sensory nerves. It was widely applied in dentistry as a local anesthetic but has been substituted by safer drugs. Nevertheless, it served as a lead substance for different local anesthetics and painkillers. Procaine was the first major analogue of cocaine which was mainly used in dentistry. Nowadays, more potent local anesthetics are available, and so, its use has declined. A huge disadvantage of procaine is instability of the ester to hydrolysis. Tetracaine, a further development of procaine, is used for minor face surgeries and in ophthalmology. It is almost 10-times more potent than procaine, however, its toxicity increased proportionately to its potency (Dewick, 2002). Lidocaine is an amino amide analogue to the ester type of cocaine and was synthesized in 1943 by the Swedish chemists Nils Löfgren and Bengt Lundqvist (Sinatra et al., 2010). Its advantages are the better stability towards hydrolysis in aqueous solution or esterase catalysis (Dewick, 2002). Beside its local anesthetic properties, it is used as an Ib type antiarrhythmic medication due to its positive cardiovascular effects.

1.3.3. Calystegine derived drugs

Until now, no drug products derived from calystegines are available, although the inhibition of mammalian glucosides by these compounds may be a promising lead in the development of new active pharmaceutical ingredients.

1.4. TROPANE ALKALOID BIOSYNTHESIS

1.4.1. Early steps in tropane alkaloid biosynthesis – a united way

The different classes of TAs cocaine, scopolamine/hyoscyamine, and the calystegines share a common precursor biosynthetic route (Figure 1-3), beginning with the amino acids L-ornithine (Orn) and L-arginine (Arg). *In planta*, Orn and Arg are formed from glutamate (Glu), an amino acid which is directly connected to the nitrogen assimilation. Ammonia (absorbed from the soil or synthesized from nitrate) is incorporated into Glu via the glutamine synthetase-glutamate synthase (GS-GOGAT)

pathway. Glu is the precursor in several polyamine (PA) pathways. The regulation of PAs is very complex and not fully elucidated due to their pleiotropic functions (Agostinelli, 2014) and PA concentration in plants can be used as indicators of various forms of abiotic stress.

In order to form putrescine (1,4-diaminobutane) from the amino acids Orn or Arg, Orn is decarboxylated by the ODC (ornithine decarboxylase; EC 4.1.1.17) and Arg undergoes a three-step reaction, including decarboxylation, hydrolysis of the imine functionality of guanidine, and hydrolysis of urea which is catalyzed by the enzymes ADC (arginine decarboxylase; EC 4.1.1.19), AIH (agmatine deiminase; EC 3.5.3.12), and CPA (*N*-carbamoylputrescine amidase; EC 3.5.1.53), respectively. The activities of ADC and ODC were suppressed in *Datura* plants by using the specific irreversible inhibitors DL-α-difluoromethylarginine and DL -α-difluoromethylornithine, respectively in order to probe the nature of these two routes to putrescine biosynthesis. These experiments indicated that the two routes do not act independently from each other and that the ADC exhibited a higher activity than the ODC (Richard J. Robins et al., 1991). Putrescine (tetramethylenediamine) is an intermediate in several metabolic pathways. It can be formed to spermidine by a spermidine synthase (SPDS; EC. 2.5.1.16) catalyzed reaction using S-adenosyl methioninamine (decarboxylated S-adenosyl methionine) and putrescine as substrates.

Putrescine can also be methylated to *N*-methylputrescine by the enzyme PMT (putrescine *N*-methyltransferase; EC 2.1.1.53) (Biastoff et al., 2009a) using SAM (S-adenosyl methionine). The next step in TA biosynthesis is the oxidative deamination of *N*-methylputrescine to 4-methylaminobutanal which is catalyzed by a *N*-methylputrescine oxidase (MPO; EC 1.4.3.6) (Mizusaki et al., 1972). This diamine oxidase requires copper as a cofactor. *N*-methyl-Δ^1-pyrrolinium, a central intermediate, is formed by spontaneous cyclization of *N*-methylputrescine. Chemically, this reaction is an intramolecular Schiff base formation. *N*-methyl-Δ^1-pyrrolinium cation is a branchpoint in TA and nicotine biosynthesis (Courdavault, 2010). In Figure 1-3, the joint biosynthesis is depicted. The condensation of nicotinic acid or more precisely its reactive derivative 2,5-Dihydropyrindine with *N*-methyl-Δ^1-pyrrolinium cation yields nicotine.

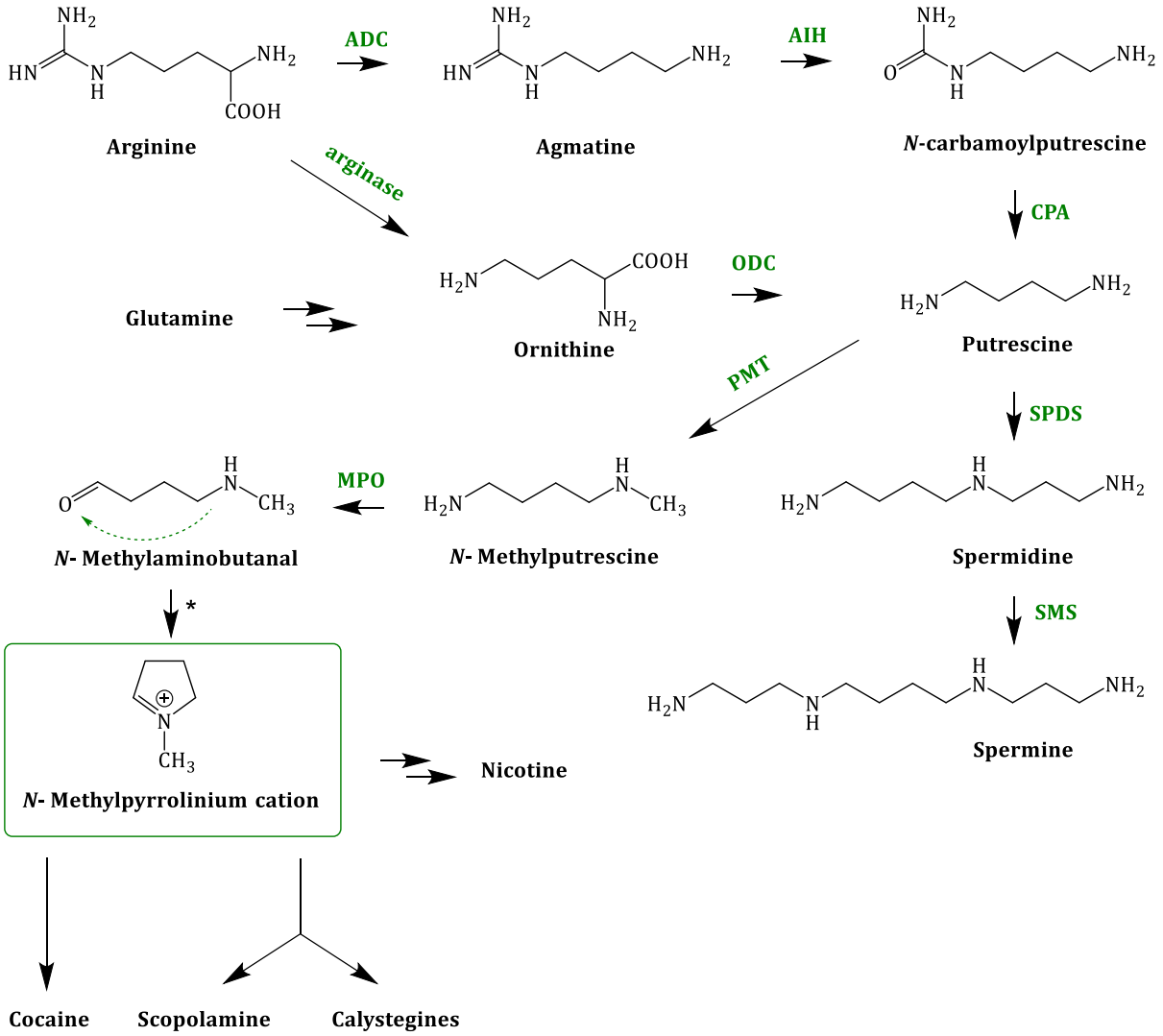

Figure 1-3: Joint steps of the early TA biosynthesis; ADC = arginine decarboxylase; AIH = agmatine deiminase; OCD = ornithine decarboxylase; CPA = *N*-carbamoylputrescine amidase; PMT = putrescine *N*-methyltransferase; SPDS = spermidine synthase; SMS = spermine synthase; MPO = *N*-methylputrescine oxidase; * = spontaneous cyclization

1.4.2. Hyoscyamine and scopolamine biosynthesis

Originating from *N*-methyl-Δ1-pyrrolinium, the next steps in the scopolamine biosynthesis (Figure 1-4) were not elucidated for a long time. Recently in 2018, Bedewitz et al., 2018 identified an atypical polyketide synthase from *A. belladonna* which catalyzes the formation of the intermediate 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoic acid. Subsequently, the formation of tropinone from this intermediate is catalyzed by a cytochrome P450 enzyme, named AbCYP82M3. Tropinone serves as substrate for two stereospecific reductases: the tropinone reductase I (TR-I; EC

1.1.1.206) and the tropinone reductase II (TR II; EC 1.1.1.236) [56]. TR-I catalyzes its reduction to tropine (3α-tropanol), whereas TR II catalyzes tropinone reduction to pseudotropine (3β-tropanol), respectively. Pseudotropine is the precursor of calystegine biosynthesis while tropine is used to produce scopolamine.

Tropine is assumed to undergo condensation with activated (*R*)-phenyllactate (phenyllactic acid-CoA), which delivers the third ring intermediate to littorine. Phenyllactate is derived from phenylalanine, an intermediate of the shikimate pathway, which is transaminated to phenylpyruvate. Bedewitz et al. (2014) discovered the coding sequence of a distinct aromatic amino acid aminotransferase (ArAT) that is coexpressed with known tropane alkaloid biosynthesis genes. Silencing of ArAT4 in *A. belladonna* disrupted scopolamine biosynthesis by reduction of phenyllactate levels. The next step, the reduction of ketone function, is catalyzed by a recently discovered phenylpyruvic acid reductase (*AbPPAR*). This reductase exhibited cell-specific expression also and was detected in root pericycle as well as the endodermis (Qiu et al., 2018).

Although no enzymatic activity had been described, it is likely that an enzyme related to the cocaine synthase may be involved (R J Robins et al., 1991; Schmidt et al., 2015a) in the formation of littorine. Littorine is rearranged via the littorine mutase/monooxygenase (CYP80F1; EC 1.6.2.4) to hyoscyamine aldehyde, which is subsequently reduced to the corresponding alcohol hyoscyamine (Li et al., 2006). Hyoscyamine is converted via the enzyme H6H (hyoscyamine 6β-hydroxylase; EC 1.14.11.11). The H6H is a 2-oxoglutarate dependent dioxygenase (Hashimoto and Yamada, 1985) which catalyzes two reactions: first, the hydroxylation of hyoscyamine to 6β-hydroxy hyoscyamine and second, the epoxidation of 6β-hydroxy hyoscyamine to scopolamine. The bifunctional dioxygenase exhibits a strong hydroxylase activity in comparison to the rate limiting epoxidase activity (Hashimoto et al., 1993a). Figure 1-4 depicts the TA pathway in Solanaceae plants, including known and unknown enzymes.

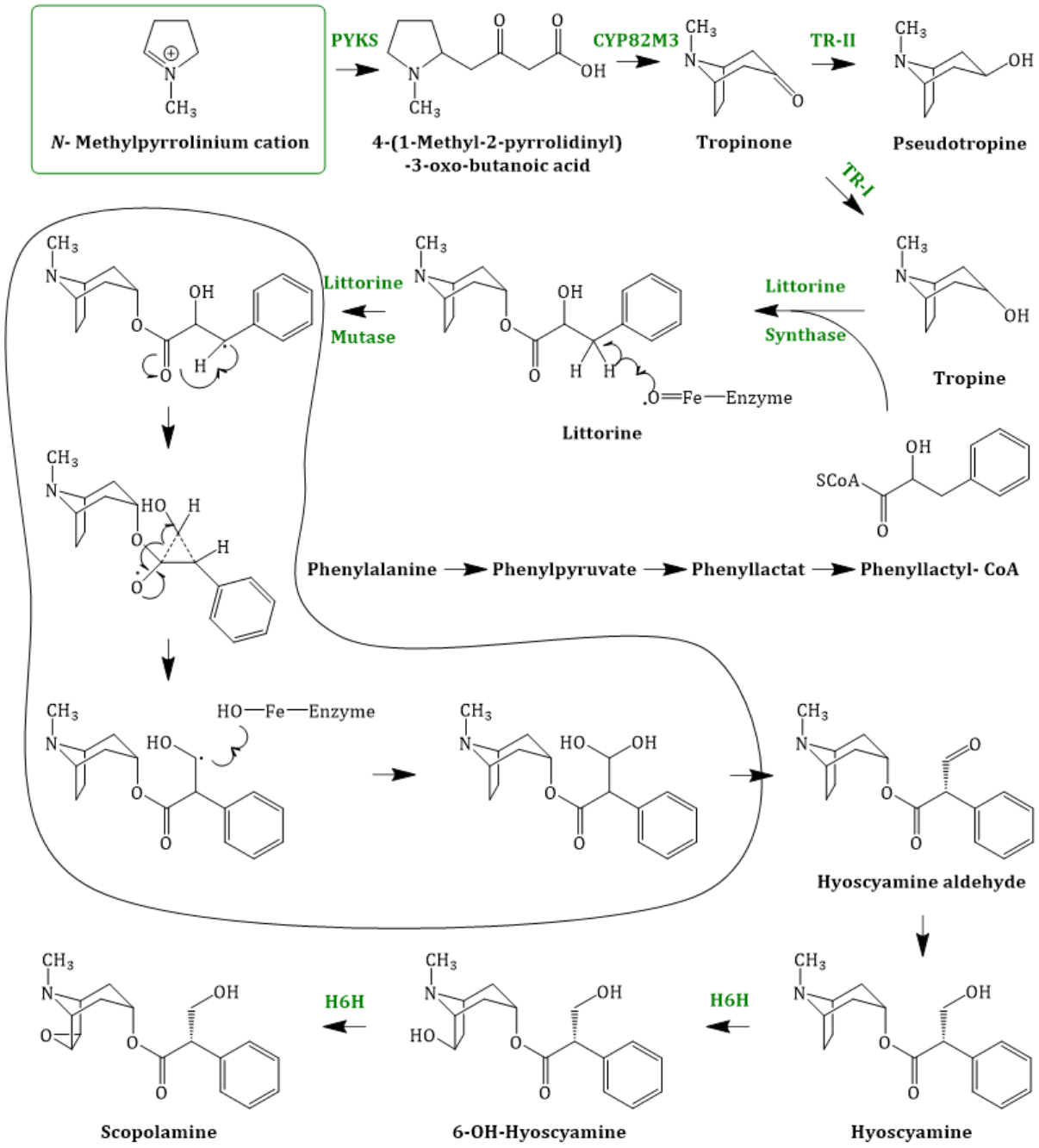

Figure 1-4: Scopolamine biosynthesis, starting with the *N*-methylpyrrolinium cation; PYKS = polyketide synthase; CYP82M3 = cytochrome P450 enzyme; TR-I/II = tropinone reductase I/II; littorine synthase (sequence not known); littorine mutase/monooxygenase (CYP80F1); (*1) = proposed mechanism of littorine rearrangement; H6H = hyoscyamine 6β-hydroxylase

1.4.2.1. Enzymes involved into scopolamine formation and their regulation

Putrescine methyltransferase

The amino acid sequence of putrescine methyltransferase (PMT) is evolutionary related to those of plant spermidine and spermine synthases (SPMS, EC 2.5.1.22; converts spermidine into spermine; cofactor: decarboxylated S-adenosyl methionine). These enzymes are grouped in the spermidine synthase family by the Prosite database and contain a polyamine biosynthesis (PABS) domain. The PABS domain consists of two subdomains: I) *N*-terminal subdomain composed of six β-strands and II) Rossmann-like *C*-terminal subdomain (Korolev et al., 2002). It is assumed that PMT evolved from SPDS (Biastoff et al., 2009b; Teuber et al., 2007). Teuber et al. (2007) performed a kinetic study of heterologous PMTs from different plants and measured K_{cat} values between 0.16 and 0.39 s^{-1}.

Tropinone Reductase I and Tropinone Reductase II

Tropinone reductase I and II (TR-I (EC 1.1.1.206) and TR-II (EC 1.1.1.236)) are small proteins belonging to the short chain dehydrogenase/reductase (SDR) family and catalyze NADPH + H^+-dependent conversion of tropinone into tropine or pseudotropine, respectively. They share the characteristic motifs of the SDR family, such as TGXXXGXG, a motif involved in NADPH binding, a NNAG motif, and the catalytic sequence motif SYK (Oppermann et al., 2003). Kushwaha et al. (2013) expressed the cDNA of *tr-I* from *Withania coagulans* in *Escherichia coli* and purified the protein to investigate its functional and catalytic properties. They investigated the pH optimum, the thermostability, substrate saturations kinetics and specificity, as well as the effect of salts. A K_{cat} value of 16.74 s^{-1} for tropinone was determined. Additional work was performed by Qiang et al. (2016) on TR-I from *Brugmansia arborea* L. and *D. stramonium*. The K_{cat} of BaTR-I for tropinone was 2.93 s^{-1} at pH 6.4 and the K_{cat} of DsTR-I was determined to be 2.40 s^{-1} at pH 6.4.

Putative littorine synthase

In 2015, Schmidt et al. published that the final step in the cocaine biosynthesis in *Erythoxylum coca*, the esterification of methylecgonine (2-carbomethoxy-3b-tropine) with benzoic acid, is catalyzed by a member of the benzylalcohol O-acetyl-, anthocyanin-O-hydroxycinnamoyl-, anthranilate-N-hydroxycinnamoyl/benzoyl-, and deacetylvindoline 4-O-acetyltransferase (BAHD) family. This cocaine synthase is a plant acyltransferase, capable of producing both cocaine and cinnamoylcocaine via the activated benzoyl- or cinnamoyl-CoA thioesters. This esterification seems to be similar to the esterification of tropine with phenyllactic acid from scopolamine biosynthesis and hence, it can be assumed that the littorine synthase may also belong to the BAHD family. Enzymes of the BAHD family utilize CoA thioesters and catalyze the formation of numerous plant metabolites. All identified members so far are monomeric, cytosolic enzymes with a molecular mass ranging from 48 to 55 kDa. The enzymes of this family share two conserved domains: The first is the HXXXDG domain, which is located near the center portion of each enzyme and is responsible for the utilization of CoA thioesters. The second highly conserved region is a DFGWG motif that is localized near the carboxyl terminus. These two motifs were identified in almost every functional enzyme of the BAHD family (D'Auria, 2006).

Littorine mutase/monooxygenase // CYP80F1

After the esterification of tropine with phenyllactic acid, the (*R*)-littorine formed is rearranged to (*S*)-hyoscyamine. Although the substrates for this isomerization were already identified in 1995 (Chesters et al., 1995), the enzyme involved and its mechanism remained unknown until recently. Due to the similarity of this step to rearrangement reactions of comparable substances, it was speculated that this reaction is a coenzyme-B12 mediated isomerization. As no traces of vitamin B12 have ever been found in plants, this idea has been rejected (Ollagnier et al., 1998). Moreover, it was discovered that SAM is involved in the rearrangement of littorine to hyoscyamine. In 2006, Li et al. demonstrated *in vitro* that CYP80F1 (EC 1.6.2.4) converts littorine mainly to hyoscyamine aldehyde. Moreover, they showed that the

suppression of the CYP80F1 gene by virus-induced gene silencing and RNAi results in the accumulation of littorine and reduction of hyoscyamine levels *in planta*.

Mechanistically, it is proposed that the rearrangement of littorine to hyoscyamine aldehyde is a cytochrome P450-catalyzed free radical reaction that includes two hydrogen abstraction steps, an optional rearrangement and is followed by an oxygen rebound. (Li et al., 2006)

Hyoscyamine 6β-hydroxylase

Hyoscyamine 6β-hydroxylase (H6H) is assumed to be the determining factor in many plants that accumulate hyoscyamine instead of scopolamine. H6H (EC 1.14.11.11) is a monomeric α-ketoglutarate dependent dioxygenase and the final enzyme of the TA pathway. This enzyme catalyzes a two-step reaction, the hydroxylation of L-hyoscyamine to 6-hydroxy hyoscyamine and the epoxidation of 6-hydroxy hyoscyamine to scopolamine, exhibiting low epoxidase activity compared to hydroxylase activity (Pramod et al., 2010a). The enzyme has an average molecular mass of 41 kDa and exhibits maximum activity at pH 7.8. L-hyoscyamine, oxygen and α-ketoglutarate are required for the enzyme activity, with respective K_m values of 35 μM and 43 μM. Iron ions (Fe^{2+}), catalase, and ascorbate (as a reductant) increase reaction catalysis. H6H is inhibited by EDTA and completely by other divalent cations, including Ca^{2+}, Cd^{2+}, Co^{2+}, Cu^{2+}, Hg^{2+}, Mn^{2+}, Ni^{2+}, Zn^{2+}, as well as by Fe^{3+}. Several alkaloids which are structurally related to L-hyoscyamine have also been shown to be hydroxylated at the C-6 position of the tropane moiety by H6H. This enzyme also epoxidizes 6,7-dehydrohyoscyamine to scopolamine (K_m 10 μM) (Hashimoto and Yamada, 1987).

1.4.2.2. Localization and organization of scopolamine biosynthesis *in planta*

The spatial localization of TA biosynthesis and their organization is diverse and complex. In Solanaceae plants, TA biosynthesis takes place in the roots and the alkaloids are then transported to the aerial parts where they are stored. Not much information regarding the transport and the transport form is available, but it is

assumed that the TAs are transported through the xylem. Cell-specific compartmentalization of scopolamine biosynthesis was previously observed in the root tissue pericycle, where expression of the genes *pmt* in *A. belladonna* L. (Suzuki et al., 1999) and *h6h* in *Hyoscyamus niger* L. (Kanegae et al., 1994) were detected. The enzyme TR-I, however, resides in the endodermis and nearby cortical cells in *H. niger* (Hashimoto et al., 1992; Nakajima and Hashimoto, 1999). In potatoes, the TR-II, which provides pseudotropine for calystegine biosynthesis, was detected in the cortex and phloem parenchyma of roots and stolons; in tuber spouts, the protein was detected in companion cells. TR-I, whose function in potatoes is not yet elucidated, was also detected in protein extracts of tuber tissue, however, in quantities too low to permit localization to single cells (Kaiser et al., 2006). The enzyme PMT also catalyzes the first step in nicotine biosynthesis (discussed above). In *Nicotiana sylvestris*, a nicotine producing plant, *pmt* is expressed in the endodermis, outer cortex, and found in root xylem (Shoji et al., 2000). This compartmentalization in biosynthesis *in planta* may complicate future attempts at heterologous production in single-celled microbial systems (discussed below). It may be that eukaryotic host cells such as yeasts or microalgae may be suitable host organisms for their biosynthesis as these cells exhibit compartmentalization of organelles and have been used for effective metabolic engineering of complex metabolites (Lauersen, 2019).

1.4.3. Cocaine biosynthesis

Cocaine biosynthesis (Figure 1-5), past its branch point with common intermediates shared with other TAs, is still under investigation and not fully elucidated. In literature, two different possibilities of the pathway towards cocaine biosynthesis have been reported. According to the classical hypothesis, the bridgehead carbon atom C-1 of methylecgonine is derived from an *N*-methyl-Δ^1-pyrrolinium cation and that of C-2 originates from acetoacetate. However, feeding experiments with labelled *N*-methyl-Δ^1-pyrrolinium cation were inconclusive *in planta* and could not confirm this theory. It was, therefore, suggested that the observed regiochemistry of incorporation of the labelled *N*-methyl-Δ^1-pyrrolinium cation into cocaine was compatible with the stepwise introduction of C2 units into the ecgonine skeleton,

derived from acetate (Leete et al., 1990). Consequently, this hypothesis proposes a new intermediate in cocaine biosynthesis, *N*-methyl-2-pyrrolidineacetic acid. Although this compound was detected in several plants, all attempts at incorporation of it into the ester or thioester forms have been so far unsuccessful (Hemscheidt and Spenser, 1992). Chemically, nucleophilic addition of the first acetyl-CoA moiety reaction is assumed to be a Mannich-like reaction using the enolate anion; the side-chain extension occurs via Claisen condensation (Dewick, 2002). The (*S*)-enantiomer cyclizes and forms the bicyclic structure of the cocaine tropane ring skeleton by an intramolecular Mannich reaction (Hoye et al., 2000). Hydrolysis of the CoA-ester followed by SAM-dependent methylation and reduction yield methylecgonine (2-carbomethoxy-3β-tropine). Methylecgonine in its turn condenses with benzoyl-CoA, which is derived from L-phenylalanine (Leete et al., 1988), to cocaine. Schmidt et al. (2015) described an enzyme catalyzing this reaction and termed it the cocaine synthase. This synthase belongs to the BAHD family, which catalyzes the transfer of CoA-activated acyl thioesters to oxygen- or nitrogen-containing acceptor molecules (D'Auria, 2006).

Cocaine and scopolamine are structurally two related compounds, which both possess a tropanol backbone: cocaine is a 3β-tropanol, scopolamine a 3α-tropanol.

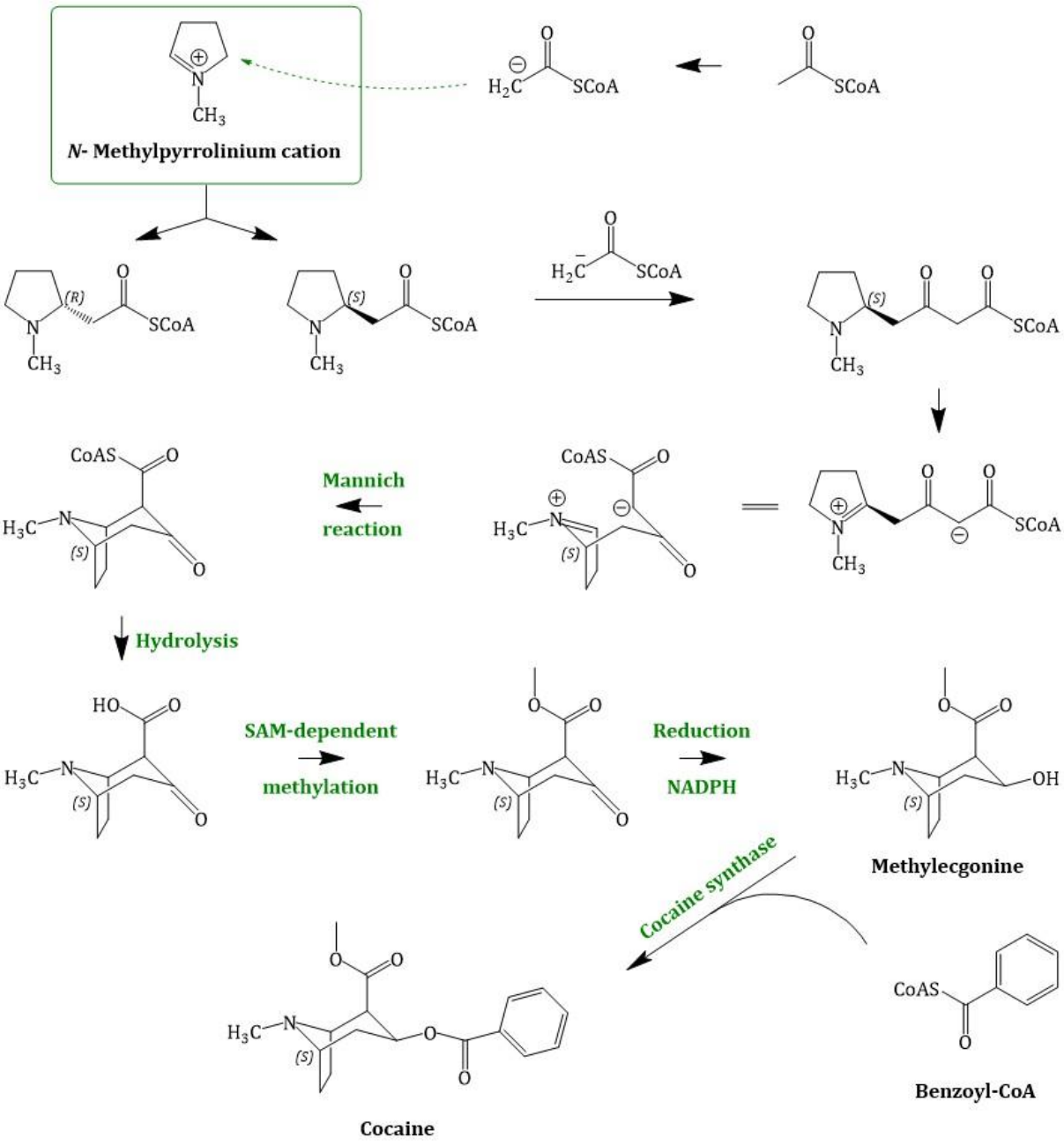

Figure 1-5: Cocaine biosynthesis, starting with the intermediate *N*-methyl-Δ¹-pyrrolinium cation; only less information regarding the enzymatically involvement is proven. The iminium cation reacts with two acetyl-CoA moieties to an intermediate that cyclizes in an intramolecular Mannich reaction. After hydrolysis, methylation and reduction methylecgonine is formed. The cocaine synthase catalyzes the last step in the pathway: the condensation of methylecgonine with benzoyl-CoA.

