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Isolation and biochemical Characterization of Cytochrome P450 Oxidoreductases in 3-years old Norway Spruce (*Picea abies*) samplings

Master Thesis

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Submitted by
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Abbreviations

ATA	Aurintricarboxylic acid
bp	basepairs
cDNA	Complementary DNA
CYP	Cytochrome P450 monooxygenase
CYP720B	Cytochrome P450 mono-oxygenase 720B subfamily
ddH_2O	Double-distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
DRA	Diterpene resin acid
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
EIC	Extracted ion chromatogram
g	gravitational force
GC-MS	Gas chromatograph-mass spectrometer
IPTG	Isopropyl-β-D-thiogalactopyranoside
Iso	Isopimaradiene
LAS	Levopimaradiene/ abietadiene
LB	Luria-Bertani broth
m/z	Mass-to-charge ratio
MeJA	Methyl jasmonate
NADPH	Nicotinamide adenine di-nucleotide phosphate
$(NH_4)_2CO_3$	Ammonium carbonate
P. abies*/*Pa	*Picea abies*
PCR	Polymerase chain reaction
qPCR	Quantitative Rea-Time PCR
RNA	Ribonucleic acid
rpm	Revolutions per minute
R_t	Retention time
S. cerevisiae	*Saccharomyces cerevisiae*
TB	Terrific broth
TBME	Tert-butyl methyl ether
TEG	TRIS EDTA glucose
TEK	TRIS EDTA potassium chloride
TES	TRIS EDTA sorbitol
TIC	Total ion chromatogram
T_m	Melting temperature
TMSH	Trimethyl sulfonium hydroxide
TPS	Terpene synthase
TRIS	Tris(hydroxymethyl) aminomethane
YPGA	Yeast extract peptone glucose adenine medium

Abstract

Through their long entire life cycle, conifers have to cope with different environmental challenges like herbivores or pathogenic pests. To protect themselves, they developed several physiological and chemical mechanisms. One group of these defense-responsible secondary metabolites are so called terpenes, which are present in the whole plant kingdom.

Most frequent representatives are monoterpenes (C_{10}), sesquiterpenes (C_{15}) and diterpenes (C_{20}). All terpenes are synthesized upon C_5 isopentenoid blocks into geranyl diphosphate, farnesyl diphosphate and geranylgeranyl diphosphate, catalyzed by different isoprenyl diphosphate synthases (IDS). These compounds are cyclized by terpene synthases (TPS) into diverse structures of monoterpenes, sesquiterpenes or diterpenes.

A characteristic of conifers is the occurrence of diterpene resin acids (DRA), which are stored in resin ducts in the phloem of the stem and in needles. This oxidized form of diterpene olefins emerge from open wounds and is able to capture invading organisms and to seal the wounding site. The three-step oxidation from diterpene olefin to the diterpene resin acids is catalyzed by Cytochrome P450 Monooxygenases (CYP) of the CYP720B subfamily.

This master thesis focuses on CYP720B enzymes in Norway Spruce (*Picea abies*). The saplings were treated with methyl jasmonate (MeJA) to analyze if there is a correlation between DRA accumulation and CYP720B transcript activation. Further, the distribution of the enzymes among the different tissues needle, roots and stem were examined. It turned out, that CYP720B1 was mostly present in needles, CYP720B11 in roots and CYP720B4 in needles and stem.

To receive additional substrates for the enzyme assays with CYP720B1 and CYP720B4, the levopimaradiene/abietadiene and isopimaradiene terpene synthases *Pa*TPS-LAS and *Pa*TPS-ISO were expressed in *Escherichia* coli (*E. coli*). These diterpene olefins were further used as substrates for enzyme assays. In the assays, CYP720B1 showed no activity during the tests, whereas CYP720B4 was active. CYP720B4 was shown to be a multifunctional, multisubstrate enzyme, able to convert abietadiene, dehydroabietadiene and dehydroabietadienol into the corresponding resin acids and aldehyde intermediate, respectively.

Through this study, there is a deeper understanding about the distribution of expression levels of Cytochrome P450 Monooxygenases within the different organs in Norway Spruce and their essential role in the DRA biosynthesis. With the methodology used in this project, it will be possible to further characterize CYP720B4 and also the other Cytochrome P450s involved in Norway Spruce DRA biosynthesis.

Zusammenfassung

Im Laufe ihres Lebens sind Koniferen einer Vielzahl unterschiedlichsten Umwelteinflüssen wie etwa Herbivorie oder pathogenen Erregern ausgesetzt. Um sich davor zu schützen, entwickelten sie verschiedenste physiologische und chemische Verteidigungsmechanismen. Eine Gruppe der für die chemische Abwehr verantwortlichen sekundären Metabolite sind Terpene, welche im gesamten Pflanzenreich zu finden sind. Die häufigsten Vertreter sind Monoterpene (C_{10}), Sesquiterpene (C_