1.4.4. Calystegine biosynthesis

Comparable to the hyoscyamine/scopolamine and cocaine pathways, the detailed processes of calystegine biosynthesis are not known. Tropinone is assumed to be involved and which should be reduced to pseudotropine, a reaction catalyzed by TR-

II. No further information regarding the biosynthesis is available so far (Scholl et al., 2001). To date, no attempts at elucidation of the hydroxylation or the demethylation of these compounds has been reported. This may be due to the relatively recent discovery of calystegines in 1990 (Dräger, 2003) and reduced interest in medical applications compared to scopolamine or cocaine. In contrast to cocaine and scopolamine, the calystegine skeleton (8-azabicyclo[3.2.1]octane) is not *N*-methylated, rather, it is polyhydroxylated. These compounds are classified into different groups, depending on the number of hydroxy groups: subgroup A consists three, subgroup B four, and subgroup C have five (Goldmann et al., 1996).

1.5. BIOTECHNOLOGICAL APPROACHES OF SCOPOLAMINE PRODUCTION

1.5.1. Scopolamine production in cell suspension and hairy root cultures

The quality of *Duboisia* spp. plant material and the quantity of scopolamine in agricultural production depends on different abiotic factors such as climate, sunlight, soil fertilization, and biotic factors (Luanratana and Griffin, 1980; Ullrich, 2016). In times of climate change, abiotic influences may become less predictable and more extreme. This in turn influences the biomass and results in variable alkaloid content and production potentials (Ullrich et al., 2017). To establish a more independent production system, different plant cell cultures have been developed – especially callus cultures, cell suspension cultures, and hairy root cultures. The advantage of these cell cultures is the possibility to control TA biosynthesis via process design in order to achieve increased or altered tropane alkaloid yields. However, to date, the produced amounts of TAs by tissue culture are not competitive to the production of scopolamine by agricultural farming of *Duboisia* hybrids. This difference in production arises due to complicated scale-up of tissue culture production and associated costs. Additionally, the cell-specific compartmentalization of TA biosynthesis as discussed in previous sections likely reduces tissue-culture specific production as callus and cell suspension cultures are totipotent, undifferentiated cells (Oksman-Caldentey and Strauss, 1986).

Hairy roots are disease manifestations developed by plants that are wounded and infected by *Agrobacterium rhizogenes* (Wang, 2007). In contrast to undifferentiated cell cultures, hairy root cultures can usually synthesize the same metabolites as unmodified roots and may also produce desired secondary metabolites (Georgiev et al., 2012). In nightshades, TA biosynthesis is localized in the root and this plant organ has been exploited for TA production. Early experiments were performed in the late 1980s and reports are continuing to be published on this process. The hairy root system itself is stable for several years with steady growth and alkaloid production rates (Maldonado-Mendoza et al., 1993), however, scale-up of this system remains technically challenging. Table 1-1 presents a brief overview of TA concentrations in engineered and untreated hairy root cultures from different plants:

Table 1-1: Overview of TA concentrations in engineered and untreated hairy root cultures from different plants. TAs content in leaves of regenerated plants; n.d. = not determined, DW = dry weight

Plant	Overexpression of	Amount		Citation
		hyoscyamine	**scopolamine**	
Atropa belladonna L.	-	0.371 ± 0.013 % DW	0.024 ± 0.010 % DW	Kamada et al., 1986
	H. niger h6h	0.02 % *)	0.45 % *)	Hashimoto et al., 1993b
	-	2.1 + 0.2 mg g^{-1} DW	n.d.	Falk and Doran, 1996
	***)*	0.31 mg g^{-1} DW	0.27 mg g^{-1} DW	Vakili et al., 2012
Hyoscyamus niger L.	-	1.6 mg g^{-1} DW	5.3 mg g^{-1} DW	Jaremicz et al., 2014
	pmt, h6h	n.d.	411 mg L^{-1}	Zhang et al., 2004
Anisodus acutangulus C.Y.WU	*h6h*	0.789 ± 0.078 mg g^{-1} DW	0.070 ± 0.003 mg g^{-1} DW	Kai et al., 2012
	tr-I	2.479 ± 0.432 mg g^{-1} DW	0.023 ± 0.004 mg g^{-1} DW	
	tr-I, h6h	2.286 ± 0.46 mg g^{-1} DW	0.072 ± 0.018 mg g^{-1} DW	
Brugmansia candida PERS. ****)*	-	0.35 ± 0.07 mg g^{-1} DW	1.05 mg g^{-1} DW	Cardillo et al., 2010
Hyoscyamus muticus L.	*h6h*	287.7 mg L^{-1}	14.41 mg L^{-1}	Jouhikainen et al., 1999
Duboisia myoporoides R.BR.	*pmt*	no increase observed		Moyano et al., 2002
	h6h	n.d.	24.93 mg g^{-1} DW	Palazón et al., 2003

*) TAs content in leaves of regenerated plants **) chromium treatment ***) *B. candida* hairy roots grown in a special bioreactor

A further development of the hairy roots cultures is the exploitation of TAs by "milking the plant". After stimulation of aeroponically cultivated plants, roots were "extracted" by putting the roots into physiological extraction medium without harming or destroying them, and desired secondary metabolites were isolated. After "milking", plants are returned to their cultivation apparatus to regenerate and produce more secondary metabolites which can be subsequently extracted in further cycles. This

promising approach still needs to be optimized to be economically competitive (Bourgaud et al., 2013).

Transgenic plants have also been generated and cultivated for TA production. Recently, Xia et al. (2016) overexpressed *pmt* from *Nicotiana tabacum* (NtPMT) and *h6h* from *H. niger* (HnH6H) in *A. belladonna* and reached high scopolamine levels (2.94 – 5.13 mg g^{-1} DW) in field conditions. Almost thirty years previously, Wang et al. (1985) also overexpressed *pmt* and *h6h* in *A. belladonna*, although a scopolamine concentration of only 1.2 mg g^{-1} DW was achieved.

To date, neither cell suspension cultures, callus cultures, nor hairy root cultures have been demonstrated to be competitive for TA production in comparison to agricultural means. In comparison to these alternative production options, the conventional field cropping of *Duboisia* species in Australia provides up to 15 tons fresh leaves per hectare, with three harvests annually. The total TA concentration of these plants is about 2 – 4 % (equivalent to 20-40 mg g^{-1} DW) with ca. 60 % scopolamine (Grynkiewicz and Gadzikowska, 2008). Obtaining these yields in terms of concentration and total amount biotechnological approaches are not yet competitive to the agricultural field production.

1.5.2. Microbial production of scopolamine and enzyme engineering approaches

Plants and plant cell suspension cultures are often slow growing and difficult to handle. In comparison, microbial cultures such as bacteria, e.g. *E. coli*, or yeast, e.g. *Saccharomyces cerevisiae*, are straightforward to cultivate and are well characterized model organisms with fully developed molecular toolkits. Cultivation of these organisms can be readily scaled in existing fermentation infrastructure, which makes their cultivation more economically favorable than plant tissue culture. Therefore, heterologous production of TAs such as scopolamine in these hosts may represent an attractive alternative given transfer of the molecular pathways is possible.

Most research in this area has been performed on understanding and optimizing H6H by metabolic engineering. Cardillo et al. (2017) expressed recombinant *Brugmansia*

candida h6h in *S. cerevisiae* and performed bioassays using isolated enzymes. Untagged H6H was able to produce 83.3 % 6β-hydroxy hyoscyamine and 7.6 % scopolamine from hyoscyamine after 15 h of incubation. Additionally, specific hydroxylase and epoxidase activity: 2.60 ± 0.19 nKat mg^{-1} and 0.24 ± 0.02 nKat mg^{-1} for these two compounds were observed, respectively. The H6H from *Anisodus acutangulus* was cloned and expressed in *E. coli* fused with either a His- or GST-tag at the *N*-terminus (Kai et al., 2011). A bifunctional assay revealed that both recombinant enzymes converted up to 80 % of fed hyoscyamine to scopolamine, however, reaction kinetics were not analyzed. Li et al., (2012) expressed *h6h* from *A. belladonna* (AbH6H) in *E. coli* and determined that the K_m value for hyoscyamine under optimal conditions was 52.1 ± 11.5 µM. Compared with former experiments it revealed that the K_m of *Ab*H6H is higher than that of *Hn*H6H from *H. niger* (35 µM; (Hashimoto and Yamada, 1987)) and from *A. tanguticus* AtH6H (15.1 ± 0.3 µM; (Liu et al., 2005)), which implies that *Ab*H6H has lower affinity for the substrate than *Hn*H6H. Furthermore, it has been shown that epoxidation is slower than hydroxylation by this enzyme (Hashimoto et al., 1993a). Pramod et al. (2010) characterized the H6H from *D. metel* and obtained K_m values for hyoscyamine and 2-oxoglutarate to be 50 µM each. In 2018, Fischer et. al., published results of SUMO-tagged H6H from *Brugmansia sanguinea* to have a K_m value of ~60µM.

First promising results concerning protein engineering of H6H were published in 2015. Cao et al. (2015) used random mutagenesis and site-directed saturation mutagenesis to increase the hydroxylation activity of H6H from *A. acutangulus*. They developed a double mutant, *Aa*H6HM1 (S14P/K97A), which has a 3.4-fold increased hydroxylation and 2.3 times higher epoxidase activity than the native enzyme, a conversion rate of 97 % was achieved *in vitro*.

The main challenge of the heterologous TA production is that the native biosynthesis of most target compounds *in planta* are not fully elucidated to date. It is not known if the condensation of tropine with phenyllactic acid-CoA reacts spontaneously or is enzymatically catalyzed. Therefore, it is currently not yet possible to engineer the complete biosynthesis heterologously. The goal of microbial production of scopolamine is still in its early stages and will require complete pathway elucidation before it can be seriously considered as an alternative to conventional farming

practices. Future efforts will require intense bioinformatic analysis of genomes and transcriptomic data to aid in identification of the complete biochemical pathways towards TA biosynthesis. Once identified, the pathways can be engineered into heterologous hosts and optimized for the generation of these desired products.

1.5.3. Additional methods of scopolamine production

To increase the scopolamine level *in planta*, polyploid plants have been developed. An impressive example was recently published by Dehghan et al. (2017). The authors produced stable tetraploid hairy root lines of *H. muticus* that exhibited lower biomass production than diploids, however, higher scopolamine (13.87 mg L^{-1}) and hyoscyamine levels (107.7 mg L^{-1}), up to 200 % more scopolamine than in diploid plants. However, the total yield of scopolamine from these plants was rather low due to the slow growth rates and results were only reported for growth conditions in optimized Murashige and Skoog (MS) or Gamborg's B5 media which are not competitive to conventional field cultivation. Nonetheless, these initial trials are promising and polyploid plants of other species such as *D. myoporoides* may be interesting alternatives. In order for this strategy to realize economic potential, a polyploid clone must be able to be cultivated under the same conditions as the current plants, produce the same (or higher) biomass, and be genetically is stable over a period of at least 10 years.

In 2017, Naik et al. published the first report regarding TA producing endophytes, namely *Colletotrichum boninense*, *Phomopsis* sp., *Fusarium solani*, *Colletotrichum incarnatum*, *Colletotrichum* siamense and *Colletotrichum gloeosporioides*, that are found in *D. metel* and possess the enzymes PMT, TR-I, and H6H. It was reported that these fungi produce a remarkable amount of scopolamine (4.1 mg L^{-1}) and hyoscyamine (3.9 g L^{-1}). Perhaps independent cultivation of these fungal species may represent a natural alternative to heterologous hosts or agricultural cultivation. It may also be possible to identify the biosynthetic pathways of TAs in these hosts, which could either be optimized in the fungi themselves and enhanced or transferred into heterologous microbial hosts." (Kohnen-Johannsen and Kayser, 2019)

1.6. SCOPE OF THIS THESIS

The work presented in this thesis is aimed at elucidation of the molecular stages of late tropane alkaloid biosynthesis in *Duboisia myoporoides* R.BR. plants. The overall biosynthesis in *Duboisia* plants as well as the tropane alkaloid biosynthesis in detail are not understood and described. For greater biotechnological optimization or pathway transfer into microbial hosts, profound knowledge of the biochemical pathways is required. In this thesis, focus was set on two main fields in detail. On the one hand, the time-dependent development of TA biosynthesis in *D. myoporoides* was elucidated and on the other hand, the isolation and identification of the last unknown enzyme in TA biosynthesis, the littorine synthase, was investigated.

Hence, the aims of this thesis are:

I. Investigation of the time-dependent biosynthesis of TAs in *Duboisia myoporoides* R.BR., comparing young, intermediate, and mature plants which were grown under controlled conditions. In addition to TA profiles and gene expression analyses, special attention is given to the spatial distribution of TA biosynthesis in all plant organs during development.

II. Investigation of candidate genes as potential candidates for the last uncharacterized, biosynthetic step in late TA biosynthesis, the littorine synthase. Presented here are the first investigations of functional expression and activity testing of potential the littorine synthase gene candidates that were identified through bioinformatic analyses.

CHAPTER 2

MATERIALS AND METHODS

Parts of this chapter were published in

Kohnen, K. L.; Sezgin, S.; Spiteller, M.; Hagels, H.; Kayser, O. Localization And Organization Of Scopolamine Biosynthesis In *Duboisia myoporoides* R. BR., *Plant Cell Physiol.* 2017, *0*, 1–12, doi:10.1093/pcp/pcx165.

2.1. CHEMICALS AND PLANT MATERIAL

2.1.1. Chemicals

Chemicals were purchased from Sigma-Aldrich (Darmstadt, Germany), Invitrogen (Karlsruhe, Germany), Merck (Darmstadt, Germany), Carl Roth (Karlsruhe, Germany), and VWR (Darmstadt, Germany) if not stated otherwise.

"Reference compounds of the alkaloids littorine (94.2% pure), hyoscyamine sulfate (99.7 % pure), 6-hydroxy hyoscyamine hydro-bromide (97.4 % pure) and scopolamine (99.3 % pure) were received from Boehringer Ingelheim Pharma. In addition, the internal standard scopolamine-D3 hydro bromide (99 % pure) was ordered from EQ Laboratories GmbH. The identity of the reference alkaloids was confirmed via ESI-MS: littorine, $[M+H]^+$, *m/z* 290.18; hyoscyamine, $[M+H]^+$, *m/z* 290.18; 6-hydroxy hyoscyamine, $[M+H]^+$, *m/z* 306.17; and scopolamine, $[M+H]^+$, *m/z* 304.15." (Kohnen et al., 2017)

NucleoSpin® Plasmid (NoLid) Kit used for *E. coli* plasmid isolation as well as the NucleoSpin® Gel and PCR Clean-Up Kit were purchased from Macherey- Nagel GmbH & Co. KG, Düren, Germany. Restriction enzymes were purchased from New England BioLabs® GmbH (NEB, Frankfurt am Main, Germany).

2.1.2. Plant material

"Cuttings of *D. myoporoides* were received by Boehringer Ingelheim Pharma (Germany). Voucher specimens (M3/1 20-24) were kept at the Technical University (TU) Dortmund, Department of Biochemical and Chemical Engineering. Cuttings were cultivated on hydro culture at the TU Dortmund, Germany, in a CLF PlantMaster indoor plant chamber walk-in room (CLF Plant Climatics) at 25°C in a 12 h day/night cycle (light intensity 110mmol photons m^{-2} s^{-1}; lamp Eye Cera Arc PAR36, 3,500 K) with humidity set to 75 %. Plants were harvested on three specific dates: after 6 weeks, 3 months and 6 months. At each time point, four replicates were taken: three biological replicates for the quantitative LC-MS- and transcriptomic analyses as well as for the MALDI -MS imaging experiments." (Kohnen et al., 2017)

2.2. METHODS

2.2.1. Extraction protocol and LC-MS analysis

"For quantitative analysis, plant material was harvested, weighed and dried at 60°C for 24 h. Then the dry mass was determined. Dried material was ground, using a mortar and pestle, then sieved (mesh size 0.63 mm). Sample preparation (plant number: 6W_M1-M4, 3M_M5-M6+M9-M10, 6M_M8+M14-M16) and measurement were performed according to Ullrich et al. (2016). For extraction, 50 ± 0.5 mg of ground plant material was weighed out and 10.0 mL of 0.5 % H_3PO_4, pH 1.6–1.7, was added to the samples. After vortexing and ultrasonic bath treatment, the solution was incubated for 18 h at 30°C, 200 rpm. The extract was then filtered, 250 mL of the filtrate was diluted with 200 mL of 0.5 % H_3PO_4 and 50 mL of internal standard solution of scopolamine-D3 (final concentration: 0.7 mg L^{-1}) was added. For each biological sample, three technical replicates were prepared and measured. An Agilent HPLC Infinity 1260 consisting of a 1260 Bin Pump, a 1260 Degasser, a 1200 ALS Autosampler, a 1260 TCC column oven and a 1260 DAD diode-array detector manufactured by Agilent Technologies was used. The Kinetex Core Shell C18 column (100 x 2.1 mm, 2.6 mm) by Phenomenex (Germany) was heated to 30°C with the flow rate set to 350 mL min^{-1}. The mobile phase consisted of aqueous formic acid (0.1 %, v/v; solvent A) and MeOH+20 % acetonitrile (solvent B). The gradient program was as follows: 0–1 min (90 % A/10 % B), 1–9.1 min (90 % A/10 % B–60 % A/40 % B) and 9.1– 17 min (90 % A/10 % B). A Micro-TOF-Q MS-system (Bruker Daltonik GmbH) was coupled to the LC system, running in positive ion mode from *m/z* 90 to 700. TAs were detected by the *m/z* ratio and quantified by $[M+H]^+$,normalized to the internal standard scopolamine-D3. LC-MS measurements of the alkaloids were performed in biological triplicates with a 3-fold determination (technical replicates). The statistical evaluation by means and SD was performed using Microsoft Office Excel. One-way analysis of variance (ANOVA) was used to determine statistically significant differences between the means (α=0.05).

2.2.2. Sample preparation for MALDI imaging and experimental setup

Fresh plant material (plant number 6W_M1-M4, 3M_M5-M6+M9- M10, 6M_M8+M14-M16) was tailored to small explants. The sample holder was prepared with 5 % carboxymethyl cellulose (CMC) gel on which the explants were placed in the CMC gel and covered in CMC. The sample was frozen and sectioned using cryostat Microm™ HM550 (Thermo Scientific). The theoretical thickness was set to 20 µm. After sectioning, the tissue section was transferred onto a glass slide and dried in a pre-cooled dry freezer for 2–4 h until the section was dry. DHB [2.5-dihydroxybenzoic acid; 30 g L^{-1} in 1:1 acetone–water (v/v); 0.1 % formic acid added] was sprayed onto the dried sample using the matrix-deposition device SMALDI Prep (TransMIT GmbH) for 2 x 15 min with a matrix flow rate of 15 mL min^{-1} and dry gas (nitrogen) flow rate of 4 L min^{-1}.

2.2.3. MALDI imaging instrumentation

MALDI-MSI experiments were performed using an AP-SMALDI ion source imagine10 (TransMIT GmbH), operating with a nitrogen laser (337.1 nm) at a pulse frequency of 60 Hz and coupled to a high-resolution mass spectrometer Q Exactive (Thermo Scientific GmbH). All measurements were recorded in positive ion mode; the mass range was set to *m/z* 90–700 while an internal lock mass calibration was performed orienting towards the lock mass *m/z* 237.03936, which corresponds to the matrix ion $[2M\text{-}2H_2O+H]^+$ of DHB. Scans were conducted at the mass resolution 140,000 at *m/z* 200. Spray voltage was adjusted at 2 kV and maximum injection time at 300 ms. The beam attenuation level of the ion source was set at 20°. Pixel resolutions were fixed in the range 20–35 mm." (Kohnen et al., 2017)

2.2.4. Evaluation of the MALDI-MSI experiments

Root, stem and leaf tissues of the respective plant samples were prepared and analyzed by MALDI-MSI. Optical images of tissue samples were obtained before matrix application with a Leica S8AP0 microscope (Leica Microsystems). For data processing, the imaging software ImageQuest (v. 1.1.0; Thermo Scientific GmbH) was used. Ion

density images, which were created within a mass tolerance window of ±2 ppm, contain pixels with mass information that were extracted by typing the theoretical masses of the investigated compounds (hyoscyamine aldehyde, hyoscyamine/ littorine, 6-hydroxy hyoscyamine, scopolamine and scopolamine glucoside; for the individual ppm values see Figure 3-2 - Figure 3-5) into the program.

The background of the measured ion density images was removed and overlaid onto the optical image. Signal intensities are visualized by a color-coding system, starting from low intensity (blue) to high intensity (red). The quality of the received results was visually compared and evaluated.

2.2.5. "Total RNA isolation and transcription into cDNA

Total RNA was extracted from flash-frozen plant material (roots, stem tissue and leaves, in biological triplicates; plant number: 6W_M1-M4, 3M_M5-M6+M9- M10, 6M_M8+M14-M16) with spin-columns (NucleoSpin RNA Plus, Machery-Nagel) and Spectrum™ Plant Total RNA Kit (Sigma Aldrich, Steinheim, Germany), respectively, according to the manufacturer's standard protocols. RNA quality and quantity were determined using Nanodrop™ One (Thermo Fisher). All the samples used for the study were pure and high in quality ($A_{260}/A_{280} \geq 1.8$; $A_{260}/A_{230} \geq 1.9$). An 800 ng aliquot of RNA was used for reverse transcription in 20 µl reactions using the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (epicentre) and oligo$(dT)_{21}$ primers. Reactions were carried out according to the manufacturer's protocols, briefly 1 h at 37°C and terminated by incubation for 5 min at 85°C. cDNA reaction was diluted with 20 mL of RNase-free water to a final concentration of 20 ng μl^{-1}." (Kohnen et al., 2017) Samples used for transcriptomic analyses were sent to IGATS, Udine, Italy in a RNAstable 96-Well Plate (Biomatrica, San Diego, USA) for sequencing. The RNA used for the generation of cDNA, was also isolated as described above and then transcribed into cDNA by the MMLV Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (epicentre).

2.2.6. Quantitative real-time-PCR

Gene expression of *D. myoporoides* genes *pmt* (GenBank accession No. KY350178), *tr-I* (KY350177), *cyp80f1* (KY350176) and *h6h* (KY350175) was quantified by qPCR experiments. Reactions were performed in a 20 µL volume with 10 µL of Fast SYBR® Green Master Mix (Thermo Fisher Scientific), 0.4 µL of 10 mM primer mixture, 1 µL of cDNA (20 ng μl^{-1}) with 8.6 µl of nuclease-free water. Amplification was performed under the following conditions: 0.5 min denaturing at 95°C; 40 cycles of 10 s denaturing at 95°C, 30 s annealing and extension at 60°C. Melting curves were recorded after running 40 cycles. The house keeping gene *gapdh* (EC 1.2.1.12.) was used as the endogenous control. Neither cross-hybridization nor fluorescent signals exceeding the baseline threshold were observed during this assay. The threshold was set to 1, and the corresponding C_t values were calculated by the qPCR software StepOne V2.2.2 (Applied Biosystems).

2.2.7. Statistical evaluation of the qPCR experiments

Quantification of the gene expression, indicated as fold change in the target genes normalized to *gapdh* and relative to the gene expression of sample 6W_M1B (calibrator which contained the lowest expression of the investigated transcripts), was calculated for each sample using the comparative $\Delta\Delta C_t$ method (Pfaffl et al 2004).

$$\Delta CP = CP\ (gene\ of\ interest) - CP\ (gapdh)$$

$$\Delta\Delta\ CP = \Delta\ CP\ (sample) - \Delta CP\ (calibrator\ 6W\ M1B)$$

$$fold\ change = 2^{-\Delta\Delta CP}$$

As with the LC-MS experiments, the measurements were performed in biological triplicates. The statistical evaluation by means and SD was performed using Microsoft Office Excel. One-way analysis of variance (ANOVA) was used to determine statistically significant differences between the means (αa=0.05).

2.2.8. Primer design

Primer pairs were designed with the program Clone Manager 9 Professional Edition. Primer length was fixed between 18 and 22 bp and amplicons were amplified within the target sequence having a length of 70–100 bp. The T_m was set to 60–65°C. Primers used for qPCR are listed in Table 3-1.

2.2.9. Candidate gene identification and phylogenetic analysis

For candidate gene identification, we probed our internal cDNA library of the 3-month-old leaves (3Mo-M10B) for the HXXXDG and DFGWG amino acid motifs. The open reading frames of these contigs were aligned using MEGA (Molecular Evolutionary Genetics Analysis) software, version 7. To increase the dataset, all described enzymes of the BAHD family (D'Auria, 2006) were used to construct a phylogenetic tree. Putative littorine synthases are suspected to be members of the BAHD family clade III, filtering these 22 candidates for those of this clade reduced the candidate genes to 6 possible gene sequences.

2.2.10. Plasmid construction with Gibson Assembly

Expression plasmids were constructed for each of the five cloneable contigs using Gibson Assembly (Gibson et al., 2011). Prior to the assembly, overlaps of the inserts and high-copy T7 *E. coli* expression vector pET32a were created. The individual contigs were amplified from cDNA by polymerase chain reactions (PCR) which were set up as follows: 12.5 µL Q5 High-Fidelity 2X Master Mix (NEB), 1.25 µL 10 µM forward primer, 1.25 µL 10 µM reverse primer (see Table A-), 2 µL cDNA template (*D. myoporoides* cDNA of 3-months-old leaves) or 100 ng vector (pET32a) and 25 µL water. The vector PCR was digested with 1 µL of *Dpn*I (NEB) overnight at 37 °C to remove the empty vector template. The concentrations of the purified (NucleoSpin Gel and PCR Clean-up; Machery Nagel, Düren, Germany) vector possessing the overlaps and the PCR amplicons of the inserts were determined with NanoDrop™ One (Thermo Fisher, Germany). For Gibson assembly, 100 ng of vector amplicons and an equimolar amount (or more) of insert were added to 5 µL Gibson Assembly Master Mix 3.2x and

the appropriate amount of water. The reaction was incubated for 60 min at 50 °C, and subsequently stored on ice for at least 5 min prior to transformation into *E. coli*.

2.2.11. Transformation of the vector constructs into *E. coli* DH5α chemical competent cells

Chemically competent *E. coli* DH5α (LifeTechnologies) cells were thawed on ice and 1 µL of each Gibson Assembly reaction was added. To control the suitability of the cells, 1 µL of water was added to one tube as negative control. The cells were incubated for 30 min on ice and heat-shock transformed afterwards by placing them for 45 sec at 42 °C. 900 µL of SOC medium was added and the cells were incubated again for 1h at 37 °C. The cells were pelleted by centrifugation for 5 min and 700 µL SOC medium was removed. After resuspension, the cells were plated on LB-agar plates with contain 1 µL mL^{-1} kanamycin and incubated for 16 – 18 h at 37 °C.

2.2.12. Expression of the littorine synthase candidate genes in *E. coli* strain BL21(DE3)

Five to ten colonies were picked for colony PCR and amplified using the primer pair T7 and T7 term, which bind on the T7 promotor and terminator sequence of the pET32a vector system (Table A-2). Colonies without the correct insert show amplicons in the 1 % agarose stained with ethidium bromide at 318 bp, colonies with the right insert show higher bands, respectively. Colonies with the correct insert were inoculated for plasmid isolation, (NucleoSpin Plasmid - plasmid Miniprep kit; Machery Nagel) and sequenced at Seqlab, Göttingen. The correct plasmids containing desired contigs were transformed into *E. coli* BL21(DE3; Invitrogen) by heat shock (see above) and the bacterial culture was grown in LB medium supplemented with 50 mg L^{-1} of kanamycin at 37 °C with shaking at 200 rpm until an OD_{600} of 0.4 to 0.5 was reached. Protein expression was induced by addition of 1 mM isopropyl β-D-1-thiogalactopyrano- side (IPTG) or 1mM lactose, respectively, with further cultivation at 18 °C for 24 h.

2.2.13. Protein extraction, SDS-PAGE, and Western Blot

E. coli cells corresponding to 100 mg CDW were resuspended 100 µL sample buffer (150 mM Tris/HCl, pH 6.8, 1.2 % sodium dodecyl sulfate (SDS), 30 % glycerol, 15 % β-mercaptoethanol and 0.0018 % bromophenol blue in H_2O). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Samples were loaded per slot on a gel together with a protein marker (Page Ruler™ Plus Prestained Protein Ladder, Thermo Scientific) and stained afterwards using Coomassie Blue staining solution (2.5 g Coomassie Brilliant Blue R-250 in a mixture of 450 mL MeOH, 100 mL acetic acid and 400 mL dH_2O). Western Blot was performed by transferring proteins to a nitrocellulose membrane at 0.8 mA/cm2 for 1 h. His-tagged proteins were detected according HisDetector™ Western Blot Kit, AP Colorimetric, SeraCare following manufacturers' protocols.

2.2.14. Functional activity testing of potential littorine synthase candidates

After protein expression, the cells were harvested by centrifugation, the supernatant was discarded, and the cell pellets were resuspended in lysis buffer (50 mM Bis-Tris buffer, pH 8, supplemented with 10 % (v/v) glycerol and 5 mM dithiothreitol (DTT)) and disrupted by sonication. The lysate was centrifuged at max-speed and 4 °C for 15 min and the soluble fractions were tested for enzymatic activity. The activity assay was performed analogously to Schmidt et al., (2015) using an assay buffer consisting of 50 mM Bis-Tris propane buffer, pH 8.0, supplemented with 10 % (v/v) glycerol, 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 1 mM tropine, 1 mM phenyllactic acid-CoA, and 1 mL of protein extract in a total volume of 10 mL The assay was stopped after 4 h with 10 mL of 1 M HCl. Supernatants were analyzed after centrifugation by HPLC using the method for alkaloid detection described above.

2.2.15. Chemical Synthesis of phenyllactic acid-CoA

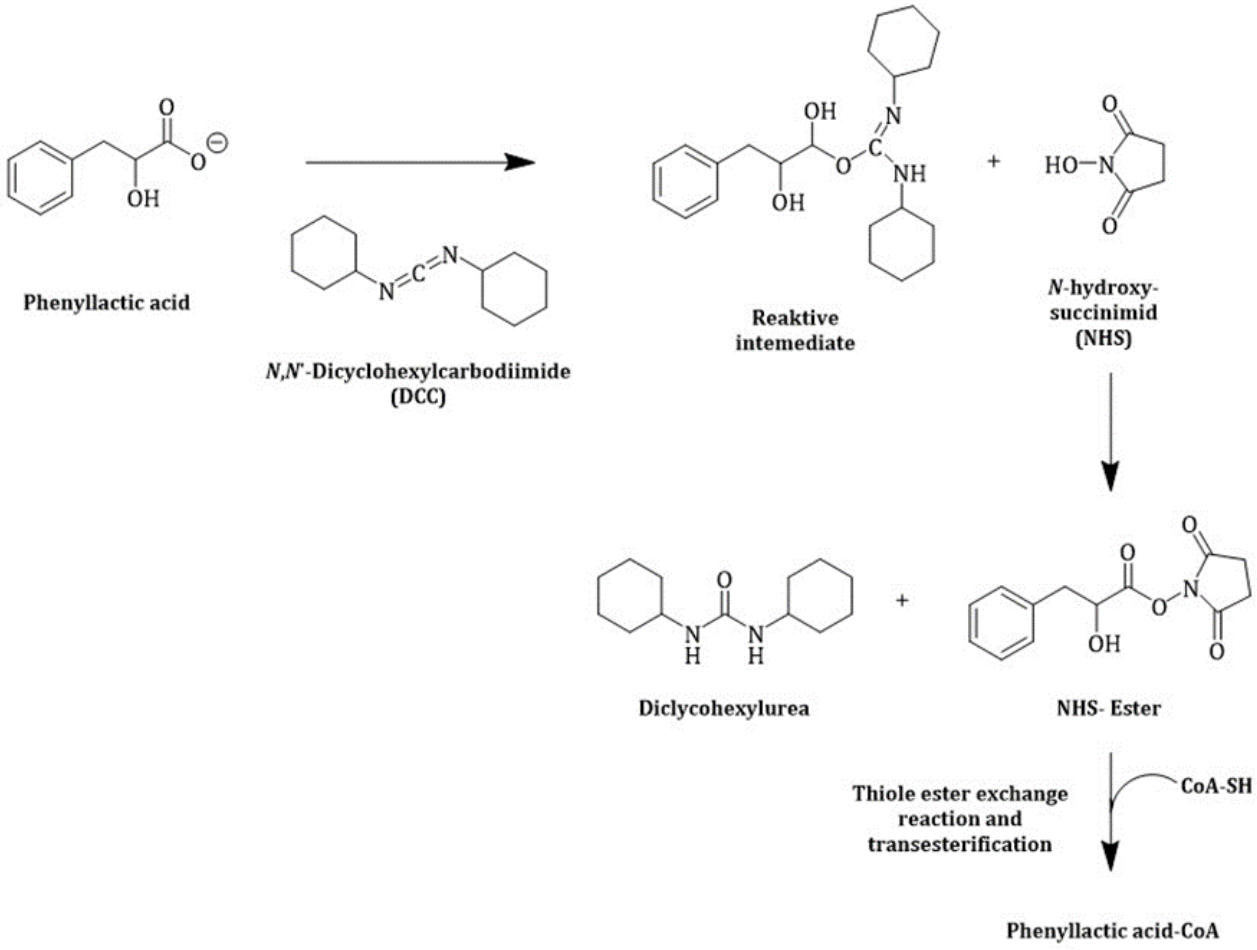

Figure 2-1: Reaction mechanism of phenyllactic acid-CoA synthesis according to Stöckigt and Zenk (1975)

Synthesis modified from Stöckigt and Zenk (1975). In a first step, the *N*-hydroxy succinimide ester of phenyllactic acid was prepared. 15 mmol phenyllactic acid and 15 mmol *N*-hydroxy succinimide were dissolved in 200 mL ethyl acetate while heating and allowed to cool to room temperature. *N*, *N*'-dicyclohexylcarbodiimide (DCC) was heated to 40 °C. until it has liquefied. 17 mmol DCC were added to the above solution until a precipitate (dicyclohexylurea) is formed, while the NHS-ester remains in the filtrate. The solvent is evaporated, and the NHS ester crystallizes. In the next steps, thiol ester exchange and transesterification are performed. For this, 25.2 mg $NaHCO_3$ are dissolved in 6.0 mL water and flushed with nitrogen for 15 min. Thereafter, 23 mg of Coenzyme-A are added, and the solution is flushed with N_2 for further 15 min. The previously prepared NHS ester is dissolved in 0.5 mL of acetone and is added dropwise

to the CoA solution. The solution is left overnight, and the volume reduced with SpeedVac vacuum concentrators. The identity was checked by ^{1}H NMR (compare Figure A-1).

2.2.16. Media and solutions

Table 2-1: Overview of the composition of LB- and SOC-medium

Medium	Composition	
SOC medium	2 %	Tryptone
	0,5 %	Yeast extract
	10 mM	NaCl
	2.5 mM	KCl
	10 mM	$MgCl_2$
	10 mM	$MgSO_4$
	20 mM	Glucose
		in water; pH 7
LB medium	10 %	Tryptone
	10 %	NaCl
	5 %	Yeast extract
		in water; pH 7

CHAPTER 3

RESULTS AND DISCUSSION

Parts of this chapter were published in

Kohnen, K. L.; Sezgin, S.; Spiteller, M.; Hagels, H.; Kayser, O. Localization And Organization Of Scopolamine Biosynthesis In *Duboisia myoporoides* R. BR., *Plant Cell Physiol.* 2017, *0*, 1–12, doi:10.1093/pcp/pcx165.

The “Results and discussion” chapter is divided into two sub-chapters:

I.) Localization and organization of tropane alkaloid biosynthesis in *Duboisia myoporoides* R.BR.

II.) Identification and isolation of the littorine synthase in *Duboisia myoporoides* R.BR.

The first part deals about the TA biosynthesis in *D. myoporoides*. The time-dependent biosynthesis of TAs *in planta*, comparing young, intermediate, and mature plants which were grown under controlled conditions is investigated. In addition to the quantitative TA profiles and gene expression analyses, special attention is given to the spatial distribution of TA biosynthesis in all plant organs during development.

In the second section, the littorine synthase candidate genes are examined as potential candidates for the last uncharacterized, biosynthetic step in late TA biosynthesis. First investigations of functional expression and activity testing of potential the littorine synthase gene candidates that were identified through bioinformatic analyses are presented.

3.1. LOCALIZATION AND ORGANIZATION OF TROPANE ALKALOID BIOSYNTHESIS IN *DUBOISIA MYOPOROIDES* R.BR.

3.1.1. Quantification of tropane alkaloids in different organs of *D. myoporoides* during plant development

“Aqueous phosphoric acid extracts of 6-week-, 3-month and 6-month-old *D. myoporoides* plants were prepared for each of the three plant organs: roots, stem tissue and leaves. Alkaloids were identified using reference compounds via LC-MS. The four major alkaloids littorine, hyoscyamine, 6-hydroxy hyoscyamine (also known as anisodamine) and scopolamine (also known as hyoscine) were quantified. Root, stem and leaf extracts exhibited different distributions of the investigated TAs during plant development. In Figure 3-1, the concentration of TAs in different plant tissues during plant growth is depicted.

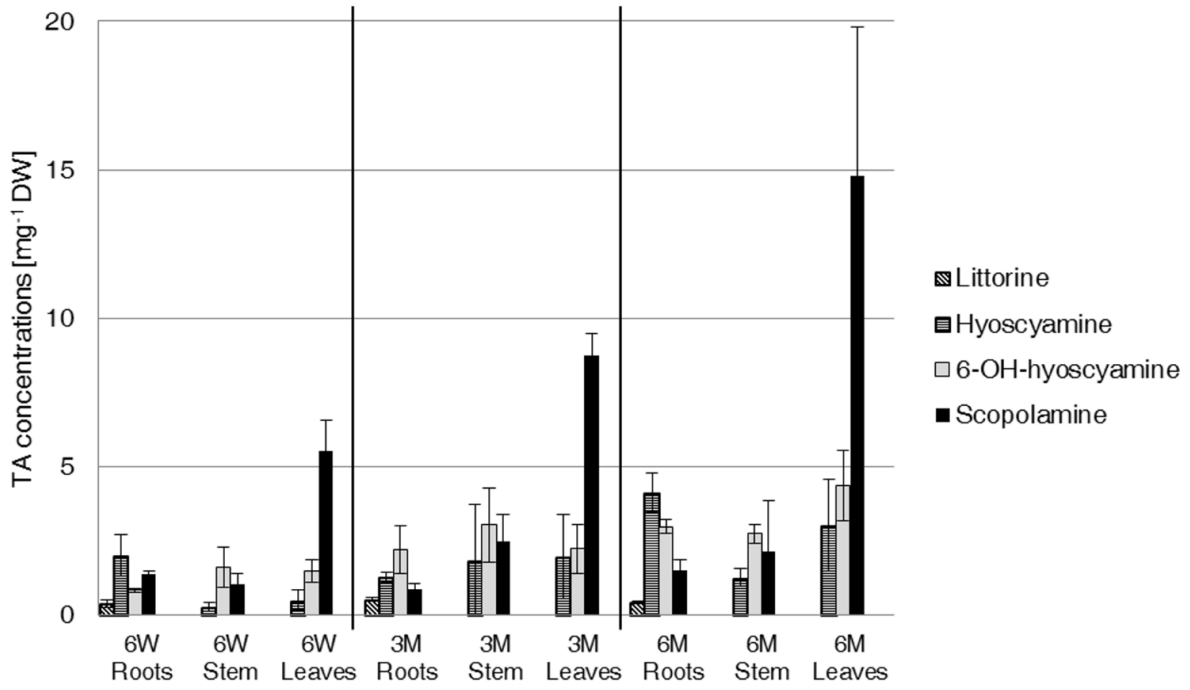

Figure 3-1: Tropane alkaloid concentrations in *D. myoporoides* (mg g^{-1} DW). Triplicate LC-MS quantification of the TAs littorine, hyoscyamine, 6-hydroxy hyoscyamine and scopolamine in the plant organs roots, stem and leaves at three different developmental stages (6W=6 weeks, 3M=3 months and 6M=6 months).

Littorine, an early precursor in the pathway, was only found in root tissue regardless of the growth stage of the plant (0.38–0.54mg g^{-1} DW). The ratio of the remaining TAs hyoscyamine, 6-hydroxy hyoscyamine and scopolamine in roots varied over time, with the total remaining low (4.22– 8.57mg g^{-1} DW). The stem contained hyoscyamine, 6-hydroxy hyoscyamine and scopolamine, which were found in an equal ratio during plant development (6 weeks–6 months): hyoscyamine was the lowest in abundance of all TAs (0.26–1.83 mg g^{-1} DW), followed by scopolamine (1.03–2.46 mg g^{-1} DW), and 6- hydroxy hyoscyamine was the most abundant TA in stem tissue (1.59–3.02 mg g^{-1} DW). Although certain trends were observed in the abundance of TAs in these tissues, no statistically significant changes were found. In the leaf tissue, scopolamine increased in abundance significantly during the growth phase from 5.5 mg g^{-1} DW in young plants (6-weeks-old) to 14.8 mg g^{-1} DW in mature plants (6-months-old) ($p=0.015$).” (Kohnen et al., 2017) Besides the significant increase of the scopolamine concentration during plant development, divergence in the measurement values increases. The main reason for this reveal in the biological samples themselves. In order to make the results as comparable as possible, vegetative propagated plant

material of *D. myoporoides*, which accordingly the same genetic information, was used. In addition, the growing conditions were standardized and controlled in order to obtain more valid results. Nonetheless, different growth and shading through their own leaves as well as thereof resulting different TA concentration which occur between the individual plants, have an influence on the measurement and the concerning bias. Another source of bias is the difficult sampling of larger plants. An attempt is made to draw a representative pattern of the respective plant, which is tricky with biological material such as plants. Young and intermediate plants were harvested completely. The plant material was divided into three parts, which were used for the RNA isolation, MALDI-MSI experiments as well as LC-MS quantitation, respectively. Representative samples of the matured plant were taken. The measured TA concentration depends greatly on the sampling of the biological plant material; hence this may result in a larger bias. To minimize this factor, sampling was performed according the protocol but nevertheless, the differences in the TA concentrations were highest in matured plants.

“All plant extracts were examined for scopolamine glucoside, which is purported to be the transported form of scopolamine. No reference substance was available and, hence, the mass of the quasi molecule ion was calculated ($[M+H]^+$; *m/z* 466.21), and abundance was verified via MS/MS in roots, stems and leaves. Root and stem extracts contained only traces of scopolamine glucoside. The highest amount of scopolamine glucoside was detected in leaf extracts; but in comparison with scopolamine the amount was approximately 150- fold lower.

3.1.2. Localization of tropane alkaloids in different organs of *D. myoporoides* during plant development

Duboisia myoporoides R.BR. plant organs were analyzed with the objective of localizing TAs within the plant to elucidate the location of biosynthesis, transport throughout development, and accumulation of scopolamine as wells as its precursors. To investigate localization of TAs in *D. myoporoides*, we studied tissue sections of the different plant organs; roots, stem tissue and leaves, using MALDI-MSI. Samples were chosen according to LC-MS analyses of plants at different ages (6 weeks, 3 months and 6 months). The advantage of MALDI-MSI, specifically the determination of different analytes within thin tissue sections through direct analysis in a single measurement, was used here to detect hyoscyamine aldehyde, hyoscyamine / littorine, 6-hydroxy hyoscyamine, scopolamine and scopolamine glucoside simultaneously in the same tissue section. The TAs hyoscyamine aldehyde, littorine/hyoscyamine, 6-hydroxy hyoscyamine and scopolamine were detected in all plant organs during plant development. No individual determination of the abundance of isomers for littorine and hyoscyamine by MALDI-MSI was possible as these isomers exhibit the same mass-to-charge ratio. As depicted in the quantitative data (Figure 3-1), in the stem and leaves, only hyoscyamine was present. In the roots, hyoscyamine and littorine were present simultaneously and, hence, the MALDI-MSI signals could not be assigned to hyoscyamine or littorine. Figure 3-2 depicts the qualitative distribution of TAs in the roots over time.

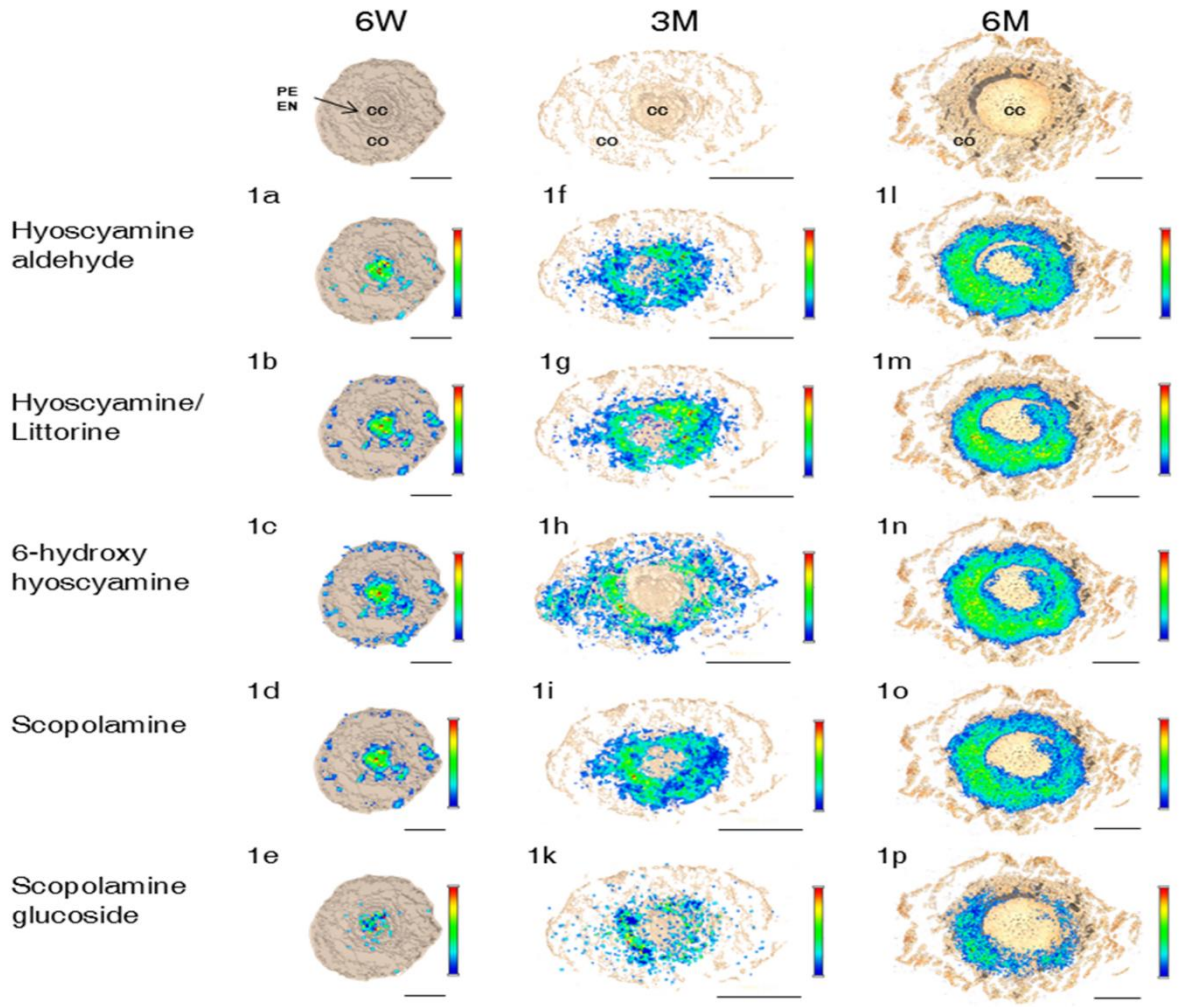

Figure 3-2*:* Ion images showing the localization and spatial distribution of tropane alkaloids in *D. myoporoides* roots tissue, cross-section (1a–1p). Localization of hyoscyamine aldehyde ($[M+H]^+$, *m/z* 288.1594), littorine/hyoscyamine ($[M+H]^+$, m/z 290.1750), 6-hydroxy hyoscyamine ($[M+H]^+$, *m/z* 306.1699), scopolamine ($[M+H]^+$, *m/z* 304.1543) and scopolamine glucoside ($[M+H]^+$, *m/z* 466.2071) at three different developmental stages (6W=6 weeks, 3M=3 months and 6M=6 months). MSI data were measured within the mass range *m/z*=90–700. Signal intensities are visualized by a color-coding system, starting from low intensity (blue) to high intensity (red). Scale bar=1mm. PE, pericycle; EN, endodermis; CC, central cylinder; CO, cortex.

Figure 3-2, 1a–e, depicts the young root tissue at the age of 6 weeks, 1f–k the root tissue at the age of 3 months and 1l–p the mature roots (6 months). In the young root, TAs were found in the inner cortex and central cylinder. The young roots at the age of 6 weeks did not undergo secondary growth; the pericycle and endodermis were close together. These tissues are purported to contain TA biosynthetic genes (enzymes) and xylem is assumed to be responsible for the TA transport. In these young roots, this spatial proximity and the small size of the central cylinder, especially the xylem, may

have contributed to the observation that all investigated TAs were found in the center of the root. After the root underwent secondary growth, the localization of the TAs was altered (Figure 3-2, 1f–p). Associated with secondary growth, the xylem as well as the central cylinder expanded, and the distribution of TAs changed to the outer part of the vascular tissue as well as the inner part of the cortex. No TAs were found in the inner central cylinder at this period in the plant's life cycle. Scopolamine glucoside signals were observed to be of lower abundance (Figure 3-2, 1e, 1k, 1p) than the other alkaloids.

Figure 3-3 and Figure 3-4 depict longitudinal and cross-sections of the stem. The highest signal intensities of all TAs were located in the outer cortex and epidermis, and within the pith; weak signal intensity was observed from the xylem. The precursors hyoscyamine aldehyde Figure 3-3, 2aFigure 3-4, 3a) and hyoscyamine Figure 3-3, 2b; Figure 3-4, 3b) were found distributed in the pith area adjacent to the xylem in young plants. In mature plants, the distribution shifted towards a predominant occurrence of hyoscyamine aldehyde Figure 3-3, 2l; Figure 3-4, 3l) and hyoscyamine Figure 3-3, 2m, Figure 3-4, 3m) in the outer cortex and epidermis. 6-Hydroxy hyoscyamine, however, was distributed equally in the outer cortex and epidermis as well as in the pith adjacent to the xylem in young plants Figure 3-3, 2c; Figure 3-4, 3c). In mature plants, the highest signal intensity was detected in the outer cortex and epidermis Figure 3-3, 2n; Figure 3-4, 3n). Scopolamine was predominantly present within the outer cortex Figure 3-3; 2d, 2i, 2o; Figure 3-4; 3d, 3i, 3o) at all ages of the plant. Only a few signals were received that were attributable to its glucoside Figure 3-3, 2e, 2k, 2p; Figure 3-4, 3e, 3k, 3p).

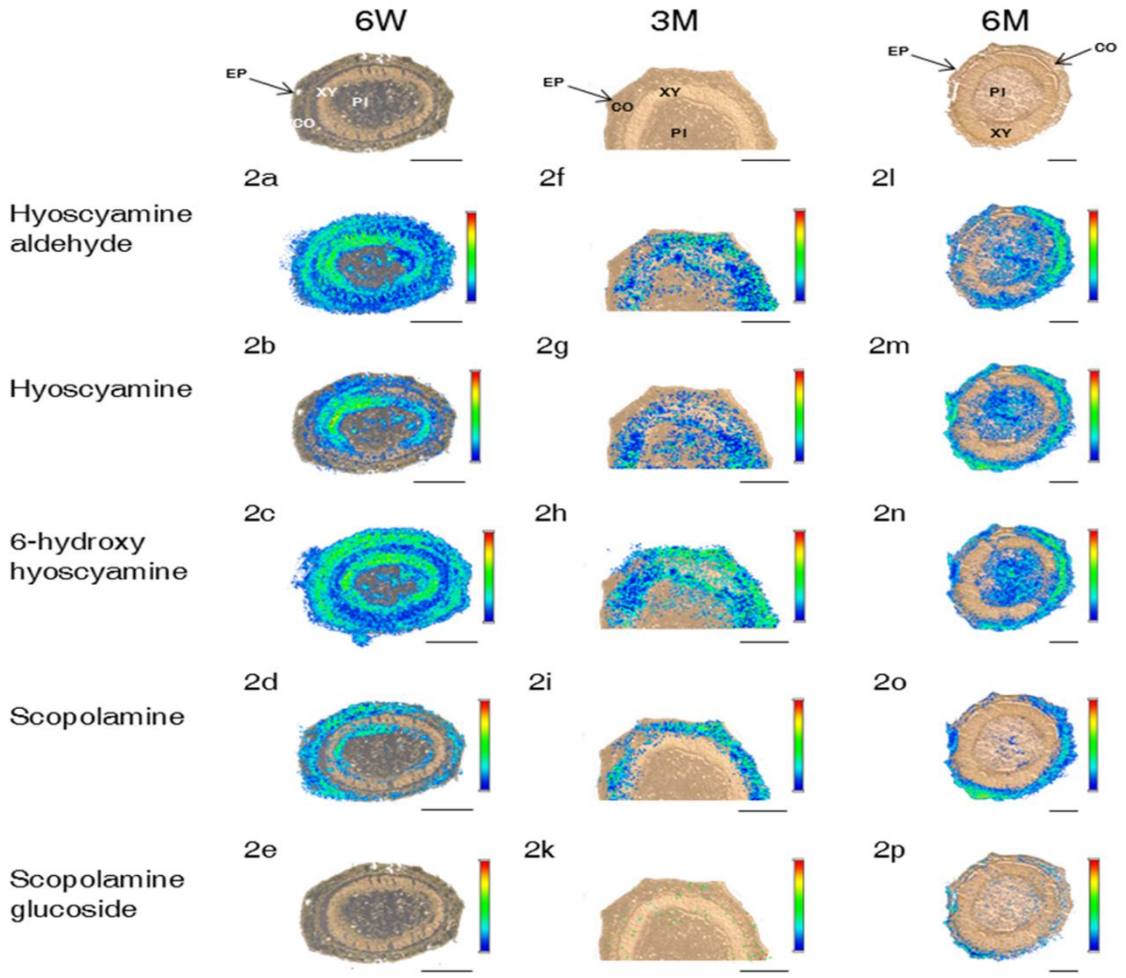

Figure 3-3*:* Ion images showing the localization and spatial distribution of tropane alkaloids in *D. myoporoides* stem tissue, cross-section (2a–2p). Localization of hyoscyamine aldehyde ($[M+H]^+$, *m/z* 288.1594), littorine/hyoscyamine ($[M+H]^+$, *m/z* 290.1750), 6-hydroxy hyoscyamine ($[M+H]^+$, *m/z* 306.1699), scopolamine ($[M+H]^+$, *m/z* 304.1543) and scopolamine glucoside ($[M+H]^+$, *m/z* 466.2071) at three different developmental stages (6W=6 weeks, 3M=3 months and 6M=6 months). MSI data were measured within the mass range *m/z*=90–700. Signal intensities are visualized by a color-coding system, starting from low intensity (blue) to high intensity (red). Scale bar=1mm. EP, epidermis; CO, cortex; XY, xylem; PI, pith.

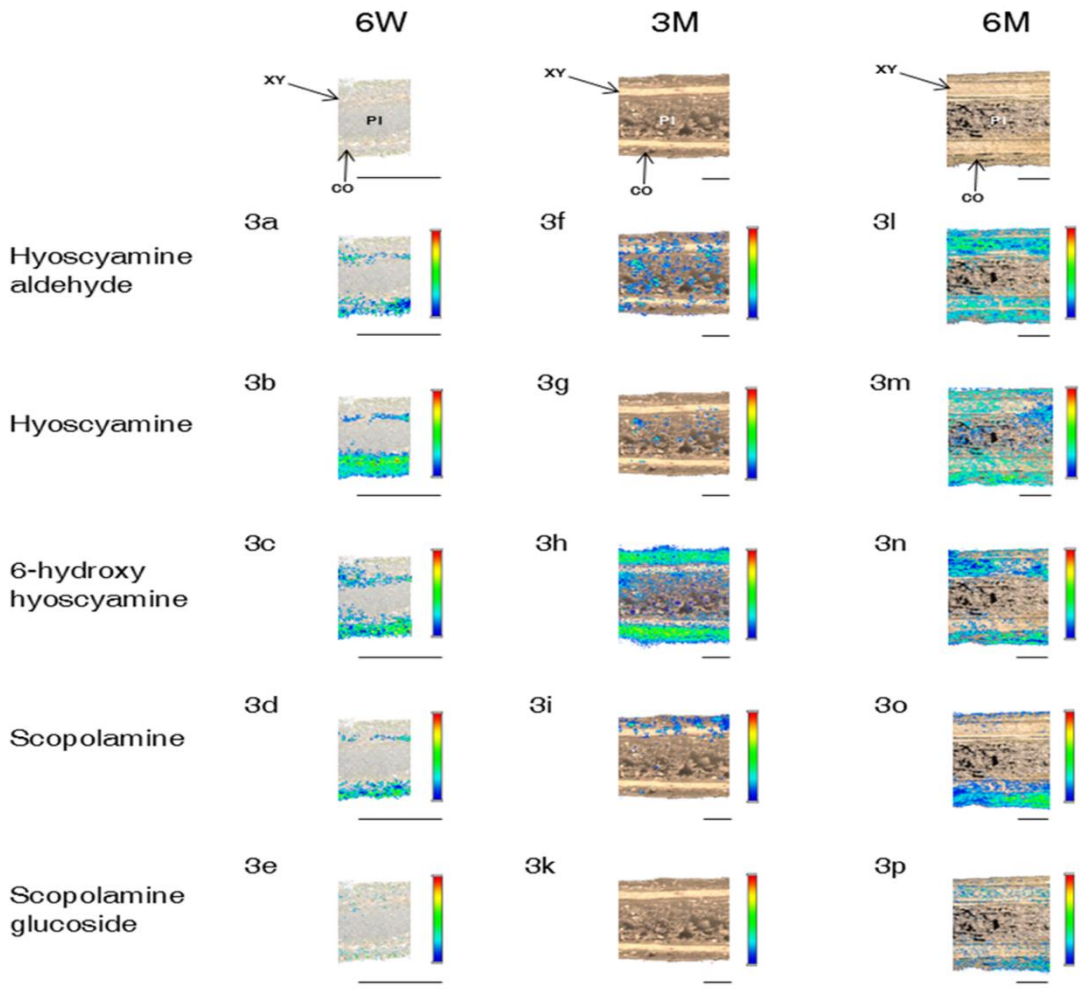

Figure 3-4: Ion images showing the localization and spatial distribution of tropane alkaloids in *D. myoporoides* stem tissue, longitudinal section (3a–3p). Localization of hyoscyamine aldehyde ($[M+H]^+$, *m/z* 288.1594), littorine/hyoscyamine ($[M+H]^+$, *m/z* 290.1750), 6-hydroxy hyoscyamine ($[M+H]^+$, *m/z* 306.1699), scopolamine ($[M+H]^+$, *m/z* 304.1543) and scopolamine glucoside ($[M+H]^+$, *m/z* 466.2071) at three different developmental stages (6W=6weeks, 3M= 3months and 6M=6months). MSI data were measured within the mass range *m/z* = 90–700. Signal intensities are visualized by a color-coding system, starting from low intensity (blue) to high intensity (red). Scale bar=1mm. CO, cortex; XY, xylem; PI, pith.

In Figure 3-5, the spatial distribution of the tropane alkaloids within the leaves over different growth stages is depicted. Scopolamine (Figure 3-5; 4d, 4i, 4o) and scopolamine glucoside (Figure 3-5; 4e, 4k, 4p) were absent in the vascular tissue; however, they were present in the adjacent area of the lamina blade. In the leaves, the precursors in the TA pathway were observed to accumulate in the same distributional pattern as scopolamine and, additionally, were found in the vascular tissue surrounding the cortex.

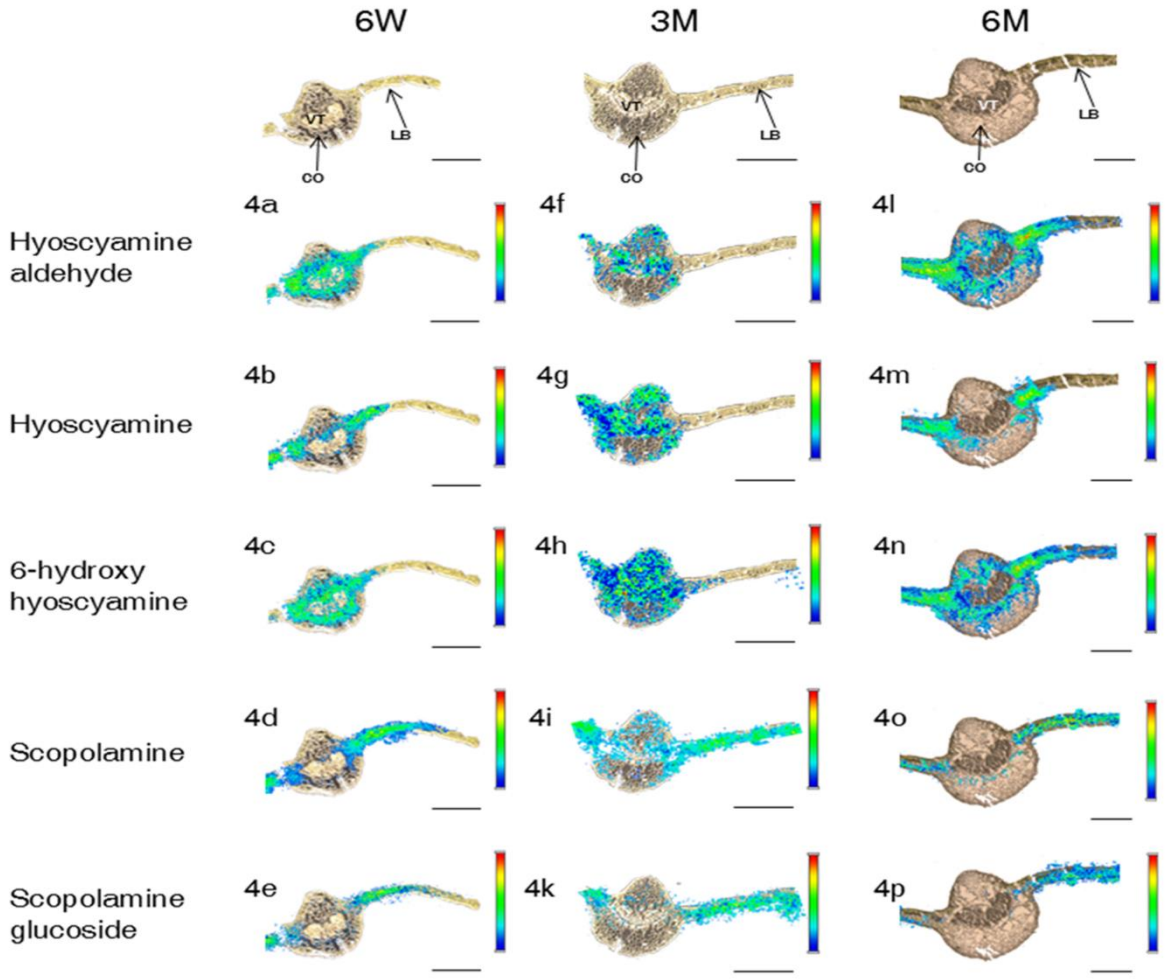

Figure 3-5*:* Ion images showing the localization and spatial distribution of tropane alkaloids in *D. myoporoides* leaf tissue (4a –4p). Localization of hyoscyamine aldehyde ($[M+H]^+$, *m/z* 288.1594), littorine/hyoscyamine ($[M+H]^+$, *m/z* 290.1750), 6-hydroxy hyoscyamine ($[M+H]^+$, *m/z* 306.1699), scopolamine ($[M+H]^+$, m/z 304.1543) and scopolamine glucoside ($[M+H]^+$, *m/z* 466.2071) at three different developmental stages (6W=6 weeks, 3M=3months and 6M=6months). MSI data were measured within the mass range *m/z*=90–700. Signal intensities are visualized by a color-coding system, starting from low intensity (blue) to high intensity (red). Scale bar=1mm. VT, vascular tissue; CO, cortex; LB, leaf blade.

3.1.3. Quantitative analysis of gene expression of different organs of *D. myoporoides* during plant development

By comparing known sequences of other TA-producing Solanaceae plants with our in-house cDNA library, the respective sequences for *D. myoporoides* were identified. The relative gene expression of four gene sequences encoding enzymes involved in the TA pathway, namely *pmt*, *tr-I*, *cyp80f1* and *h6h* (Figure 3-6 – 3-10), was determined by applying quantitative real-time PCR (qPCR).

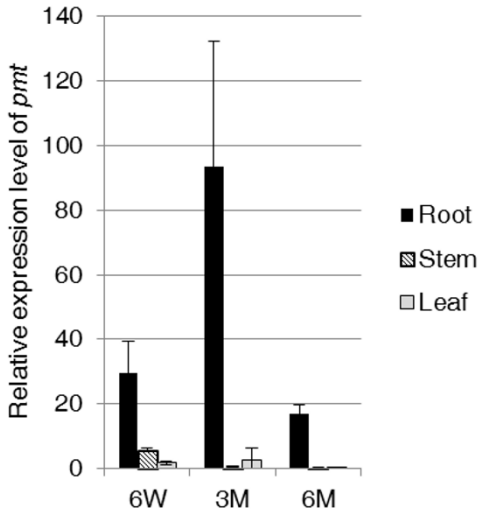

Figure 3-6: Gene expression of *putrescine methyltransferase* from *D. myoporoides* in leaf, stem and root tissue at three different develop- mental stages (6W=6 weeks, 3M=3 months and 6M=6 months). Relative expression level, normalized to *gapdh*, was calculated using the comparative $\Delta\Delta C_t$ method.

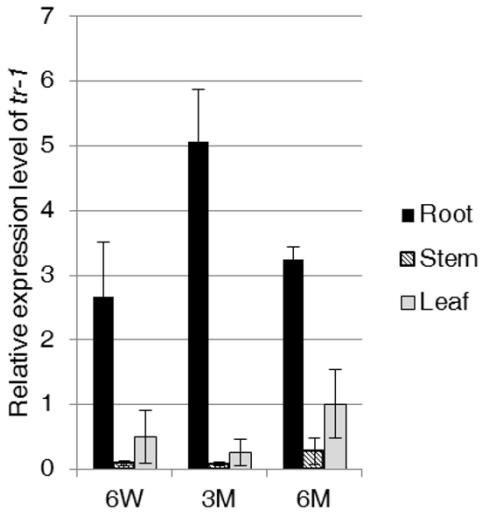

Figure 3-7: Gene expression of *tropinone reductase I* from *D. myoporoides* in leaf, stem and root tissue at three different develop- mental stages (6W=6 weeks, 3M=3 months and 6M=6 months). Relative expression level, normalized to *gapdh*, was calculated using the comparative $\Delta\Delta C_t$ method.

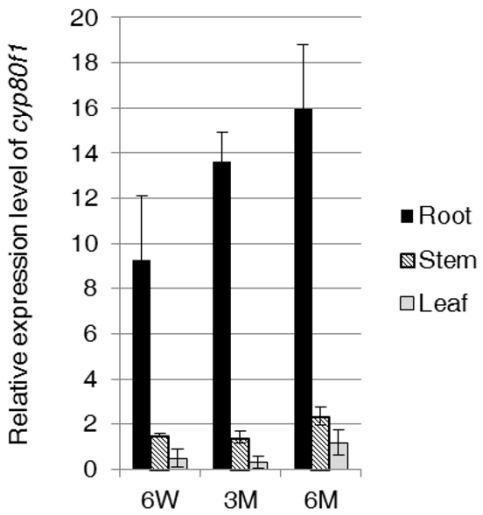

Figure 3-8: Gene expression of *cyp80f1* (*littorine mutase*) from *D. myoporoides* in leaf, stem and root tissue at three different develop- mental stages (6W=6 weeks, 3M=3 months and 6M=6 months). Relative expression level, normalized to *gapdh*, was calculated using the comparative $\Delta\Delta C_t$ method.

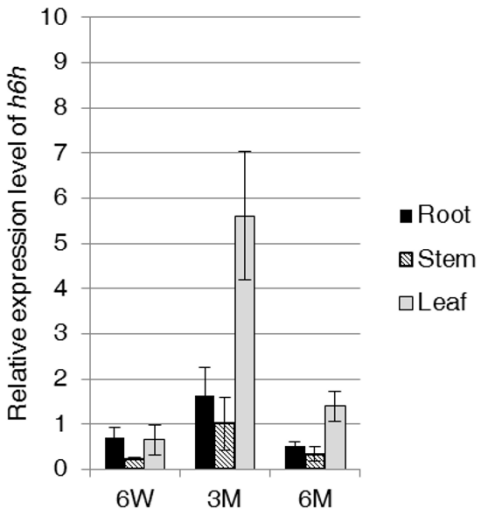

Figure 3-9: Gene expression of *hyoscyamine 6 -hydroxylase* from *D. myoporoides* in leaf, stem and root tissue at three different develop- mental stages (6W=6 weeks, 3M=3 months and 6M=6 months). Relative expression level, normalized to gapdh, was calculated using the comparative $\Delta\Delta C_t$ method.

According to the LC-MS and MALDI-MSI analyses, cDNA of *D. myoporoides* root, stem and leaf tissue of 6-week-, 3-month and 6-month-old plants was studied. Normalization was performed using *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) as an endogenous control. The primer pairs (Table 3-1) used for qPCR were tested, amplicons were separated and the melting curves for each target were determined.

Table 3-1: Primer sequences used for qPCR

Gene	Forward Primer (5` – 3`)	Reverse Primer (5` – 3`)
DmPMT for qPCR	TCCAATCGGTCCTGCTAAA	ACAACTCCTCCTGGTCTAAG
DmTR1 for qPCR	ATGGAGAAGACGGAGGAAGATG	TGCCTTTAGAGCCACCAGTAAC
DmCYP80F1 for qPCR	AAAGACTTCGGGATGAAATCTC	ACAACTCCTCCTGGTCTAAG
DmH6H for qPCR	CTTCTCAGCCAAGACAATCC	ACCAGCATCATAGCCTGAC
DmGAPDH for qPCR	AAGGAGGAATCAGAGGGTAAG	GACCTGCTATCACCAACAAAG

The gene expression of *pmt*, *tr-I*, *cyp80f1* and *h6h* demonstrated differences at the transcriptional level between the different organs - leaves, stem tissue and roots- during plant growth. The transcript levels of *pmt* (Figure 3-6) and *tr-I* (Figure 3-8) were highest in the roots and varied in a time-dependent manner. In the roots of 3-month-old plants, the transcript level of *pmt* was almost 3- to 4-fold higher compared with 6- week-old and 6-month-old plants. The transcript level of *tr-I* in the roots of 3-month-old plants was almost doubled compared with 6-week-old and 6-month-old plants. Leaf and stem tissue were observed to have significantly lower expression levels of these genes (*pmt*, $p = 0.0149$; *tr-I*, $p = 0.0182$). The expression level of *cyp80f1* (Figure 3-8) was highest in the roots and was increased 1.2-fold between young and intermediate and between intermediate and mature plants. Stem and roots had 7- to 10-fold lower expression of this gene (statistically significant; $p = 0.0096$). The expression level of *h6h* (Figure 3-9) was highest in leaves of 3-month-old plants. At this time point, a 3.5-fold higher expression level of *h6h* was detected in the leaves compared with the roots. In 6-week-old plants, comparable expression levels were observed for this gene in root and leaf tissue. Six-month-old plants exhibited a slightly higher expression level of *h6h* in the leaf tissue than in the root tissue. The expression level of *h6h* was lowest in all stem tissue samples." (Kohnen et al., 2017)

3.1.4. Discussion

"Consistent with other TA-producing plants such as *Atropa* spp., *Datura* spp. or *Hyoscyamus* spp. (Ziegler and Facchini, 2008), we confirmed that in *D. myoporoides* TAs were biosynthesized in roots and subsequently accumulated in leaves. The alkaloid pattern observed in root tissue (Figure 3-1) exhibited slightly higher hyoscyamine and 6-hydroxy hyoscyamine concentrations in 6-month-old plants compared with 6-week-old plants. The amount of littorine and scopolamine in the roots remained constant during plant development. This implied a marginal enrichment of the last precursors but no accumulation of scopolamine in the roots. Figure 3-2 depicts differences regarding the spatial distribution of TAs in young roots and roots after secondary growth. As reported before, the genes *pmt* (Suzuki et al., 1999) and *h6h* (Kanegae et al., 1994) were found tissue specifically in the pericycle, and the enzyme TR-I (Nakajima and Hashimoto, 1999) was located in the endodermis and cortex. This tissue-specific localization of the metabolites concerned could not be detected due to the limits of resolution of the MALDI-MSI, but differences between 6-week-old roots and roots with secondary growth (3 and 6 months old) were observed. In young roots, the endodermis, pericycle and the not yet fully developed central cylinder were close together. This spatial proximity and, potentially, short diffusion paths may have led to an equal distribution of all investigated TAs in these three tissues. Associated with secondary growth of the roots, the structure of the root changed, and the xylem grew. This led to an altered spatial localization of the investigated TAs. In intermediate and matured roots, the investigated TAs were found in the outer part of the central cylinder and the inner part of the cortex, but not in the innermost part of the vascular tissue. The stem tissue was investigated in consideration of the possible transportation of TAs. Quantitative data show the absence of littorine in the stem tissue. The concentration of the other three TAs remained unaltered during plant development (Figure 3-1). The alkaloid localization, however, changed from 6-week-old to 6-month-old plants. MALDI-MSI data showed a predominant accumulation of TAs in the pith adjacent to the xylem in 6-week-old plants; this pattern shifted towards a predominant distribution of TAs in the outer cortex and epidermis in 6-month-old plants (Figure 3-3 and Figure 3-4). Transportation of the TAs probably occurs through the xylem. The results at first

glance may be contradictory to the idea that TAs are transported through the xylem, as no TAs were found in the xylem of roots with secondary growth (Figure 3-2) or stem tissue (Figure 3-3 and Figure 3-4). Transportation of the alkaloids is a slow process, and the 20 mm thin tissue section is only a snap- shot of the entire stem length. The detection of high amounts of TAs in the tissue through which the transportation takes place was unlikely. Wink (1998) and Pakdeechanuan et al. (2012) proposed that the xylem is the transportation tissue for other alkaloid-producing plants. Here, neither quantitative LC-MS nor MALDI-MSI data revealed which particular alkaloids were transported from the site of biosynthesis to the aerial parts; however, scopolamine glucoside can be excluded as a specific transport form due to its low abundance in comparison with the other alkaloids.

In the leaves, the plant organ where TAs and especially scopolamine are accumulated, significant differences regarding alkaloid distribution during plant development were observed (Figure 3-1). Contrary to Xia et al. (2016), who reported that 6-hydroxy hyoscyamine concentration was higher in underground than in aerial parts of *A. belladonna*, our data show an equal distribution of 6-hydroxy hyoscyamine in the different plant organs of *D. myoporoides*. This may indicate the genus-specific differences of the accumulation of TAs in plants. As presented in Figure 3-5, TAs were distributed from the vascular tissue to the leaf tips. Due to higher concentrations in leaf blades than in the vascular tissue, simple diffusion of TAs from the xylem to the leaf tips seems to be unlikely. Although no TA-specific transporter mechanism has been reported, we postulate that there may be an active transport of these molecules by a yet unknown mechanism. Alkaloid transporter systems are poorly investigated to date. The few available studies deal with the nicotine alkaloid transporter system and its respective processes (Shoji et al. 2008; Shitan et al. 2009; Pakdeechanuan, et al. 2012).

The investigation of relative expression for TA-related genes presented here increases our understanding of organ- and time- dependent development of TA biosynthesis. We were interested in the changes of the expression patterns that the four investigated enzymes undergo in various organs during plant development. The gene transcripts of *pmt*, *tr-I*, *cyp80f1* and *h6h* were detected in all plant organs during growth. In agreement with previous reports (Nakajima and Hashimoto, 1999), we

were able to show that *D. myoporoides* constitutively expressed gene transcripts involved in TA biosynthesis. From this we can infer that TAs are important for the plants, probably due to their reported effect against herbivores and vertebrates (Wink, 2010). The enzyme PMT is a junction in the biosynthesis, which connects primary metabolism with the secondary metabolism that yields TAs. The higher gene expression of *pmt* in roots compared with leaves implies that the methylation of putrescine could probably be catalyzed in the roots. The amount of *tr-I* transcripts was highest in the roots during early plant development (6- week- to 3-month-old plants) and equally distributed among the three organs of mature plants, indicating that, at least in young plants, the reduction of tropinone to tropine was performed in roots. As the *cyp80f1* transcript was approximately 40-fold higher in the roots than in the leaves, regardless of plant age, it can be assumed that this reaction also takes place in the roots throughout the plant's life cycle. The last gene involved in TA biosynthesis, *h6h*, showed a different expression pattern. In 6-week- and 6-month-old plants, equal amounts of transcripts were detected in the roots and leaves, whereas the amount of *h6h* was 3.4-fold higher in leaves of 3-month-old plants than in roots. Previous studies reported that no active H6H was found with the exception of in the roots of *D. myoporoides* (Hashimoto et al., 1991) and *Datura metel* L. (Pramod et al., 2010b).Hashimoto et al. did not investigate the respective gene transcript and, therefore, no data regarding the presence or concentration of *h6h* in leaves are available. Our data, however, do not determine whether the presence of *h6h* transcript in leaves leads to an active H6H. Quantification of alkaloids in leaves shows that hyoscyamine and 6-hydroxy hyoscyamine, two substrates of H6H, were present in this organ. Based on our data, no final answer can be given regarding the expression and activity of the enzyme encoded by *h6h*. Further investigation on proteome level and activity testing of H6H is recommended to address the question of whether H6H is functionally expressed and active in the leaves as well." (Kohnen et al., 2017)

3.2. IDENTIFICATION AND ISOLATION OF THE LITTORINE SYNTHASE IN *DUBOISIA MYOPOROIDES* R.BR.

3.2.1. Putative candidate littorine synthase gene isolation and phylogenetic analysis

Screening of an in-house transcriptome database made from *D. myoporoides* three-month old leaf tissues for BAHD family motifs HXXXDG and DFGWG yielded 22 sequence hits. It is assumed that the cocaine synthase belongs to clade III of the BAHD super family and a hypothetical littorine synthase should share a similar mechanism to the cocaine synthase, therefore, these 22 sequences were analyzed in a phylogenetic tree for those belonging to this superfamily. Phylogenetic analysis were performed using MEGA7 and used for comparing all known BAHD sequences (D'Auria, 2006; Eudes et al., 2016) with our 22 sequences. Six of these sequences were found to group in clade III of the BAHD superfamily: contig#5915, contig#50965, contig#168348, contig#176922, contig#178702, and contig#191374.

Figure 3-10 shows the classification of the BADH family into the five major clades. Enzymes of clade I are involved in the modification of phenolic glucosides, predominantly anthocyanins. Only two members of clade II are known, the Glossy2 from *Zea mays* and CER2 from *Arabidopsis thaliana*, which are involved in the extension of long-chain epicuticular waxes. The members of clade III like the cocaine synthase and the putative littorine synthase accept a vast range of alcohol substrates and utilize acetyl-CoA as the major acyl donor. The only member of clade IV is the agmatine coumaroyl transferase (ACT). Clade V consists of two major subgroups: enzymes of the first (group a) catalyze the biosynthesis of volatile esters; the second (group b) are involved in the production of the compound paclitaxel (D'Auria, 2006; Eudes et al., 2016; Suzuki et al., 2007). Figure 3-11 shows clade III in more detail.

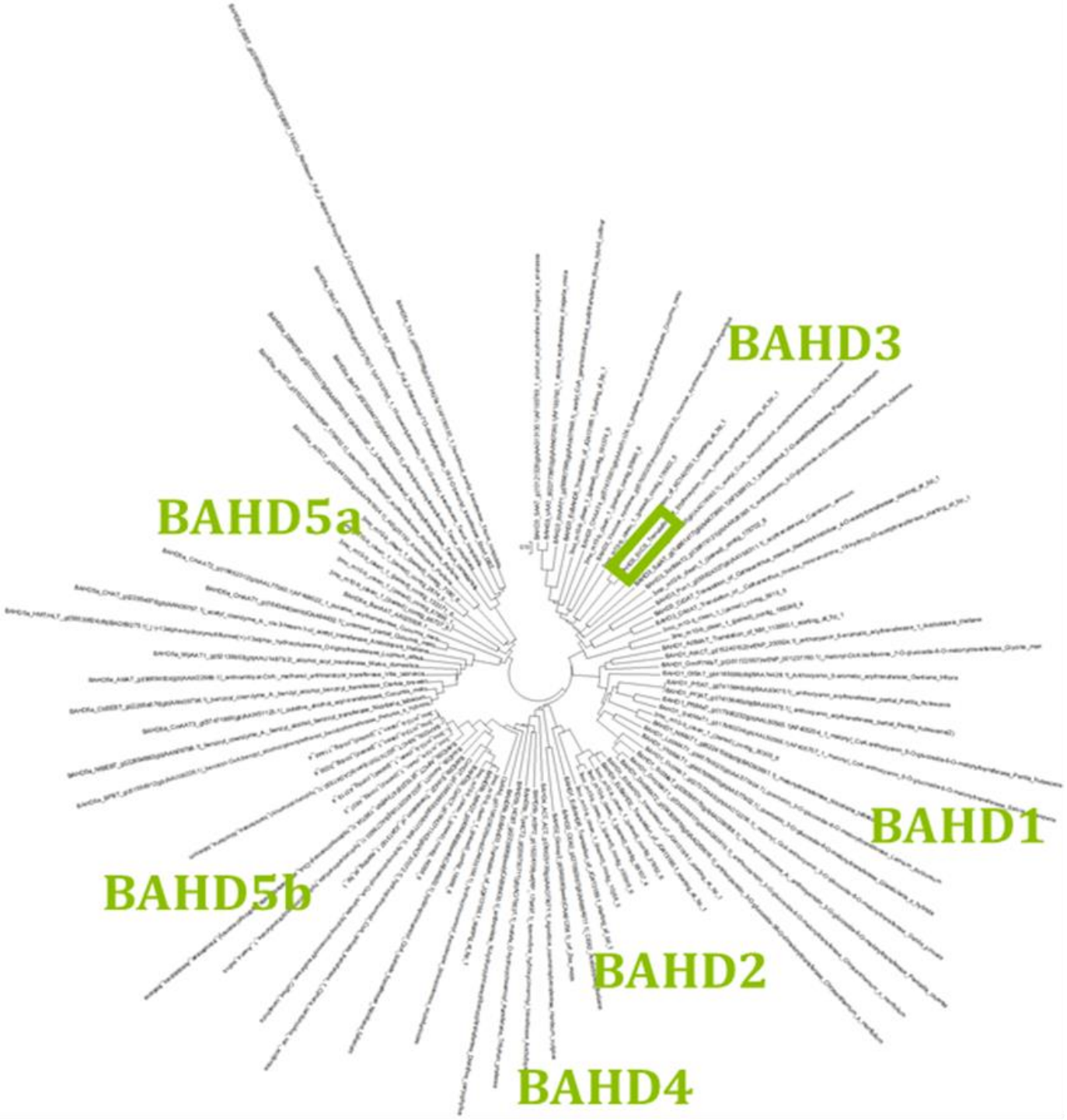

Figure 3-10: Evolutionary relationships of members of the BAHD family. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerkandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. The analysis involved the amino acid sequences of all known members of the BAHD family as well as the 22 putative sequences of *Duboisia* BAHD family members. Evolutionary analyses were conducted in MEGA7. Green box: cocaine synthase.

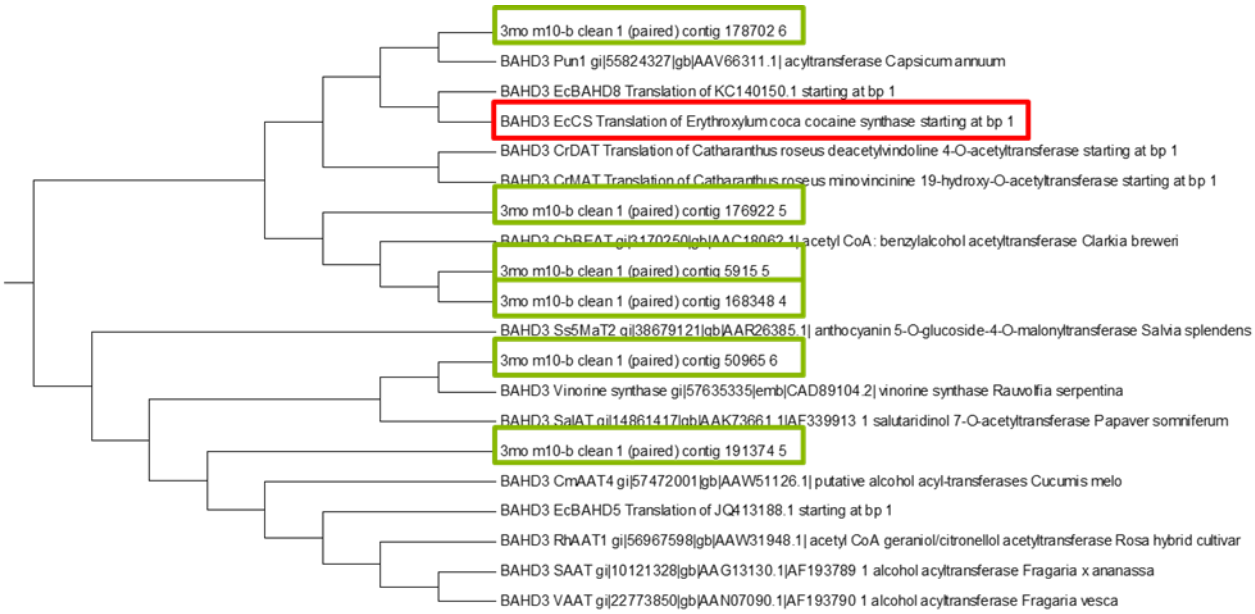

Figure 3-11: Enlargement of Clade III of the BAHD family showing the relationship between the cocaine synthase (red box) and the putative littorine synthase (green boxes; contig#178702, contig#176922, contig#5915, contig#168348, contig#50965, contig#191374); sample name of the sequenced *D. myoporoides* leaf tissue: 3mo m10-b clean

From these six putative littorine synthases, five exhibit integral open reading frames, whereas one sequence (contig#176922) is incomplete. Neither computational or *in vitro* experiments (3'RACE PCR) were successful in amplifying this gene candidate and it was subsequently omitted from further experiments.

The alignment of the five putative littorine synthase candidate gene and the *E. coca* cocaine synthase is displayed in Figure 3-12.

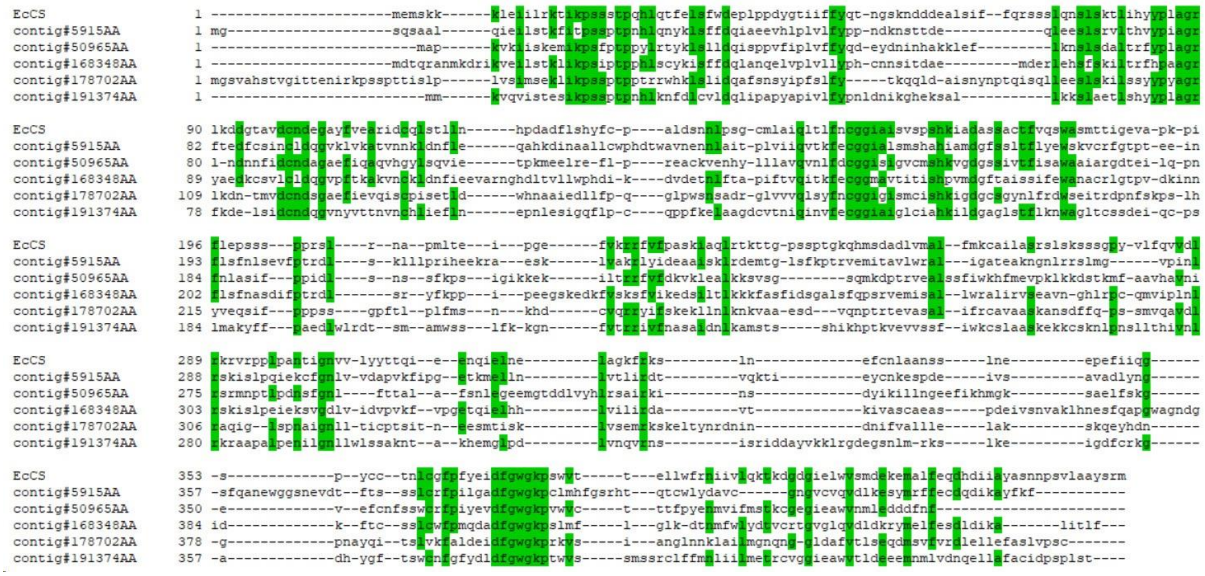

Figure 3-12: Amino acid sequence alignment of EcCS and the five putative candidate gene sequences. The alignment was generated using the software Clone Manager 9 Professional Edition (Sci-Ed Software, Denver, USA). Matching amino acids within the alignment are colored in green.

The amino acid sequences of EcCS and contig#5915 share 27% of their sequences, the sequence alignment of EcCS and contig#50965 resulted in a sequence identity of 31%. EcCS and contig#168348 26%, EcCS and contig#178702 30%, while EcCS and contig#191374 32%. The six sequences are all about approximately the same length (EcCS: 437 amino acids (AA), contig#5915: 442 AA, contig#50965: 413 AA, contig168348: AA 458, contig#178702: 456, contig#91374: 441 AA). All sequences share the two-family specific motifs (see above), but besides that, the homology of the compared sequences is on a low level. This presence of the family specific motifs in combination with the low consistency of homology indicate that the putative candidate gene sequences belong to the BAHD superfamily, but the probability that one of these sequences catalyzes a condensation similar to cocaine condensation is rather low. To verify this hypothesis, the putative candidate genes were expressed heterologously and testes regarding activity.

3.2.2. Heterologous expression of the putative littorine synthase candidate genes in *Escherichia coli*

To test the candidate genes regarding their ability to produce littorine, the concerning proteins were expressed heterologously. The sequences of contig#5915, contig#168348, contig#191374 obtained through cloning were completely identical to the *in silico* determined sequences. The sequences of contig#50965 and contig#178702 show slight differences compared to those used in transcriptomics. The sequence of contig#50965 has a silent mutation and an exchange of leucine to glutamine (L329Q) and one exchange of valine to glutamine (V391L). The sequence of contig#178702 exhibit threonine instead of serine (S1119T) and shows a further silent mutation. Leaf cDNA of different *D. myoporoides* plants was tested and these exchanges were found in all samples, therefore, they were used for heterologous protein expression in *E. coli*.

Recombinant expression of these five candidates was conducted in *E. coli* BL21(DE3). Plasmids for expression of candidate genes were generated as described in the Material and Methods section. The induction of expression was determined for pET32a_contig#163848 using either 1mM lactose or isopropyl β-D-1-thiogalactopyranoside (IPTG) and compared to non-induced conditions to determine promoter leakage and degree of inducibility. IPTG was determined to have the best induction effect for this construct and was subsequently used for all further expression induction experiments (Figure 3-13).

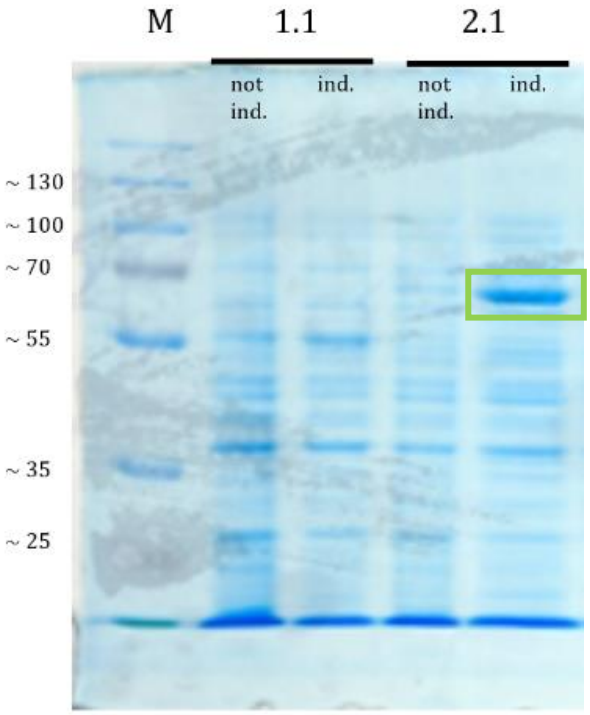

Figure 3-13: Coomassie stained SDS gel showing the different induction conditions. Culture 1.1, empty vector pET32a; Culture 2.1, vector construct (pET32a) carrying insert contig#168438. ind: induced, not ind.: not induced. M, marker, size in kDa; Induction by 1mM IPTG. Green box: Overexpressed protein of pET32a_contig#168438, induced by IPTG.

The expression of each target protein was low and consequently difficult to analyze by SDS PAGE and Coomassie staining (

Figure 3-14). Western Blot and immunodetection of each target protein were performed using the 6x histidine (His) tag on the C-terminus of each expressed protein using anti-His antibodies.

Figure 3-15 depicts the Western blot and immunodetection signals obtained from each of the five constructs expressed in *E. coli.* Each of the putative littorine synthase candidate genes were able to be expressed and could be detected due to their His tag, although only very weak in candidate gene contig#5915.

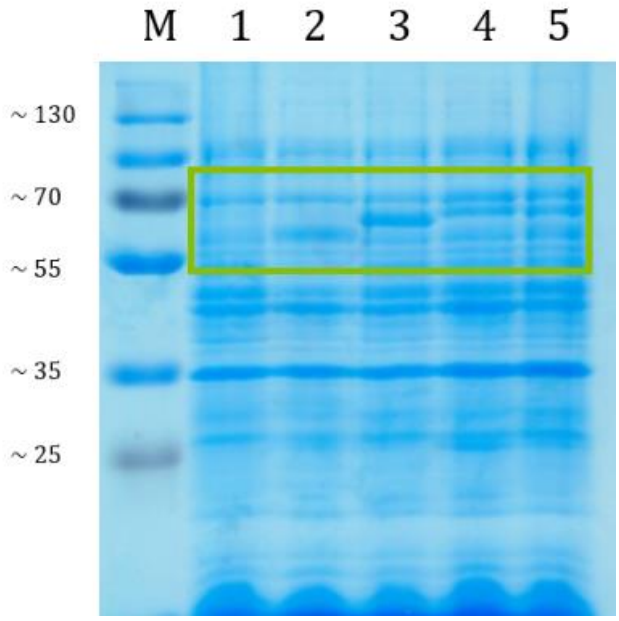

Figure 3-14: Coomassie stained SDS gel showing the expression of the different candidate genes: M, marker, size in kDa; line 1, contig#5915; line 2, contig#50965; line 3, contig#168348; line 4, #178702; line 5, contig#191374. Induction by 1mM IPTG. Green box: overexpressed enzymes of each contig.

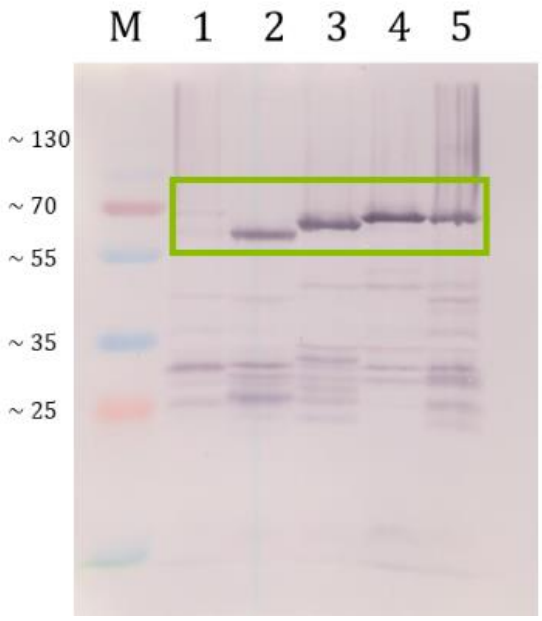

Figure 3-15: Western Blot of the concerning SDS gel (
Figure 3-14). M, marker, size in kDa; line 1, contig#5915, 66 kDa; line 2, contig#50965, 64 kDa; line 3, contig #168348, 68 kDa; line 4, contig #178702, 67 kDa; line 5, contig #191374, 66 kDa. Green box: overexpressed enzymes of each contig.

3.2.3. Littorine synthase activity testing

In order to test the activity of each littorine synthase candidate gene, we assumed that tropine is condensed with activated phenyllactate to form littorine. It is likely that the activated chemical form of phenyllactate is an CoA-derivate, phenyllactic acid-CoA. This compound is not commercially available, therefore, we synthesized it according to the procedure described by Stöckigt & Zenk (1975; Supplementary Data). After cell lysis, cell debris were removed by centrifugation and assay buffer was added to the crude cell soluble extracts. In addition, the educts tropine and phenyllactic acid-CoA were also added and samples were taken at 1.5 and 4 hours after incubation for analysis. No littorine was detected in these assays (compare Figure A-3 - Figure A-8), therefore, no conversion from tropine and phenyllactic acid-CoA to littorine was achieved.

3.2.4. Discussion

The formation of littorine is an important step in the biosynthetic pathway of Solanaceae TAs. The final goal, the production of TAs in heterologous host, is only feasible if all involved enzymes are known and their respective activities characterized. This research gives first indications of the identity of genes which could be potentially responsible for this step. The screening of our internal *D. myoporoides* transcriptomic database successfully revealed 22 putative sequences of enzymes sharing BAHD motifs. Phylogenetic alignment and analysis of all known BAHD family members (D'Auria et al., 2007; Eudes et al., 2016) with our 22 putative *D. myoporoides* sequences indicated six possible candidate genes which could function as littorine synthases. We assume that the littorine synthase belongs into clade III of the BAHD family as does the cocaine synthase. Only five of this six candidate genes exhibit complete open reading frames. All approaches to determine the missing base pairs of this sixth candidate were unsuccessful and it may be an artefact of the transcriptomics database. It may also be that this specific target gene was not successfully amplified from the cDNA library due to target shearing during RNA isolation, this gene was not expressed during the phase used for RNA isolation, or cDNA synthesis failed to amplify this target for an unknown reason. Nevertheless, it was not possible to isolate target 6 and only 5 targets were used for expression analysis here.

The *in silico* determined candidate gene sequences were isolated from *D. myoporoides* cDNA of three months old leaves. Three candidate gene sequences were identical to transcriptomic predictions (contig#5915, contig#168348, contig#191374), while two (contig#50965, contig#191374) exhibited slight differences. The L329Q exchange of contig#50965 may have an effect due to the polarity of the amino acids – from nonpolar to polar while it is unlikely that the V391L exchange has an effect as these are similar amino acids. The S119T of contig#178702 likely does not have any effect on proposed protein activity due to the similarity of the amino acids. As leaf cDNA of different *D. myoporoides* plants were tested and the exchanges were found in all samples and the plants were the same for the generation of transcriptomic data, these substitutions are likely the native plant sequences and the variances were artefacts in the construction of the transcriptomic library. It may also be that there are different isoforms of these contigs *in planta*, for example, *D. myoporoides* also has various

isoforms of the enzyme H6H. Besides these differences between the isoforms, the homology between the cocaine synthase and the putative littorine candidate gene is rather low. This may indicate, that the *in silico* determined sequences belong to the BAHD family but are not closely related to the cocaine synthase. The phylogenetic analysis displays the closest relationship between these sequences and the cocaine synthase, so these sequences were tested.

To receive more information, the candidate gene sequences were heterologously overexpressed in *E. coli* to generate protein for enzyme activity assays. Induction by IPTG at a lower-than-optimal temperature for *E. coli* (18 ˚C) was determined to exhibit more robust protein expression than induction by lactose. This was tested as previous experiments with other plant enzymes reveal that the induction by lactose may be favorable.

The expression of these 5 enzymes was determined to be weak even with these conditions which required Western Blot and immunodetection for induction confirmation. Of all candidate genes, contig#5915 exhibited the lowest levels of expression in *E. coli*. Both SDS PAGE and Western blots only detect the expression of the enzymes, but no information about the correct folding or about their activities. Activity testing was attempted by incubation of each enzyme with the substrates tropine and phenyllactic acid-CoA. It would be expected that if these enzymes were expressed and correctly folded, incubation of the cell extract with these two compounds should result in littorine formation. In these experiments, no littorine could be detected. There are many potential reasons for this result: the enzymes may be non-functional when expressed in the bacterial host due to lack of co-factors or proper folding, none of the five tested genes are actually littorine synthases, or enzyme titers were not high-enough to generate catalysis in lysate samples. In addition, the chemical synthesis of phenyllactic acid-CoA was adopted from the protocol of Stöckigt & Zenk, (1975) which is designed for hydroxycinnamoyl-CoA derivates. Theoretically, this protocol should also be suitable for the synthesis of phenyllactic acid-CoA esters, however, it was not tested before. After synthesis, the substance was checked by ^{1}H NMR, which does not lead to an unambiguous verification of the correct constitution in this substance. It is therefore not clear if the phenyllactic acid-CoA used here was truly this substance. Further NMR experiments, such as coupled methods like HSQC

(Heteronuclear Single Quantum Correlation)/ HMQC (Heteronuclear Multiple Quantum Correlation) or HMBC (Heteronuclear Multiple Bond Correlation) are required to test the chemical species generated in this procedure. Unfortunately, the amounts generated here were not sufficient to conduct these analyses. An enzymatic approach to the production and the phenyllactic acid-CoA is also conceivable. The enzyme 4-coumarate-CoA ligase (4-CL), the last reaction of the general phenylpropanoid pathway, catalyzes ATP depend the following reaction:

$$\text{4-coumarate} + \text{CoA} \rightleftharpoons \text{4-coumaroyl-CoA}$$

It is described that it can also use other hydroxycinnamic acid derivatives as substrates (Ehlting et al., 2001) and may represent an alternative to chemical synthesis of phenyllactic acid-CoA. Moreover, it is still possible that the missing contig#176922 is the littorine synthase. However, there was no intact ORF in this sequence, so it had to be excluded from further experiments and could later be generated by DNA synthesis for expression.

The six potential candidate genes identified by *in silico* modelling and phylogenetic relationship analysis are a good starting point for further characterization experiments to determine if one of these candidates exhibits littorine synthase activity. For future experiments, increased yields of the recombinant products, synthetic generation of an expression construct of the missing 6th gene, and the confirmation of phenyllactic acid-CoA production for substrate testing will enable this elucidation.

Another possibility, which is the most likely one, is that due to the low degree of similarity between the EcCS and the *in silico* determined sequences, the littorine synthase does not belong to the BAHD family. The similar reaction mechanism firstly raises the assumption that the littorine and the cocaine synthase may be related. The low degree of homology rather excludes this possibility. To elucidate the educts and mechanism of the condensation of tropine and activated phenyllactic acid, the kind of reaction must be determined in more details.

3.3. DISCUSSION

Tropane alkaloids (TAs), especially hyoscyamine and scopolamine, are important precursors for antispasmodic and anticholinergic drugs which are currently in growing demand worldwide. To date, these compounds are obtained at commercial scale from conventional agricultural cultivation of *Duboisia myoporoides* x *D. leichhardtii* crossed hybrids (Luanratana and Griffin, 1980). Climate change and increases in extreme weather events are resulting in unpredictable yields from traditional agriculture. These factors have prompted efforts to generate more sustainable production processes for these important medicinal compounds. In order to generate TAs by alternative processes, their biosynthesis (*in planta*) must first be fully elucidated. The insights gained from the investigations presented in this thesis add to the growing body of knowledge surrounding the biosynthesis of TAs and may assist future efforts for their production.

A key feature of TA biosynthesis *in planta* is the differential locations of their synthesis and storage. TAs are synthesized in the roots and stored in the leaves where they likely perform a protective role for the plant against herbivores (Wink, 2010). This trafficking was shown in TA-producing plants such as *Atropa* spp., *Datura* spp. or *Hyoscyamus* spp. (Ziegler and Facchini, 2008), and it was also found to be true in the scopolamine producing *D. myoporoides* in this thesis. Moreover, the biosynthetic genes *pmt* (Suzuki et al., 1999) and *h6h* (Kanegae et al., 1994) were found specifically in the pericycle, and the enzyme TR-I (Nakajima and Hashimoto, 1999) was located in the endodermis and cortex. This is in agreement with subsequent biosynthetic steps occurring in these different tissues. We could not detect this tissue-specific localization of the TAs due to the limits of resolution of MALDI-MSI experiments performed in this work. However, differences in TA biosynthesis between 6-week-old roots and roots with secondary growth (3- and 6-months-old) were clearly detected. Xylem tissues were observed to grow in tandem with thickening roots which led to an altered spatial localization of TAs *in planta*.

Stem tissue was investigated for possible transport forms of TAs. Quantitative data found an absence of littorine in stem tissue while the concentration of the other three TAs remained unaltered. MALDI-MSI revealed that no TAs were found in the xylem of roots with secondary growth and only to a lesser extent were these observed in the

stem. Nevertheless, we assume that TA transport occurs through the xylem. Although this may seem contradictory to the presented results, the transportation of alkaloids is a slow process and tissue sections used in metabolite imaging can only provide a snap-shot of the actual flow going through this tissue. The detection of high amounts of TAs in xylem tissues was predicted to be unlikely and confirmed in these efforts. Neither quantitative LC-MS nor MALDI-MSI data revealed which particular alkaloids were the predominant transport form, however, scopolamine glucoside can be excluded due to its low abundance in comparison with the other alkaloids.

In the leaves, TAs were distributed from the vascular tissue to the leaf tips. Therefore, passive diffusion of TAs from the xylem to the leaf tips seems to be unlikely due to higher concentrations in leaf blades than in the vascular tissue. We postulate that there may be active transport of these molecules mediated by an as of yet unknown mechanism. To date, alkaloid transporter systems are poorly investigated (Shoji et al. 2008; Shitan et al. 2009; Pakdeechanuan, et al. 2012).

The investigation of the relative gene expression of TA-related genes increases our understanding of organ- and time- dependent development. We observed the expression patterns of four enzymes in various organs during plant development. The gene transcripts of *pmt*, *tr-I*, *cyp80f1*, and *h6h* were detected in all plant organs during growth. This constitutive gene expression shows the importance of TAs for the plants. The last gene involved in TA biosynthesis and most likely a bottleneck in the pathway, the *h6h*, showed an interesting expression pattern. In young as well as mature plants, equal amounts of transcripts were detected in the roots and leaves, whereas the amount of *h6h* was 3.4-fold higher in leaves of intermediate plants than in roots. Previous studies reported that no active H6H was found in the roots (Hashimoto et al., 1991; Pramod et al., 2010a). Our data cannot be used to determine in which extent the presence of *h6h* transcript in leaves leads to an active H6H. Therefore, no final answer can be given regarding whether the expression and activity of the enzyme encoded by *h6h* are correlated. Further investigation on proteome level and activity testing of H6H is recommended to address this question.

After consideration of late biosynthesis in the whole plant, efforts were directed towards the identification of the littorine synthase. The screening of our internal

Duboisia transcriptomic database successfully revealed 22 putative sequences of enzymes sharing the BAHD acyltransferase family motifs DFGWG and HXXXDG. It was postulated that the cocaine synthase, which is a member of the BAHD superfamily, should share a similar mechanism to the yet-identified littorine synthase. Six possible candidate genes of the littorine synthase were identified in this work by phylogenic comparison to the BAHD consensus motifs and the specific clade of the cocaine synthase. Five of these genes could be cloned, while a sixth was not possible due to a lack of complete starting template for amplification. These five genes were expressed in *E. coli* and the enzyme extracts used for *in vitro* enzymatic assays. However, these assays failed to determine whether any of the five could function as the putative littorine synthase *in planta*. Limitations were apparent in this strategy, as the proposed enzyme substrate required for testing was produced here by a modified chemical synthesis procedure used for a closely related molecule. Due to time constraints it was not possible to scale this procedure to generate sufficient titers which could be used to confirm its purity and identity. Expression titers of the target enzymes were also low, and their heterologous expression as well as purification could certainly be improved in future analyses. The sixth sequence may also be interesting to investigate. Although it could not be generated by cDNA synthesis and PCR here, it may be possible to use its *in silico* derived coding sequence to synthetically design a gene for expression in bacteria. It may also be prudent to express these genes in a yeast or algal chloroplast, which may provide more favorable environments for the folding of plant derived enzymes than the bacterial cell. Within this work, the identification of these potential littorine synthase gene candidates provides a platform for future analysis of this currently unknown portion of TA biosynthesis. Other investigations will be able to conduct focused experiments in the characterization of these enzymes and hopefully elucidate this final black box and key bottleneck reaction in the late stages of TA biosynthesis.

CHAPTER 4

CONCLUSION

Parts of this chapter were published in

Kohnen, K. L.; Sezgin, S.; Spiteller, M.; Hagels, H.; Kayser, O. Localization And Organization Of Scopolamine Biosynthesis In *Duboisia myoporoides* R. BR., *Plant Cell Physiol.* 2017, *0*, 1–12, doi:10.1093/pcp/pcx165.

4.1. LOCALIZATION AND ORGANIZATION OF TROPANE ALKALOID BIOSYNTHESIS IN *DUBOISIA MYOPOROIDES* R.BR.

"*Duboisia* hybrids are currently the main source of scopolamine and have been commercially cultivated since the late 1950s (Boffa et al., 1996). Plant-derived TAs are still a major source to produce the compound scopolamine, the most valuable TA for medicinal applications. MSI is an advanced method enabling temporal and spatial profiling of target molecules in various biological samples (Cornett et al., 2007). In our study, we analyzed the time-dependent spatial distribution of TAs in different organs of *D. myoporoides*. In particular, we focused on root tissue, where biosynthesis takes place, stem tissue, the organ responsible for transportation, and leaves, the storage organ. In addition, quantification data supported the MSI data by providing the alkaloid pattern. Furthermore, we established qPCR experiments to determine the transcript levels of *pmt*, *tr-I*, *cyp80f1* and *h6h*. In combining the results of spatial distribution with those regarding the quantity and pattern of the TAs as well as gene expression analyses during plant development, we were able to draw a comprehensive picture showing the organization of the biosynthesis and, in particular, highlighting the time-dependent flow and accumulation of alkaloids from roots to leaves. Our data indicate a low probability that scopolamine glucoside is used as the transport form due to its low occurrence in the stem. Yet, it is obvious that scopolamine and its precursors hyoscyamine and 6-hydroxy hyoscyamine are transported. To determine whether this is a limiting factor, the transportation processes and corresponding proteins must be investigated further. Although scopolamine was observed to be accumulated in leaves over time, no cell compartments have been detected where the alkaloids were stored. Thus, in order to determine which variables and whether the transporter system and storage procedures contribute to an increased, time-dependent accumulation, further studies have to be performed. By investigating the location of TA biosynthesis throughout different plant organs, we were able to elucidate details of the biosynthetic processes leading to TA accumulation in *D. myoporoides*. The results presented in this study may aid improved crop breeding, engineering and cultivation approaches for commercial production of scopolamine." (Kohnen et al., 2017)

4.2. IDENTIFICATION AND ISOLATION OF THE LITTORINE SYNTHASE IN *DUBOISIA MYOPOROIDES* R.BR.

The biosynthetic steps of late stages in TA formation are well described and characterized in many aspects, except for littorine formation. This work makes an important contribution to the identification of the littorine synthase in *D. myoporoides*. Due to the similarity of the condensation reaction, it is assumed that littorine synthase is a cocaine synthase-related enzyme which esterifies 2-carbomethoxy-3β-tropine (methylecgonine) with activated benzoyl- or cinnamoyl-CoA thioester, while the littorine synthase should esterify tropine with phenyllactic acid-CoA. It is assumed that a littorine synthase will share sequence similarities with BAHD family clade III enzymes like the cocaine synthase. Screening of a *D. myoporoides* cDNA library in this work for BAHD acyltransferase motifs and subsequent phylogenetic studies revealed six candidate gene sequences which may be putative littorine synthases. Although the function of these genes could not be determined in this work, the identification of these six candidates is a major step forward to support future characterization trials

4.3. OUTLOOK

Tropane alkaloids (TAs) are interesting secondary plant metabolites which occur in various plant families. The TA scopolamine has a large economic impact due to its numerous pharmacological applications. This work contributes to increasing understanding of the late stages of TA biosynthesis in *Duboisia* plants. Work presented in this thesis has described how TA biosynthesis adapts during plant development with focus on TA biosynthesis in all plant tissues using a global, whole-plant, view through new metabolite spectral imaging techniques. From this study, further questions arise, which in the future can be solved by further analytical methods, such as deep examination of the plant proteome during development. The second section of this thesis sought to identify the elusive littorine synthase. Although no conclusive identification could be generated, the field of possible gene candidates has been significantly narrowed by the identification of a handful of potential genes which may play this role. This investigation will certainly provide a foundation for future studies which should be able to elucidate this enzymatic step in TA biosynthesis. There is a strong desire to produce TA products in heterologous hosts in order to increase space-time yields and process reliability, especially for target compounds such as scopolamine. Further research is required to fully elucidate the last remaining missing pieces of TA biosynthesis and enable metabolic engineering of these products in heterologous hosts.

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Chapter 3 Results and discussion	

References

Agostinelli, E., 2014. Polyamines and transglutaminases: biological, clinical, and biotechnological perspectives. Amino Acids 46, 475–485. https://doi.org/10.1007/s00726-014-1688-0

Barnes, P.J., 2000. The Pharmacological Properties of Tiotropium. Chest 117, 63S-66S. https://doi.org/10.1378/chest.117.2_suppl.63S

Bedewitz, M. a., Gongora-Castillo, E., Uebler, J.B., Gonzales-Vigil, E., Wiegert-Rininger, K.E., Childs, K.L., Hamilton, J.P., Vaillancourt, B., Yeo, Y.-S., Chappell, J., DellaPenna, D., Jones, a. D., Buell, C.R., Barry, C.S., 2014. A Root-Expressed L-Phenylalanine:4-Hydroxyphenylpyruvate Aminotransferase Is Required for Tropane Alkaloid Biosynthesis in *Atropa belladonna.* Plant Cell 26, 3745–3762. https://doi.org/10.1105/tpc.114.130534

Bedewitz, M.A., Jones, A.D., D'Auria, J.C., Barry, C.S., 2018. Tropinone synthesis via an atypical polyketide synthase and P450-mediated cyclization. Nat. Commun. 9, 5281. https://doi.org/10.1038/s41467-018-07671-3

Berger, H., 1931. Archiv Für Psychiatrie Und Nervenkrankheiten. Clin. Neurophysiol. 95–132.

Biastoff, S., Brandt, W., Dräger, B., 2009a. Putrescine *N*-methyltransferase - The start for alkaloids. Phytochemistry 70, 1708–1718. https://doi.org/10.1016/j.phytochem.2009.06.012

Biastoff, S., Reinhardt, N., Reva, V., Brandt, W., Dräger, B., 2009b. Evolution of putrescine *N*-methyltransferase from spermidine synthase demanded alterations in substrate binding. FEBS Lett. 583, 3367–3374. https://doi.org/10.1016/j.febslet.2009.09.043

Boffa, J.-M., Yarnéogo, G., Nikiéma, P., Knudson, D.M., 1996. Domestication and commercialization of non-timber forest products in agroforestry systems, Domestication and crop development of *Duboisia* spp. (Solanaceae). Foo.

Bourgaud, F., Benoit, M., Picardie, U. De, Verne, J., 2013. Développement d ' un nouveau procédé de production d ' actifs pharmaceutiques à partir de plantes médicinales : la technologie des plantes à traire, in: Les Rencontres Du Végétal. p. 2013.

Cao, Y., He, Y., Li, H., Kai, G., Xu, J., Yu, H., 2015. Efficient biosynthesis of rare natural product scopolamine using E . coli cells expressing a S14P / K97A mutant of hyoscyamine 6b -hydroxylase. J. Biotechnol. 211, 123–129. https://doi.org/10.1016/j.jbiotec.2015.07.019

Cardillo, A.B., Otálvaro, A.Á.M., Busto, V.D., Talou, J.R., Velásquez, L.M.E., Giulietti, A.M., 2010. Scopolamine, anisodamine and hyoscyamine production by *Brugmansia candida* hairy root cultures in bioreactors. Process Biochem. 45, 1577–1581. https://doi.org/10.1016/j.procbio.2010.06.002

Cardillo, A.B., Talou, J.R., Giulietti, A.M., 2008. Expression of *Brugmansia candida* Hyoscyamine 6beta-Hydroxylase gene in *Saccharomyces cerevisiae* and its potential use as biocatalyst. Microb. Cell Fact. 7, 17. https://doi.org/10.1186/1475-2859-7-17

Chesters, N.C.J.E., O'hagan, D., Robins, R.J., 1995. The biosynthesis of tropic acid: the (R)-

D-phenyllactyl moiety is processed by the mutase involved in hyoscyamine biosynthesis in *Datura stramonium*. J. Chem. Soc. Chem. Commun. 127. https://doi.org/10.1039/c39950000127

Cornett, D.S., Reyzer, M.L., Chaurand, P., Caprioli, R.M., 2007. MALDI imaging mass spectrometry: molecular snapshots of biochemical systems. Nat. Methods 4, 828–833. https://doi.org/10.1038/nmeth1094

Courdavault, V., 2010. Plant Developmental Biology, Plant Developmental Biology, Methods in Molecular Biology. Humana Press, Totowa, NJ. https://doi.org/10.1007/978-1-60761-765-5

D'Auria, J.C., 2006. Acyltransferases in plants: a good time to be BAHD. Curr. Opin. Plant Biol. 9, 331–340. https://doi.org/10.1016/j.pbi.2006.03.016

D'Auria, J.C., Reichelt, M., Luck, K., Svatoš, A., Gershenzon, J., 2007. Identification and characterization of the BAHD acyltransferase malonyl CoA: Anthocyanidin 5-O-glucoside-6″-O-malonyltransferase (At5MAT) in *Arabidopsis thaliana*. FEBS Lett. 581, 872–878. https://doi.org/10.1016/j.febslet.2007.01.060

Dehghan, E., Häkkinen, S.T., Oksman-Caldentey, K.M., Ahmadi, F.S., 2012. Production of tropane alkaloids in diploid and tetraploid plants and in vitro hairy root cultures of Egyptian henbane (*Hyoscyamus muticus* L.). Plant Cell. Tissue Organ Cult. 110, 35–44. https://doi.org/10.1007/s11240-012-0127-8

Dewick, P.M., 2002. Medicinal Natural Products. Pharm. Sci. 0471496405, 514. https://doi.org/10.1016/j.jbiosc.2010.01.005

Döbereiner, J.W., 1842. Deutsches Apothekerbuch; zum Gebrauche bei Vorlesungen und zum Selbstunterrichte für Apotheker, Droguisten, Aerzte und Medicin-Studirende. Stuttgart, Germany.

Dräger, B., 2003. Chemistry and Biology of calystegines. Nat. Prod. Rep. 211–223.

Dräger, B., 1994. Identification and Quantification of Calystegines , Polyhydroxyl Nortropane Alkaloids 6, 31–37. https://doi.org/10.1002/pca.2800060105

EFSA, 2013. Scientific Opinion on Tropane alkaloids in food and feed. EFSA J. 2013 9, 2406; 1–134. https://doi.org/10.2903/j.efsa.

Ehlting, J., Shin, J.J.K., Douglas, C.J., 2001. Identification of 4-coumarate:coenzyme A ligase (4CL) substrate recognition domains. Plant J. 27, 455–465. https://doi.org/10.1046/j.1365-313X.2001.01122.x

Endo, T., Yamada, Y., 1985. Alkaloid production in cultured roots of three species of Duboisia. Phytochemistry 24, 1233–1236. https://doi.org/10.1016/S0031-9422(00)81107-3

Eudes, A., Mouille, M., Robinson, D.S., Benites, V.T., Wang, G., Roux, L., Tsai, Y.L., Baidoo, E.E.K., Chiu, T.Y., Heazlewood, J.L., Scheller, H. V., Mukhopadhyay, A., Keasling, J.D., Deutsch, S., Loque, D., 2016. Exploiting members of the BAHD acyltransferase family to synthesize multiple hydroxycinnamate and benzoate conjugates in yeast. Microb. Cell Fact. 15, 1–16. https://doi.org/10.1186/s12934-016-0593-5

Falk, L.R., Doran, P.M., 1996. Influence of inoculum morphology on growth of *Atropa belladonna* hairy roots and production of tropane alkaloids. Biotechnol. Lett. 18,

1099–1104. https://doi.org/10.1007/BF00129739

Fischer, C., Kwon, M., Ro, D., Belkum, M.J. Van, Vederas, J.C., 2018. Isolation, expression and biochemical characterization of recombinant hyoscyamine-6β- hydroxylase from *Brugmansia sanguinea* – tuningthe scopolamine production †. Med. Chem. Commun. 9, 888–892. https://doi.org/10.1039/c8md00090e

Freud, S., 1885. Über Coca.

Gadzikowska, M., Grynkiewicz, G., 2002. Tropane alkaloids in pharmaceutical and phytochemical analysis. Acta Pol. Pharm. 59, 149–160.

Gaedecke, F., 1855. Über Erythroyline. Arch. der Pharm.

Geiger, Hesse, 1833. Über das Atropin. Pharm. Cent.

Georgiev, M.I., Agostini, E., Ludwig-Müller, J., Xu, J., 2012. Genetically transformed roots: From plant disease to biotechnological resource. Trends Biotechnol. 30, 528–537. https://doi.org/10.1016/j.tibtech.2012.07.001

Goldmann, A., Message, B., Tepfer, D., Molyneux, R.J., Duclos, O., 1996. Biological Activities of the Nortropane Alkaloid , Calystegine B 2 , and Analogs : Structure - Function Relationships. J. Nat. Prod. 59, 1137–1142.

Griffin, W.J., Lin, G.D., 2000. Chemotaxonomy and geographical distribution of tropane alkaloids. Phytochemistry 53, 623–637. https://doi.org/10.1016/S0031-9422(99)00475-6

Grinspoon, L., Bakalar, J.B., 1981. Coca and cocaine as medicines: An historical review. J. Ethnopharmacol. 3, 149–159. https://doi.org/10.1016/0378-8741(81)90051-9

Grynkiewicz, G., Gadzikowska, M., 2008. Tropane alkaloids as medicinally useful natural products and their synthetic derivatives as new drugs. Pharmacol. Reports 60, 439–463.

Gyermek, L., 2002. Structure-activity relationships among derivatives of dicarboxylic acid esters of tropine. Pharmacol. Ther. 96, 1–21. https://doi.org/10.1016/S0163-7258(02)00296-6

Hardegger, E., Ott, H., 1954. Konfiguration des Cocains und Derivate der Ecgoninsäure. Helv. Chim. Acta 331, 312–320.

Hashimoto, T., Hayashi, A., Amano, Y., Kohno, J., Iwanari, H., Usuda, S., Yamada, Y., 1991. Hyoscyamine 6b-Hydroxylase, an Enzyme Involved in Tropane Alkaloid Biosynthesis, Is Localized at the Pericycle of the Root. J. Biol. Chem. 266, 4648–4653.

Hashimoto, T., Matsuda, J., Yamada, Y., 1993a. Two-step epoxidation of hyoscyamine to scopolamine is catalyzed by bifunctional hyoscyamine-6β-hydroxylase. FEBS Lett. 329, 35–39.

Hashimoto, T., Nakajima, K., Ongena, G., Yasuyuki, Y., 1992. Two Tropinone Reductases with Distinct Stereospecificities from Cultured Roots of *Hyoscyamus niger*. Plant Physiol. 100, 836–845. https://doi.org/10.1104/pp.100.2.836

Hashimoto, T., Yamada, Y., 1987. Purification and characterization of hyoscyamine 6 beta-hydroxylase from root cultures of *Hyoscyamus niger* L. Hydroxylase and

epoxidase activities in the enzyme preparation. Eur. J. Biochem. 164, 277–85.

Hashimoto, T., Yamada, Y., 1985. Hyoscyamine 6b-Hydroxylase, a 2-Oxoglutarate-Dependent Dioxygenase, in Alkaloid Producing Root Cultures. Plant Physiol. 81, 619–625.

Hashimoto, T., Yun, D.J., Yamada, Y., 1993b. Production of tropane alkaloids in genetically engineered root cultures. Phytochemistry 32, 713–718. https://doi.org/10.1016/S0031-9422(00)95159-8

Heinrich, M., Jäger, A.K., 2015. Ethnopharmacology.

Hemscheidt, T., Spenser, I.D., 1992. Biosynthesis of 6.beta.-hydroxytropine in *Datura stramonium*: nonregiospecific incorporation of [1,2-13C2]acetate. J. Am. Chem. Soc. 114, 5472–5473. https://doi.org/10.1021/ja00039a095

Hoye, T.R., Bjorklund, J. a, Koltun, D.O., Renner, M.K., 2000. *N*-methylputrescine oxidation during cocaine biosynthesis: study of prochiral methylene hydrogen discrimination using the remote isotope method. Org. Lett. 2, 3–5. https://doi.org/10.1021/ol990940s

Jaremicz, Z., Luczkiewicz, M., Kokotkiewicz, A., Krolicka, A., Sowinski, P., 2014. Production of tropane alkaloids in *Hyoscyamus niger* (black henbane) hairy roots grown in bubble-column and spray bioreactors. Biotechnol. Lett. 36, 843–853. https://doi.org/10.1007/s10529-013-1426-9

Jocković, N., Fischer, W., Brandsch, M., Brandt, W., Dräger, B., 2013. Inhibition of human intestinal b-glucosidases by calystegines. J. Agric. Food Chem. 61, 5550–5557. https://doi.org/10.1021/jf4010737

Jouhikainen, K., Lindgren, L., Jokelainen, T., Hiltunen, R., Teeri, T.H., Oksman-Caldentey, K.-M., 1999. Enhancement of scopolamine production in *Hyoscyamus muticus* L. hairy root cultures by genetic engineering. Planta 208, 545–551. https://doi.org/10.1007/s004250050592

Kai, G., Liu, Y., Wang, X., Yang, S., Fu, X., Luo, X., Liao, P., 2011. Functional identification of hyoscyamine 6β-hydroxylase from *Anisodus acutangulus* and overproduction of scopolamine in genetically-engineered *Escherichia coli*. Biotechnol. Lett. 33, 1361–1365. https://doi.org/10.1007/s10529-011-0575-y

Kai, G., Zhang, A., Guo, Y., Li, L., Cui, L., Luo, X., Lin, C., Xiao, J., 2012. Enhancing the production of tropane alkaloids in transgenic *Anisodus acutangulus* hairy root cultures by over-expressing tropinone reductase I and hyoscyamine-6β-hydroxylase. [WWW Document]. https://doi.org/10.1039/c2mb25208b

Kaiser, H., Richter, U., Keiner, R., Brabant, A., Hause, B., Dräger, B., 2006. Immunolocalisation of two tropinone reductases in potato (*Solanum tuberosum* L.) root, stolon, and tuber sprouts. Planta 225, 127–137. https://doi.org/10.1007/s00425-006-0335-8

Kamada, H., Okamura, N., Satake, M., Harada, H., Shimomura, K., 1986. PlantCell Reports Alkaloid production by hairy root cultures in *Atropa belladonna*. Plant Cell Rep. 239–242.

Kanegae, T., Kajiya, H., Amano, Y., Hashimoto, T., Yamada, Y., 1994. Species-dependent expression of the hyoscyamine 6 beta-hydroxylase gene in the pericycle. Plant

Physiol. 105, 483–490. https://doi.org/10.1104/pp.105.2.483

Kanto, J., Kentala, E., Kaila, T., Pihlajamäki, K., 1989. Pharmacokinetics of scopolamine during caesarean section: relationship between serum concentration and effect. Acta Anaesthesiol. Scand. 33, 482–6. https://doi.org/10.1111/j.1399-6576.1989.tb02950.x

Kohnen-Johannsen, K., Kayser, O., 2019. Tropane Alkaloids: Chemistry, Pharmacology, Biosynthesis and Production. Molecules 24, 796. https://doi.org/10.3390/molecules24040796

Kohnen, K.L., Sezgin, S., Spiteller, M., Hagels, H., Kayser, O., 2017. Localization And Organization Of Scopolamine Biosynthesis In *Duboisia myoporoides* R. Br. Plant Cell Physiol. 0, 1–12. https://doi.org/10.1093/pcp/pcx165

Korolev, S., Ikeguchi, Y., Skarina, T., Beasley, S., Arrowsmith, C., Edwards, A., Joachimiak, A., Pegg, A.E., Savchenko, A., 2002. The crystal structure of spermidine synthase with a multisubstrate adduct inhibitor. Nat. Struct. Biol. 9, 27–31. https://doi.org/10.1038/nsb737

Kushwaha, A.K., Sangwan, N.S., Tripathi, S., Sangwan, R.S., 2013. Molecular cloning and catalytic characterization of a recombinant tropine biosynthetic tropinone reductase from Withania coagulans leaf. Gene 516, 238–247. https://doi.org/10.1016/j.gene.2012.11.091

Ladenburg, A., 1879. Künstliches Atropin. Berichte der Dtsch. Chem. Gesellschaft 12, 941–944. https://doi.org/10.1002/cber.187901201241

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–5.

Langmead, C.J., Watson, J., Reavill, C., 2008. Muscarinic acetylcholine receptors as CNS drug targets. Pharmacol. Ther. 117, 232–243. https://doi.org/10.1016/j.pharmthera.2007.09.009

Lauersen, K.J., 2019. Eukaryotic microalgae as hosts for light-driven heterologous isoprenoid production. Planta 249, 155–180. https://doi.org/10.1007/s00425-018-3048-x

Leete, E., Bjorklund, J. a., Sung, H.K., 1988. The biosynthesis of the benzoyl moiety of cocaine. Phytochemistry 27, 2553–2556. https://doi.org/10.1016/0031-9422(88)87026-2

Leete, E., Endo, T., Yamada, Y., 1990. Biosynthesis of nicotine and scopolamine in a root culture of *Duboisia leichhardtii*. Phytochemistry 29, 1847–1851. https://doi.org/10.1016/0031-9422(90)85026-C

Li, J., Van Belkum, M.J., Vederas, J.C., 2012. Functional characterization of recombinant hyoscyamine 6β-hydroxylase from *Atropa belladonna*. Bioorganic Med. Chem. 20, 4356–4363. https://doi.org/10.1016/j.bmc.2012.05.042

Li, R., Reed, D.W., Liu, E., Nowak, J., Pelcher, L.E., Page, J.E., Covello, P.S., 2006. Functional genomic analysis of alkaloid biosynthesis in *Hyoscyamus niger* reveals a cytochrome P450 involved in littorine rearrangement. Chem. Biol. 13, 513–20. https://doi.org/10.1016/j.chembiol.2006.03.005

Liu, T., P, Z., KD, C., C, M., HX, H., 2005. Molecular cloning, expression and characterization of hyoscyamine 6beta-hydroxylase from hairy roots of *Anisodus tanguticus*. Planta 71, 249–53. https://doi.org/10.1055/s-2005-837825

Lossen, W., 1869. Ueber das Atropin. Ann. der Chemie und Pharm. https://doi.org/18641310103

Luanratana, O., Griffin, W.J., 1980. Cultivation of a *Duboisia* Hybrid - Part A. J. Nat. Prod. 43, 477–491.

Maldonado-Mendoza, I.E., Ayora-Talavera, T., Loyola-Vargas, V.M., 1993. Establishment of hairy root cultures of *Datura stramonium*. Characterization and stability of tropane alkaloid production during long periods of subculturing. Plant Cell. Tissue Organ Cult. 33, 321–329. https://doi.org/10.1007/BF02319018

Mizusaki, S., Tanabe, Y., Noguchi, M., Tamaki, E., 1972. *N*-methylputrescine oxidase from tobacco roots. Phytochemistry 11, 2757–2762. https://doi.org/10.1016/S0031-9422(00)86509-7

Moyano, E., Fornalé, S., Palazón, J., Cusidó, R.M., Bagni, N., Piñol, M.T., 2002. Alkaloid production in Duboisia hybrid hairy root cultures overexpressing the *pmt* gene. Phytochemistry 59, 697–702. https://doi.org/10.1016/S0031-9422(02)00044-4

Müller, J., 1998. Hexensalben und Liebestränke : ein Beitrag zur Kulturgeschichte der Nachtschattengewächse Hexensalben und Liebestränke. Gesnerus Swiss J. Hist. Med. Sci. 55.

Mutschler, E., Geisslinger, G., Kroemer, H., Ruth, P., Schäfer-Korting, M., 2008. Mutschler Arzneimittelwirkungen, 9th ed.

Naik, T., Vanitha, S.C., Rajvanshi, P.K., Chandrika, M., Kamalraj, S., Jayabaskaran, C., 2017. Novel Microbial Sources of Tropane Alkaloids: First Report of Production by Endophytic Fungi Isolated from Datura metel L. Curr. Microbiol. 75, 1–7. https://doi.org/10.1007/s00284-017-1367-y

Nakajima, K., Hashimoto, T., 1999. Two tropinone reductases, that catalyze opposite stereospecific reductions in tropane alkaloid biosynthesis, are localized in plant root with different cell-specific patterns. Plant Cell Physiol. 40, 1099–1107.

Naudé, T.W., 2007. *Datura* spp. and other related plants, in: Veterinary Toxicology. Elsevier, pp. 892–906. https://doi.org/10.1016/B978-012370467-2/50167-X

Niemann, A., 1860. Ueber eine neue organische Base in den Cocablättern. Arch. Pharm. (Weinheim). 153, 129–155. https://doi.org/10.1002/ardp.18601530202

Oksman-Caldentey, K.-M., Strauss, A., 1986. Somaclonal Variation of Scopolamine Content in Protoplast-Derived Cell Culture Clones of *Hyoscyamus muticus*. Planta Med. 52, 6–12. https://doi.org/10.1055/s-2007-969053

Ollagnier, S., Kervio, E., Rétey, J., 1998. The role and source of 5'-deoxyadenosyl radical in a carbon skeleton rearrangement catalyzed by a plant enzyme. FEBS Lett. 437, 309–312. https://doi.org/10.1016/S0014-5793(98)01258-7

Oppermann, U., Filling, C., Hult, M., Shafqat, N., Wu, X., Lindh, M., Shafqat, J., Nordling, E., Kallberg, Y., Persson, B., Jörnvall, H., 2003. Short-chain dehydrogenases/reductases (SDR): The 2002 update. Chem. Biol. Interact. 143–144, 247–253.

https://doi.org/10.1016/S0009-2797(02)00164-3

Pakdeechanuan, P., Shoji, T., Hashimoto, T., 2012. Root-to-shoot Translocation of Alkaloids is Dominantly Suppressed in *Nicotiana alata*. Plant Cell Physiol. 53, 1247–1254. https://doi.org/10.1093/pcp/pcs065

Palazón, J., Moyano, E., Cusidó, R.M., Bonfill, M., Oksman-Caldentey, K.M., Piñol, M.T., 2003. Alkaloid production in *Duboisia* hybrid hairy roots and plants overexpressing the h6h gene. Plant Sci. 165, 1289–1295. https://doi.org/10.1016/S0168-9452(03)00340-6

Park, L., Furey, M., Nugent, A., Farmer, C., Ellis, J., Szczepanik, J., Lener, M., Zarate, C., 2018. F173. Negative Trial of Scopolamine in Major Depressive Disorder Does Not Demonstrate Neurophysiological Changes Seen With the Antidepressant Response of Ketamine. Biol. Psychiatry 83, S305–S306. https://doi.org/10.1016/j.biopsych.2018.02.787

Pfaffl, M.W., Physiologie, L., Weihenstephan, W., 2004. Real-time RT-PCR : Neue Ansätze zur exakten mRNA Quantifizierung. BIOspektrum 1/04 10. J, 92–95.

Pramod, K.K., Singh, S., Jayabaskaran, C., 2010a. Biochemical and structural characterization of recombinant hyoscyamine 6β-hydroxylase from Datura metel. https://doi.org/10.1016/j.plaphy.2010.09.003

Pramod, K.K., Singh, S., Jayabaskaran, C., 2010b. Biochemical and structural characterization of recombinant hyoscyamine 6b-hydroxylase from *Datura metel* L. Plant Physiol. Biochem. 48, 966–970. https://doi.org/10.1016/j.plaphy.2010.09.003

Qiang, W., Xia, K., Zhang, Q., Zeng, J., Huang, Y., Yang, C., Chen, M., Liu, X., Lan, X., Liao, Z., 2016. Functional characterisation of a tropine-forming reductase gene from *Brugmansia arborea*, a woody plant species producing tropane alkaloids. Phytochemistry 127, 12–22. https://doi.org/10.1016/j.phytochem.2016.03.008

Qiu, F., Yang, C., Yuan, L., Xiang, D., Lan, X., Chen, M., Liao, Z., 2018. A Phenylpyruvic Acid Reductase Is Required for Biosynthesis of Tropane Alkaloids. Org. Lett. 20, 7807–7810. https://doi.org/10.1021/acs.orglett.8b03236

Renner, U.D., Oertel, R., Kirch, W., 2005. Pharmacokinetics and pharmacodynamics in clinical use of scopolamine. Ther. Drug Monit. 27, 655–665. https://doi.org/10.1097/01.ftd.0000168293.48226.57

Robenshtok, E., Luria, S., Tashma, Z., Hourvitz, A., 2002. Adverse reaction to atropine and the treatment of organophosphate intoxication. Isr. Med. Assoc. J. 4, 535–539. https://doi.org/12120467

Robins, R J, Bachmann, P., Robinson, T., Rhodes, M.J., Yamada, Y., 1991. The formation of 3 alpha- and 3 beta-acetoxytropanes by *Datura stramonium* transformed root cultures involves two acetyl-CoA-dependent acyltransferases. FEBS Lett. 292, 293–7.

Robins, Richard J., Parr, A.J., Walton, N.J., 1991. Studies on the biosynthesis of tropane alkaloids in *Datura stramonium* L. transformed root cultures. Planta 183, 196–201. https://doi.org/10.1007/BF00197788

Rothman, R.B., Baumann, M.H., Dersch, C.M., Romero, D. V, Rice, K.C., Carroll, F.I., Partilla,

J.S., 2001. Amphetamine-type central nervous system stimulants release norepinephrine more potently than they release dopamine and serotonin. Synapse 39, 32–41. https://doi.org/10.1002/1098-2396(20010101)39:1<32::AID-SYN5>3.0.CO;2-3

Rudy, D., Cline, K., Harris, R., Goldberg, K., Dmochowski, R., 2006. Multicenter phase III trial studying trospium chloride in patients with overactive bladder. Urology 67, 275–280. https://doi.org/10.1016/j.urology.2005.08.017

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Schlagmann, C., Remien, J., 1986. Klinische Schrift Zur Behandlung der Parkinson-Krankheit. Klin. Wochenschr. 64, 939–942.

Schmidt, G.W., Jirschitzka, J., Porta, T., Reichelt, M., Luck, K., Torre, J.C.P., Dolke, F., Varesio, E., Hopfgartner, G., Gershenzon, J., D'Auria, J.C., 2015a. The Last Step in Cocaine Biosynthesis Is Catalyzed by a BAHD Acyltransferase. Plant Physiol. 167, 89–101. https://doi.org/10.1104/pp.114.248187

Schmidt, G.W., Jirschitzka, J., Porta, T., Reichelt, M., Luck, K., Torre, J.C.P., Dolke, F., Varesio, E., Hopfgartner, G., Gershenzon, J., D'Auria, J.C., 2015b. The Last Step in Cocaine Biosynthesis Is Catalyzed by a BAHD Acyltransferase. Plant Physiol. https://doi.org/10.1104/pp.114.248187

Scholl, Y., Höke, D., Dräger, B., 2001. Calystegines in Calystegia sepium derive from the tropane alkaloid pathway. Phytochemistry 58, 883–889. https://doi.org/10.1016/S0031-9422(01)00362-4

Schultze-Kraft, M., 2016. Evolution of estimated coca cultivation and cocaine production in South America (Bolivia, Colombia and Peru) and of the actors, modalities and routes of cocaine trafficking to Europe - Background paper commissioned by the EMCDDA for the 2016 EU Drug Marke.

Shitan, N., Morita, M., Yazaki, K., 2009. Identification of a nicotine transporter in leaf vacuoles of Nicotiana tabacum. Plant Signal. Behav. 4, 530–532. https://doi.org/10.1073/pnas.0812512106

Shoji, T., Inai, K., Yazaki, Y., Sato, Y., Takase, H., Shitan, N., Yazaki, K., Goto, Y., Toyooka, K., Matsuoka, K., Hashimoto, T., 2008. Multidrug and Toxic Compound Extrusion-Type Transporters Implicated in Vacuolar Sequestration of Nicotine in Tobacco Roots. Plant Physiol. 149, 708–718. https://doi.org/10.1104/pp.108.132811

Shoji, T., Yamada, Y., Hashimoto, T., 2000. Jasmonate induction of putrescine N-methyltransferase genes in the root of Nicotiana sylvestris. Plant Cell Physiol. 41, 831–9.

Sinatra, R.S., Jahr, J.S., Watkins-Pitchford, M., 2010. The Essence of Analgesia and Analgesics. Cambridge University Press, Cambridge. https://doi.org/10.1017/CBO9780511841378

Sneader, W., 2005. Drug Discovery: A History. Glasgow, UK. https://doi.org/10.1002/0470015535

Sorbe, B., Andersson, H., Schmidt, M., Söderberg, M., Högberg, T., Wernstedt, L., Janson,

E.T., Ehrnström, B., Kjaer, M., Havsteen, H., Overgaard, M., Sandberg, E., Flander, M., Heikkinen, M., Nikkanen, V., 1994. Tropisetron (Navoban) in the prevention of chemotherapy-induced nausea and vomiting - the Nordic experience. Support. Care Cancer 2, 393–399. https://doi.org/10.1007/BF00344055

Stöckigt, J., Zenk, M.H., 1975. Chemical Syntheses and Properties of Hydroxycinnamoyl Coenzyme A Derivatives. Zeitschrift für Naturforschung. C, A J. Biosci. 30, 352–358.

Suzuki, H., Nishino, T., Nakayama, T., 2007. cDNA cloning of a BAHD acyltransferase from soybean (*Glycine max*): Isoflavone 7-O-glucoside-6″-O-malonyltransferase. Phytochemistry 68, 2035–2042. https://doi.org/10.1016/j.phytochem.2007.05.017

Suzuki, K., Yamada, Y., Hashimoto, T., 1999. Expression of Atropa belladonna putrescine *N*-methyltransferase gene in root pericycle. Plant Cell Physiol. 40, 289–297.

Teuber, M., Azemi, M.E., Namjoyan, F., Meier, A.C., Wodak, A., Brandt, W., Dräger, B., 2007. Putrescine *N*-methyltransferases - A structure-function analysis. Plant Mol. Biol. 63, 787–801. https://doi.org/10.1007/s11103-006-9126-7

Ulbricht, C., Basch, E., Hammerness, P., Vora, M., Wylie, J., Woods, J., 2005. An Evidence-Based Systematic Review of *Belladonna* by the Natural Standard Research Collaboration. J. Herb. Pharmacother. 4, 61–90. https://doi.org/10.1300/J157v04n04_06

Ullrich, S.F., 2016. Influence of abiotic factors on growth and biosynthesis of secondary plant components in *Duboisia* species.

Ullrich, S.F., Averesch, N.J.H., Castellanos, L., Choi, Y.H., Rothauer, A., Kayser, O., 2016. Discrimination of wild types and hybrids of *Duboisia myoporoides* and *Duboisia leichhardtii* at different growth stages using 1H NMR-based metabolite profiling and tropane alkaloids-targeted HPLC-MS analysis. Phytochemistry 131, 44–56. https://doi.org/10.1016/j.phytochem.2016.08.008

Ullrich, S.F., Rothauer, A., Hagels, H., Kayser, O., 2017. Influence of Light, Temperature, and Macronutrients on Growth and Scopolamine Biosynthesis in *Duboisia* species. Planta Med. 83, 937–945. https://doi.org/10.1055/s-0043-106435

Vakili, B., Karimi, F., Sharifi, M., Behmanesh, M., 2012. Chromium-induced tropane alkaloid production and H6H gene expression in *Atropa belladonna* L. (Solanaceae) invitro-propagated plantlets. Plant Physiol. Biochem. 52, 98–103. https://doi.org/10.1016/j.plaphy.2011.12.003

Wang, K., 2007. Agrobacterium Protocols, Structure. https://doi.org/10.1038/nprot.2007.241

Wang, X., Chen, M., Yang, C., Liu, X., Zhang, L., Lan, X., Tang, K., Liao, Z., 2011. Enhancing the scopolamine production in transgenic plants of *Atropa belladonna* by overexpressing *pmt* and *h6h* genes. Physiol. Plant. 143, 309–315. https://doi.org/10.1111/j.1399-3054.2011.01506.x

Watts, S.W., Kanagy, N.L., Lombard, J.H., 2008. Receptor-Mediated Events in the Microcirculation, Microcirculation. https://doi.org/10.1016/B978-0-12-374530-9.00007-3

Wess, J., Duttaroy, A., Zhang, W., Gomeza, J., Cui, Y., Miyakawa, T., Bymaster, F.P., Mckinzie, L., Felder, C.C., Lamping, K.G., Faraci, F.M., Deng, C., Yamada, M., 2003. M 1

-M 5 Muscarinic Receptor Knockout Mice as Novel Tools to Study the Physiological Roles of the Muscarinic Cholinergic System. Recept. Channels 9, 279–290. https://doi.org/10.3109/10606820308262

WHO, 2015. Annex 1 19th WHO Model List of Essential Medicines.

Williams, N., 1977. Cocaine and Metabolites: Relationship between pharmacological activity and inhibitory action on dopamine uptake into struatal synaptosomes. Prog. Neuropsychopharmacol. 1, 265–269.

Wink, M., 2010. Molecular Modes of Action of Defensive Secondary Metabolites. https://doi.org/10.1002/9781444318876.ch2

Wink, M., 1998. Compartmentation of alkaloid synthesis, transport, and storage, in: Alkaloids, Biochemistry, Ecology and Medicinal Applications. pp. 239–262.

Wolffenstein, R., 1922. Die Pflanzenalkaloide.

Xia, K., Liu, X., Zhang, Q., Qiang, W., Guo, J., Lan, X., Chen, M., Liao, Z., 2016. Promoting scopolamine biosynthesis in transgenic *Atropa belladonna* plants with pmt and h6h overexpression under field conditions. Plant Physiol. Biochem. 106, 46–53. https://doi.org/10.1016/j.plaphy.2016.04.034

Yu, H., Lv, D., Shen, M., Zhang, Y., Zhou, D., Chen, Z., Wang, C., 2019. BDNF mediates the protective effects of scopolamine in reserpine-induced depression-like behaviors via up-regulation of 5-HTT and TPH1. Psychiatry Res. 271, 328–334. https://doi.org/10.1016/j.psychres.2018.12.015

Zhang, L., Ding, R., Chai, Y., Bonfill, M., Moyano, E., Oksman-Caldentey, K.-M., Xu, T., Pi, Y., Wang, Z., Zhang, H., Kai, G., Liao, Z., Sun, X., Tang, K., 2004. Engineering tropane biosynthetic pathway in *Hyoscyamus niger* hairy root cultures. Proc. Natl. Acad. Sci. U. S. A. 101, 6786–91. https://doi.org/10.1073/pnas.0401391101

Ziegler, J., Facchini, P.J., 2008. Alkaloid biosynthesis: metabolism and trafficking. Annu. Rev. Plant Biol. 59, 735–769. https://doi.org/10.1146/annurev.arplant.59.032607.092730

Zuckerkandl, E., Pauling, L., 1965. Evolutionary Divergence and Convergence in Proteins, in: Evolving Genes and Proteins. Elsevier, pp. 97–166. https://doi.org/10.1016/B978-1-4832-2734-4.50017-6

APPENDIX

LIST OF ABBREVIATIONS

ADC	Arginine decarboxylase
AIH	Agmatine deiminase
cDNA	Complementary DNA
CDW	Cell dry weight
CNS	Central nervous system
CoA	Coenzyme A
COPD	Chronic obstructive pulmonary disease
CPA	*N*-carbamoylputrescine amidase
DAD	Diode-array detector
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EC numbers	Enzyme Commission numbers
EEG	Electroencephalogram
GPCRs	G-protein-coupled receptors
GST-tag	Glutathione-S-transferase-tag
H6H/ *h6h*	Hyoscyamine 6β-hydroxylase
HIS-tag	Polyhistidine-tag
H NMR	Proton nuclear magnetic resonance spectroscopy
HPLC	High performance liquid chromatography
Km	Michaelis–Menten constant
kcat	Constant for conversion to product
M	Marker or protein ladder
mAChR	Muscarinic acetylcholine receptor
MALDI MSI	Matrix-assisted laser desorption/ionization mass spectrometry imaging
MPO	*N*-methylputrescine oxidase
MS	Mass spectrometry
m/z	Mass to charge ratio
OrnDC	Ornithine decarboxylase
PA	Polyamine
PMT / *pmt*	Putrescine *N*-methyltransferase

RNA	Ribonucleic acid
SAM	S-adenosyl methionine
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SMS	spermine synthase
SPDS	Spermidine synthase
TA	Tropane alkaloids
TCEP	Tris(2-carboxyethyl)phosphine HCl
TR-I / *tr-I*	Tropinone-reductase I
TR-II / *tr-II*	Tropinone-reductase II
TTS	Transdermal therapeutic systems
WHO	World Health Organization

SUPPLEMENTARY DATA

Gene sequences and primers used in this study.

Table A-1: Gene sequences used in this study

Name	Sequence
Contig #5915	ATGGGTTCCCAATCTGCTGCCCTACAAATTGAAATTTTGTCCAC AAAGTTCATAACACCGTCCTCACCAACCCCAAATCATTTACAGA ATTACAAGTTATCTTTCTTTGATCAAATAGCTGAAGAAGTCCA CTTGCCTCTTGTTCTTTTCTATCCACCAAACGACAAAAACTCCA CTACCGATGAACAACTCGAAGAATCCCTGTCTAGAGTATTAACC CATGTTTACCCAATAGCCGGTAGATTTACAGAGGATTTTTGCTC CATCAATTGCCTTGATCAAGGAGTTAAACTTGTAAAGGCAACT GTCAATAACAAGCTCGATAATTTTCTTGAGCAAGCACACAAGG ATATTAATGCCGCATTACTTTGTTGGCCTCACGATACATGGGCT GTTAATGAAAATAATTTGGCTATCACACCACTTGTTATTATCC AAGTAACCAAATTTGAATGTGGTGGGATTGCCCTGTCAATGAG CCATGCACACATTGCAATGGACGGATTCTCGAGTCTGACATTTT TGTACGAGTGGTCAAAAGTGTGTAGATTTGGAACTCCCACAGA AGAAATCAATTTCCTGAGTTTTAATTTGAGTGAAGTTTTTCCT ACAAGGGATTTATCTAAACTTTTACTGCCTCGTATTCATGAAG AGAAACGTGCAGAGAGCAAACTGGTTGCTAAGAGGTTATATAT TGATGAAGCTGCTATATCAAAGCTAAGAGATGAAATGACTGGT TTGAGTTTTAAACCCACGAGGGTCGAGATGATTACAGCAGTTT TATGGAGGGCTTTGATCGGTGCTACGGAAGCGAAAAATGGGAA TTTGAGACGTTCTCTAATGGGAGTCCCAATCAACTTGCGTAGTA AGATTTCTTTGCCTCAAATTGAAAAGTGTTTTGGTAATCTTGT AGTTGATGCTCCTGTAAAATTTATTCCCGGGGAGACAAAGATG GAGCTTCTTAACTTAGTGACATTGATCAGGGATACAGTGCAGA AAACTATTGAGTATTGCAACAAGGAATCACCAGATGAGATAGT TTCTGCAGTTGCTGATTTATACAATGGAAGTTTTCAAGCAAAT GAGTGGGGAGGCAGTAATGAAGTTGACACATTTACATCTTCAA GTTTGTGCAGGTTTCCTATACTAGGAGCTGATTTTGGTTGGGG AAAACCTTGCTTGATGCATTTTGGATCCAGGCATACTCAAACTT GTTGGCTGTATGATGCAGTATGTGGCAATGGTGTCTGTGTGCA GGTGGATCTCAAGGAAAGTTACATGCGCTTCTTTGAGTGTGAT CAAGATATCAAGGCTTATTTTAAGTTTTAG
Contig #50965	ATGGCTCCCAAAGTGAAGATCATATCCAAAGAAATGATCAAAC CTTCATTCCCAACGCCTCCTTACCTTAGAACTTACAAACTATCA CTTCTCGATCAAATTTCACCTCCAGTTTTCATACCTTTGGTTTT CTTTTACCAAGATGAATATGACAATATCAATCATGCAAAAAAA TTAGAGTTCTTGAAGAATTCTTTATCCGATGCTTTAACGCGAT TCTACCCATTGGCCGGAAGGCTCAATGATAACAATTTTATTGA TTGCAATGATGCTGGAGCCGAGTTCATTCAAGCTCAAGTTCAT GGTTACCTCTCACAAGTTATAGAAACTCCAAAAATGGAGGAAT TAAGAGAATTTCTCCCAAGAGAAGCATGTAAGGTAGAGAATCA TTATTTACTTCTAGCAGTCCAAGTTAATTTATTTGATTGTGGA GGGATTTCAATTGGTGTGTGCATGTCTCACAAAGTTGGTGATG

Continuation Contig #50965	GTTCTTCCATAGTGACATTCATCAGTGCGTGGGCAGCCATTGCT AGGGGAGACACCGAAATTTTGCAGCCTAATTTTAATCTGGCAA GCATTTTTCCGCCAATAGATTTGTCTAACTCAAGTTTCAAACCA TCTATTGGGATAAAGAAAGAAAAAATATTGACGAGAAGGTTTG TTTTTGACAAGGTAAAGCTTGAAGCACTCAAGAAATCAGTTTC AGGATCACAAATGAAGGATCCCACTCGCGTGGAAGCTCTCTCAT CCTTCATATGGAAGCATTTCATGGAGGTTCCAAAGTTGAAAAA AGATTCTACGAAAATGTTTGCAGCAGTGCATGCAGTGAATATA AGATCAAGAATGAATCCAACTCTACCGGATAACTCCTTTGGAA ACCTTTTTACAACTGCACTGGCATTCTCAAATCTAGAAGGTGAA GAAATGGGAACTGATGATTTGGTGTACCACTTGAGAAGTGCAA TAAGGAAAATCAACAGTGACTATATTAAGATATTACTAAACGG AGAAGAGTTTATAAAACACATGGGTAAATCAGCAGAGCTGTTC TCAAAGGGGGAAGTTGAGTTTTGCAATTTTAGCAGTTGGTGTA GGTTTCCTATTTATGAAGTGGATTTTGGATGGGGAAAGCCAGT ATGGGTGTGTACTACAACTTTTCCATATGAGAATATGGTCATC TTTATGAGTACAAAATGTGGGGAGGGAATAGAAGCATGGGTTA ACATGCTCGAGGATGATGACTTTAATTTTTGA
Contig #168348	ATGGATACTCAGCGTGCCAACATGAAGGATAGAATAAAAGTTG AGATCTTGTCCACAAAGCTCATAAAGCCATCCATACCAACTCCC CCTCACCTCTCATGTTACAAAATTTCATTCTTTGACCAATTAGC TAACCAAGAACTTGTCCCTTTAGTTCTTCTCTATCCTCATTGCA ACAACTCTATCACTGATGCAGAGATGGACGAACGACTTGAGCA CTCCTTTTCTAAGATATTAACACGTTTTCATCCAGCTGCTGGAA GGTATGCTGAAGATAAATGTTCAGTTCTTTGTCTTGATCAAGG CGTTCCTTTTACCAAAGCAAAGGTCAACTGTAAGCTGGACAAT TTCATCGAAGAAGTTGCACGCAATGGCCATGATCTGACAGTCCT CCTCTGGCCACATGACATCAAGGATGTGGATGAAACCAATCTGT TTACAGCACCAATTTTCACTGTCCAAATAACCAAGTTTGAATGT GGCGGCATGGCTGTAACTATTACTATTTCGCACCCTGTAATGGA TGGTTTCACAGCCATATCATCTATTTTTGAGTGGGCCAATGCAT GTAGACTGGGGACTCCTGTCGACAAGATCAATAATTTCTTAAG CTTCAATGCGAGCGATATTTTTCCAACAAGAGACTTATCAAGG TATTTCAAGCCTCCTATTCCAGAAGAAGGAAGCAAAGAAGATA AATTTGTCTCAAAGAGCTTTGTTATCAAAGAGGATTCTATCTT GACGCTCAAAAAAAAATTTGCAAGCTTCATTGATTCGGGTGCT TTGAGTTTTCAGCCTTCAAGAGTTGAAATGATCTCAGCATTGT TATGGAGAGCTCTTATCCGTGTTTCAGAAGCAGTAAATGGGCA CTTGAGACCATGTCAAATGGTTATTCCACTGAACTTGCGTTCTA AAATTTCTTTACCTGAAATAGAGAAGTCTGTCGGGGACTTGGT AATTGATGTTCCAGTGAAATTTGTACCTGGGGAGACACAGATA GAGTTGCACCACCTTGTAATATTGATCAGGGATGCAGTGACGA AAATTGTTGCTTCCTGTGCAGAAGCTTCACCTGACGAGATAGTT TCCAATGTGGCTAAATTACATAATGAAAGCTTTCAAGCACCAG GATGGGCAGGTAATGATGGTATTGACAAATTCACGTGTTCAAG TTTGTGCTGGTTTCCTATGCAGGATGCTGATTTTGGTTGGGGA AAACCATCCTTGATGTTTTTGGGATTAAAAGATACGAATATGT TTTGGTTGTACGATACGGTTTGTCGTACTGGTGTGGGTCTGCA

	AGTGGACTTGGACAAAAGGTACATGGAGTTATTTGAATCAGAC CTCGATATTAAGGCTCTCATCACTCTATTCTAG
Contig #176922	ATGACCAAATTAGAGATTCAAATCCAGGTAAGGAAAATGTTAA AGCCCTCAACTCCTACACCGAATCATCTTCGCATCCTTAATCTT TCATTGTTTGATCAGCTGGCTCCTCGTATATATGTACCAGTGCT GTTCCACTATTTGCCAAGCAGTGAATGGAACTCGGAAGGAATT ACTGAAAGATGCAATAAGCTGCAAAACTCTCTGGTTGAGACAT TAACCAAGTTTTACCCTCTAGCAGGAAGATTTAGAGAAGATGA TCTCTCAATTCACTGCAATGATGAAGGTGTTGAGTATGTCGAA ACCAAAGTCGACGCGGATCTTGCTGAATTTCTCCACCAAGGATC CAAGAATATTGAGCTTTTGGATGATCTTCTTCCAAAAACAGAT CTTCCATCAAGTCCATTGCTTGGAATCCAAGTGAACAAATTCAA TTGTGGAGGCTTAGTCATGGGGATAGAAATGTCACACATCCTA GCAGATGGCTTCACGTTAGGAACATTTGTCAAGGAATGGGCAA AAATTAGCCAAACCGGAACAGCAAAGGGTTTTCTCCCAAGTTT CGGTCGCTTGCCATCGCTCTTTCCTACAAGAGTGCTATCCGGAC CTTACTTTTCACCACCTTCCAACAGAGACCCTAAGATTGTCACG CGGAGGTTTGTGTTTGATGCTTCAGCAATAGCAAATCTCAAAG ACAGAATCAATTCAAATGCCACATTCACGAGACCTACTCGGGTG GTGGTCGTTATGTCGTTAATATGGAAGGTTCTTGTGGGCATTT CCTCAGCCAAGCATGGGCACTCAAGGGACTCTTATTTATTATTT CCCGTTAATTTGAGGGGGAAAACAAATTTACCATCTTTAGAAC ATGCTCTAGGGAATTTCTGCTTGGCTGGTATTGCTACACTTGAG GCAAACCAGTCAAGGAAGGAGTTAAATGACTTTGTTAACATGG TAGGAAGTACAGCAAGGGACACATCTGAAGGCATTGGTAAGGC AAGCATCGATGATATTACCTCTATGTTTGTCAACTGCAACACAC CAGGTCAGAAGGACGAGATGGACATGTATATCTGCACTAGTTG GTGCAATTTCCCCTGGTATGAAGCTGACTTTGGCTGGGGAAAA CCGTTCTGGGTGAGCGGAGTAAGAAAAACTGCGGAACTAATTT CTCTGATTGATACAAAAAATGGTGATGGAATAGAAGCATGGAT AGGTTTGAAGGAGAATGACATGGCTGAATTCGAGAAAGACCCT GAAATTTTGACACATTGTCCACCTCTGC
Contig #178702	ATGGGCTCAGTTGCACATTCAACTGTGGGAATTACTACAGAAA ATATTAGAAAACCTTCCTCTCCCACCACTATTTCTCTTCCACTT GTATCCATAATGTCTGAGAAGCTCATCAAACCATCTTCTCCAAC TCCTCCTACTAGAAGATGGCACAAGCTTTCTCTTATTGATCAAG CTTTCAGCAACTCCTATATTCCATTTTCTCTGTTCTACACCAAA CAACAACTAGATGCTATATCAAACTATAATCCTACTCAAATCTC TCAACTTCTTGAGGAATCTTTATCGAAAATCTTGTCCAGTTATT ATCCATATGCCGGAAGGCTTAAAGATAACACTATGGTTGATTG TAATGACTCCGGGGCCGAATTCATTGAAGTTCAAATCAGTTGCC CCATATCAGAAACTCTAGATTGGCACAATGCAGCTATAGAAGA TTTGCTATTTCCTCAAGGATTACCTTGGTCAAATAGTGCAGATC GTGGATTAGTTGTAGTTCAACTAAGCTATTTTAATTGTGGAGG AATAGGTATCAGTATGTGTATTTCTCATAAGATTGGAGATGGA TGCAGTGGTTACAATCTTTTTCGTGATTGGTCTGAGATAACTC GTGATCCAAATTTTTCAAAACCATCTCTTCACTATGTTGAACAA TCTATTTTTCCTCCACCATCTAGTGGTCCTTTTACTTTACCATT

Continuation Contig #178702	ATTCATGTCTAACAAACATGACTGTGTCCAAAGAAGGTACATT TTCTCTAAAGAAAAATTACTTAACCTTAAGAACAAGGTTGCTG CTGAATCAGATGTGCAAAATCCAACACGTACTGAAGTTGCCAG CGCACTTATCTTTAGATGTGCTGTTGCAGCATCAAAGGCAAACT CGGATTTTTTCCAGCCATCATCAATGGTTCAAGCAGTTGATTTA CGAGCTCAAATTGGTTTGTCTCCAAATGCTATTGGAAATCTTCT TACTATCTGTCCCACATCAATTACCAATGAAGAGAGTATGACA ATATCAAAATTGGTCAGTGAAATGAGAAAATCGAAAGAGCTAA CTTACAATAGAGACAACATCAATGACAACATATTTGTGGCTTT ATTACTTGAATTAGCTAAATCGAAACAGGAATATCACGACAAT GGTCCTAATGCTTATCAAATTACTAGCTTAGTGAAATTTGCAC TTGATGAAATTGATTTTGGATGGGGGAAGCCCAGAAAGGTGAG TATAGCAAATGGTCTGAATAACAAGTTGGCTATCTTGATGGGT AATCAAAATGGAGGACTGGATGCATTTGTGACGCTCAGTGAAC AAGATATGTCTGTGTTCGTACGTGACCTTGAGCTTCTCGAGTTT GCTTCTCTTGTTCCAAGCTGCTAG
Contig #191374	ATGATGAAAGTTCAAGTCATTTCCACAGAAAGCATTAAACCTT CATCTCCAACACCAAATCACCTCAAAAATTTTGATCTTTGTGTA TTGGATCAGCTTATTCCTGCTCCTTATGCACCCATTGTACTCTT CTATCCCAATCTTGACAATATTAAAGGCCACGAAAAATCAGCA TTGTTAAAGAAATCACTAGCTGAAACTTTGTCTCACTATTATCC CCTTGCAGGGAGGTTTAAAGATGAACTCTCCATTGATTGCAAT GATCAAGGAGTTAATTATGTCACTACCAATGTTAATTGCCATC TAATTGAATTTCTAAACGAACCAAACCTCGAATCAATTGGTCA ATTTCTTCCTTGTCAACCTCCTTTTAAGGAATTAGCTGCAGGGG ATTGTGTCACGAACATTCAAATTAATGTTTTTGAATGTGGTGG AATTGCAATTGGTTTATGTATAGCGCATAAGATTCTTGATGGA GCAGGGCTCAGCACATTCCTAAAAAATTGGGCTGGATTGACCT GCAGTTCAGATGAAATCCAATGCCCTAGTTTAATGGCAAAATA CTTCTTCCCTGCAGAGGATTTGTGGTTAAGAGACACATCCATGG CCATGTGGAGTTCATTGTTCAAGAAAGGCAATTTTGTCACAAG AAGGATTGTTTTTAATGCCTCCGCTATAGACAACCTTAAAGCT ATGTCAACTAGCTCACATATAAAACATCCTACTAAAGTTGAGG TTGTCTCTTCTTTCATATGGAAATGTTCACTAGCTGCATCCAAA GAGAAAAAATGTTCTAAAAACTTGCCTAATTCACTGCTAACAC ACATTGTGAACTTGAGAAAAAGAGCAGCACCAGCTTTACCAGA AAACATTTTGGGAAATCTTCTTTGGCTATCAAGTGCTAAGAAT ACAGCAAAACATGAAATGGGGTTGCCTGATTTGGTGAATCAAG TACGAAATTCTATATCGAGGATCGATGATGCTTATGTCAAAAA GCTACGAGGCGATGAAGGGTCTAATTTGATGAGAAAGTCTCTC AAGGAAATTGGAGATTTTTGCAGAAAAGGTGCAGATCACTATG GTTTTACTAGTTGGTGCAACTTTGGGTTCTATGATCTTGATTTT GGATGGGGAAAGCCAACATGGGTCAGCAGTATGAGTTCAAGGT GTTTATTTTTCATGAATCTTATTATTCTTATGGAAACCAGGTG TGTTGGTGGAATTGAAGCATGGGTGACATTGGATGAGGAAGAA ATGAACATGCTAGTGGATAATCAGGAACTTTTGGCTTTTGCTT GTATAGACCCTAGCCCTCTTAGTACTTGA

Table A-2: Primers used in this study

Primer name	Sequence (5'-3')
T7	TAATACGACTCACTATAGG
T7 term	TGCTAGTTATTGCTCAGCGG
Contig #5915 fw	GGTACCGACGACGACGACAAGGCCATGGGTTCCCAATCTG
Contig #5915 rv	CGAGTGCGGCCGCAAGCTTCTAAAACTTAAAATAAGCCTTGAT
Contig #50965 fw	GACGACGACGACAAGGCCATGGCTCCCAAAGTGAAGATCATATCC
Contig #50965 rv	CGAGTGCGGCCGCAAGCTTTCAAAAATTAAAGTCATCATCCTCGAGCATGT
Contig #168348 fw	GGTACCGACGACGACGACAAGGCCATGGATACTCAGCGTGCCAAC
Contig #168348 rv	CGAGTGCGGCCGCAAGCTTCTAGAATAGAGTGATGAGAGCCTTAATATC
Contig #178702 fw	GGTACCGACGACGACGACAAGGCCATGGGCTCAGTTGCACATTC
Contig #178702 rv	CTCGAGTGCGGCCGCAAGCTTCTAGCAGCTTGGAACAAGAGA
Contig #191374 fw	GGTACCGACGACGACGACAAGGCCATGATGAAAGTTCAAGTCATTTC
Contig #191374 rv	CGAGTGCGGCCGCAAGCTTTCAAGTACTAAGAGGGCTAGGG

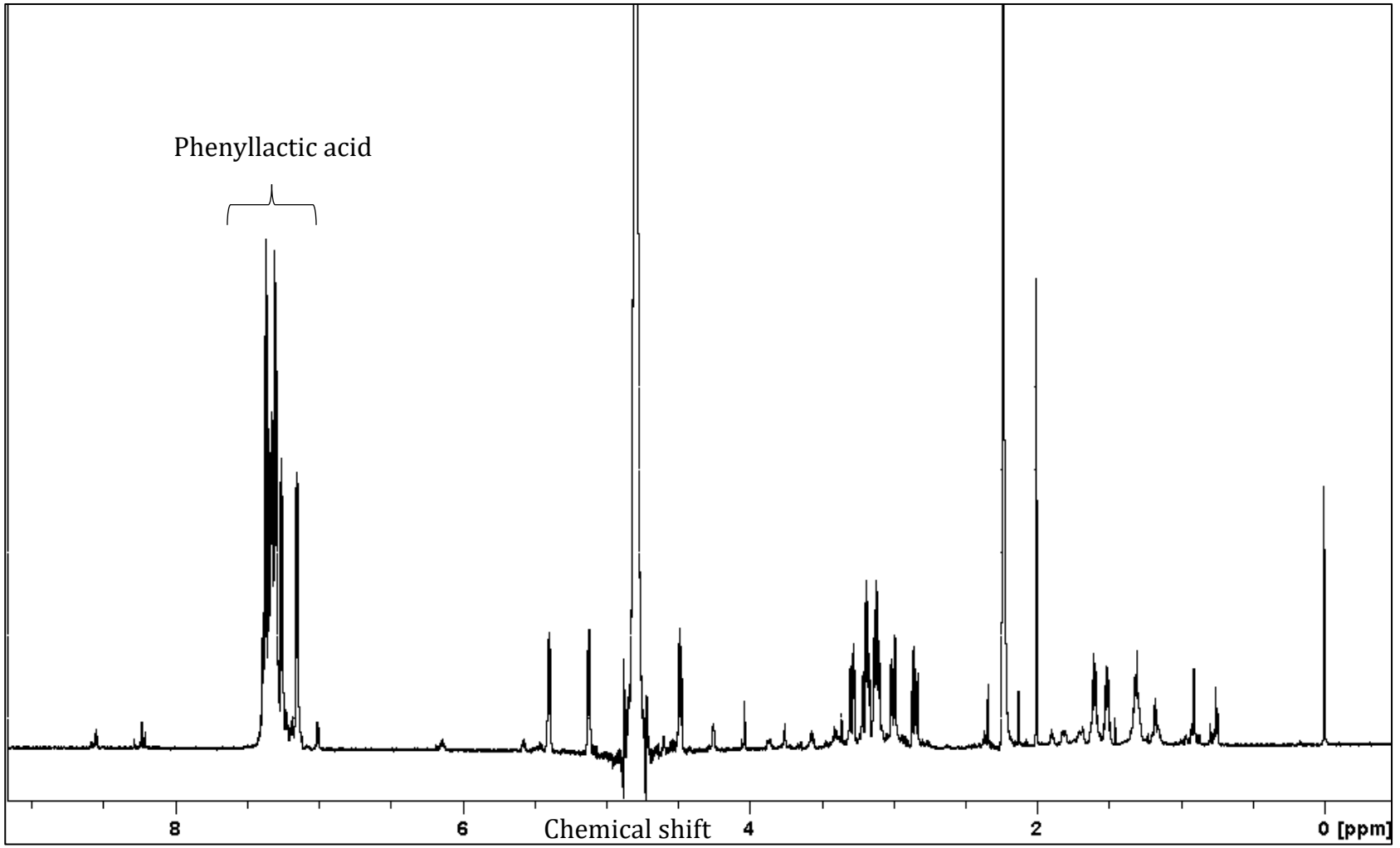

Figure A-2: ^{1}H-NMR spectra of phenyllactyl-CoA in D_2O; 600 MHz. The NMR data were processed using the ACD/NMR Processor Academic Edition software package

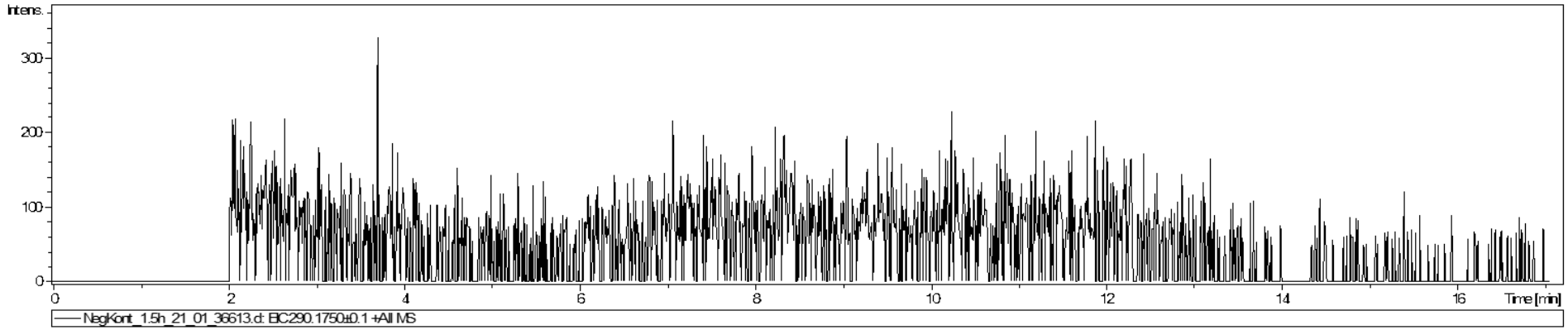

Figure A-3: LC-MS results of the negative control. The disrupted cells were incubated with 1 mM tropine and putative phenyllactyl-CoA. Extracted ion chromatograms (EIC) of *m/z* 290.18 (littorine).

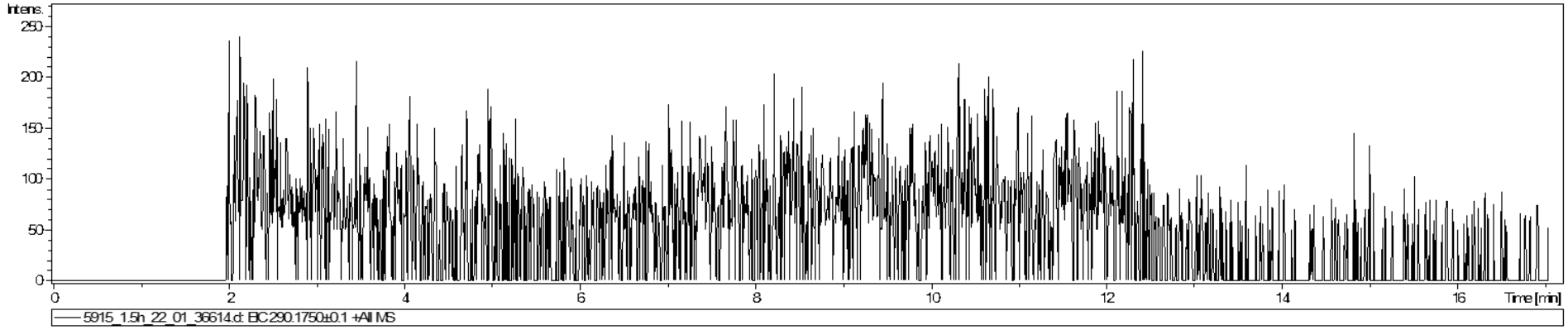

Figure A-4: LC-MS results of contig#5915. The disrupted cells were incubated with 1 mM tropine and putative phenyllactyl-CoA. Extracted ion chromatograms (EIC) of *m/z* 290.18 (littorine).

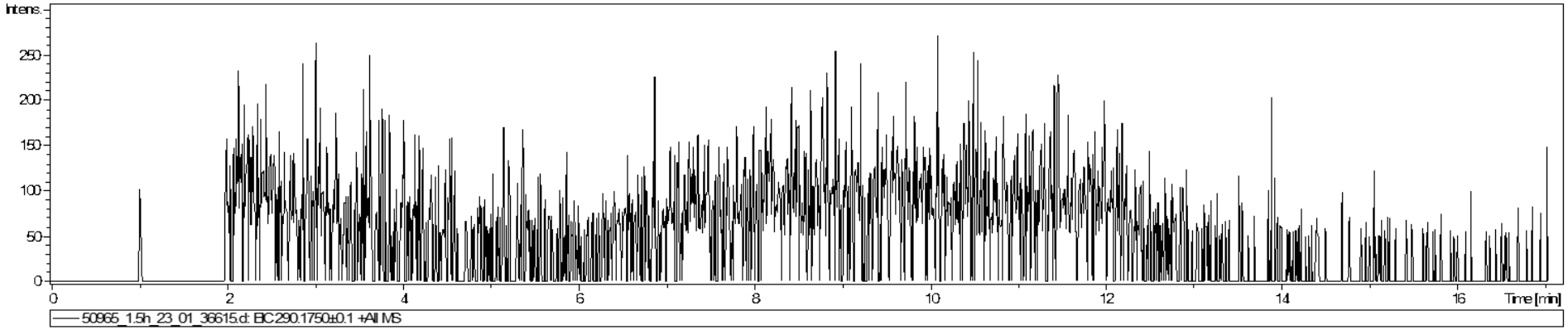

Figure A-5: LC-MS results of contig#50965. The disrupted cells were incubated with 1 mM tropine and putative phenyllactyl-CoA. Extracted ion chromatograms (EIC) of *m/z* 290.18 (littorine).

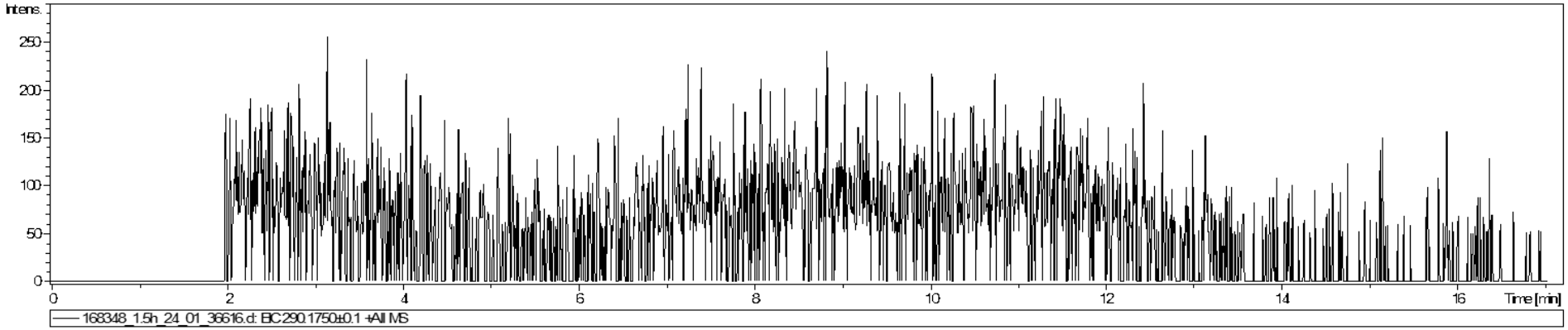

Figure A-6: LC-MS results of contig#168348. The disrupted cells were incubated with 1 mM tropine and putative phenyllactyl-CoA. Extracted ion chromatograms (EIC) of *m/z* 290.18 (littorine).

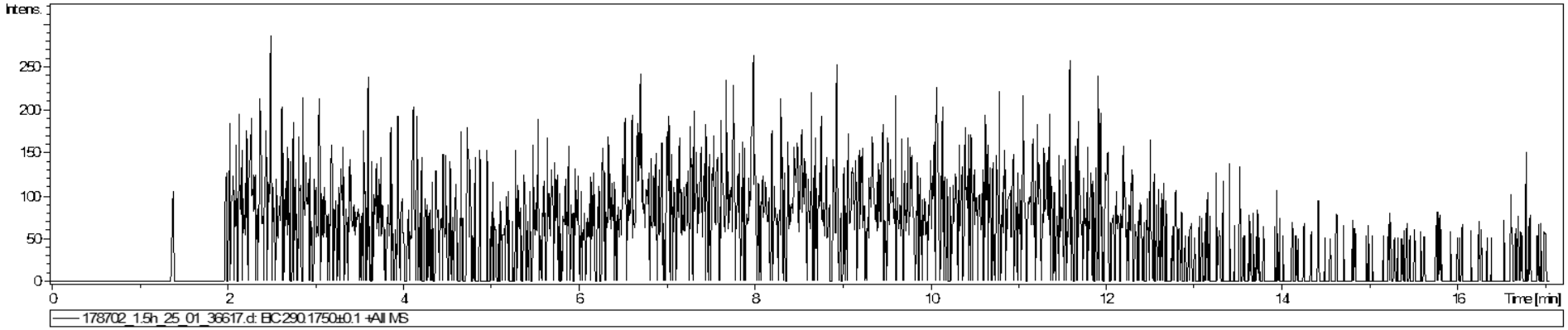

Figure A-7: LC-MS results of contig#178702. The disrupted cells were incubated with 1 mM tropine and putative phenyllactyl-CoA. Extracted ion chromatograms (EIC) of *m/z* 290.18 (littorine).

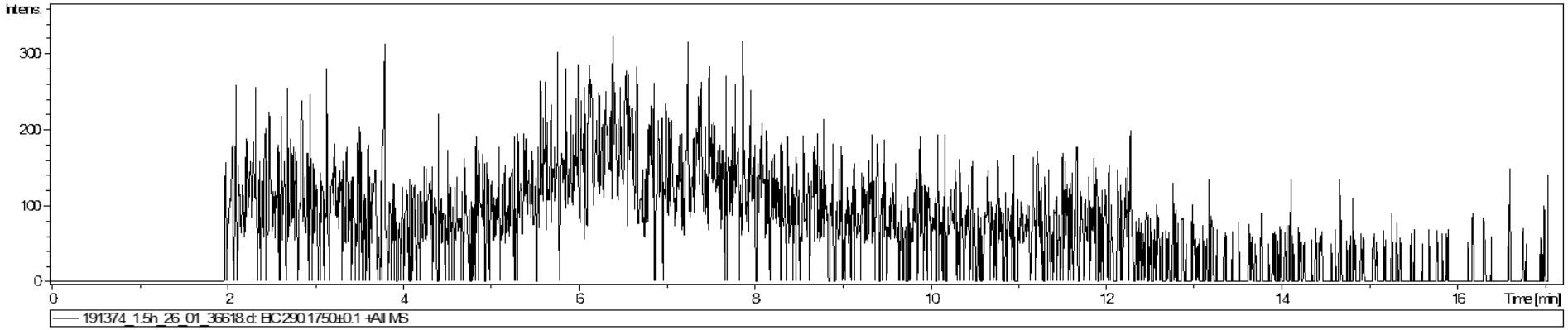

Figure A-8: LC-MS results of contig#191374. The disrupted cells were incubated with 1 mM tropine and putative phenyllactyl-CoA. Extracted ion chromatograms (EIC) of *m/z* 290.18 (littorine).

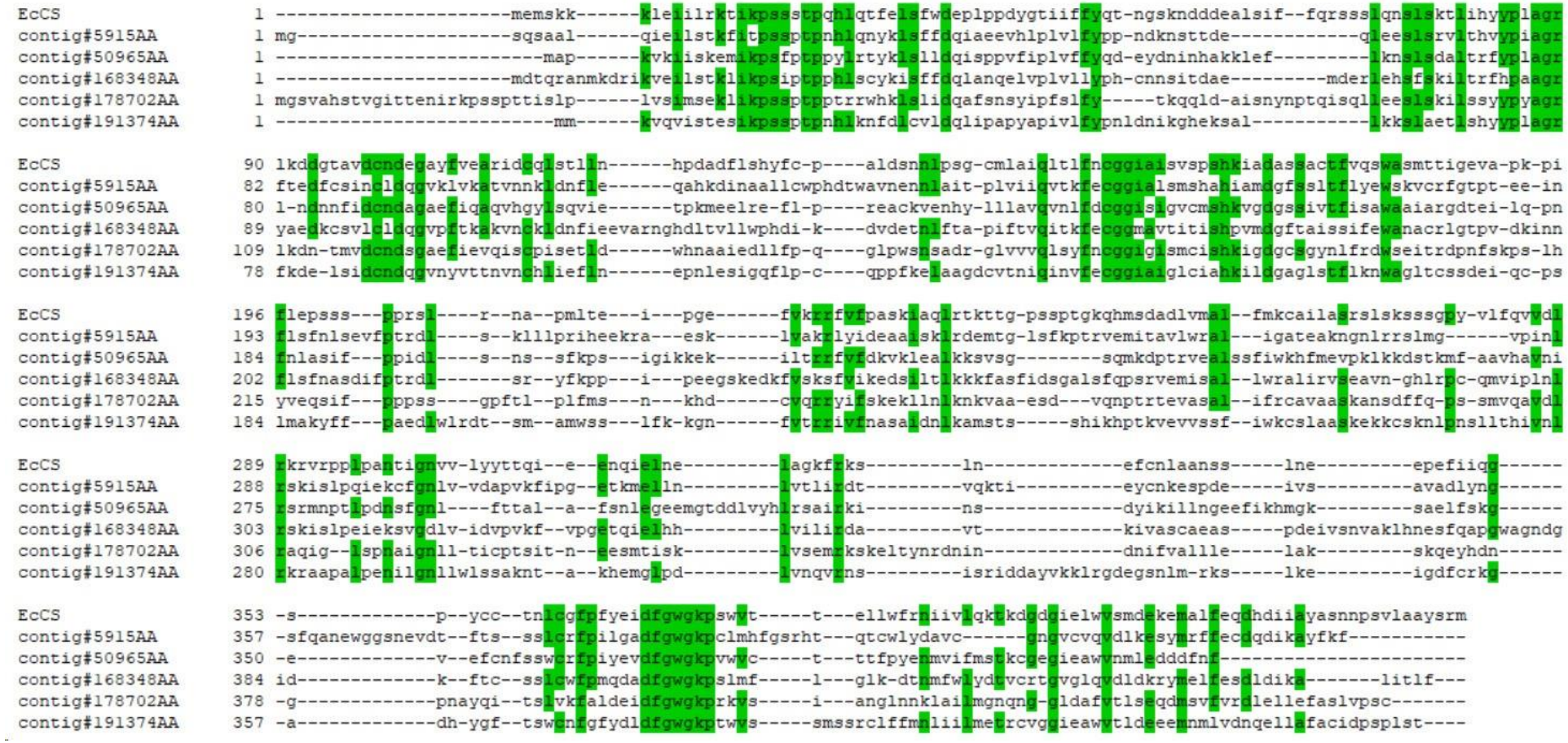

Figure A-9: Enlargement of Figure 3-12; Amino acid sequence alignment of EcCS and the five putative candidate gene sequences. The alignment was generated using the software Clone Manager 9 Professional Edition (Sci-Ed Software, Denver, USA). Matching amino acids within the alignment are colored in green.

LIST OF PUBLICATIONS

PEER REVIEWED ARTICLES

Kohnen, K. L.; Sezgin, S.; Spiteller, M.; Hagels, H.; Kayser, O. Localization And Organization Of Scopolamine Biosynthesis In *Duboisia myoporoides* R. BR., *Plant Cell Physiol.* 2017, *0*, 1–12, doi:10.1093/pcp/pcx165.

Kohnen-Johannsen, K. L., Kayser, O. Tropane Alkaloids: Chemistry, Pharmacology, Biosynthesis and Production, *Molecules* 2019, *24*, 796. https://doi.org/10.3390/molecules24040796

Oral Presentations

Kohnen, K. L., Kayser, O., Molecular elucidation of late tropane alkaloid biosyntheis., 2nd DISCO Progress & Review Meeting, 03.- 05.11.2015, Brussels, Belgium

Kohnen, K.L., Kayser, O., Biosynthesis of scopolamine, Postgraduate workshop of the section „Pflanzliche Naturstoffe",21.-23.09.2014, Nuremberg, Germany

Poster Presentations

Kohnen, K. L., Kayser, O., Quantitative and qualitative investigation of *Duboisia myoporoides* R. BR., 9th Joint Natural Products Conference (JNPC2016), 24.-27.07.2016, Copenhagen, Denmark

Kohnen, K. L., Ullrich, S.F., Averesch, N.J.H., Kayser, O., Principal Studies on Scopolamine Biosynthesis in *Duboisia* spec. for Heterologous Reconstruction of Tropane Alkaloid Biosynthesis, Metabolic Engineering 11, 26.-30.06.2016, Kobe, Japan

Kohnen, K. L., Kayser, O., Spatial localisation of intermediates involved in the tropane alkaloid biosynthesis, Tag der Chemie, TU Dortmund, 13.02.2015, Dortmund, Germany

CURRICULUM VITAE

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Place of Birth	Berlin
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Since 11/2017	Pharmacist at Koog-Apotheke, Brunsbüttel
Since 03/2014	Ph.D. student at the Department of Biochemical and Chemical Engineering (BCI), TU Dortmund University
07/2013 – 12/2013	Pharmacist (practical year), Fortunatus Apotheke, Berlin
01/2013 – 06/2013	Pharmacist (practical year), Hoffmann-La Roche, Basel, Switzerland
10/2008 – 11/2012	Christian-Albrechts-University zu Kiel, Study of Pharmacy
07/1999 – 08/2008	Gymnasium Marianum, Meppen
07/1995 – 06/1999	Clemensschule, Wesuwe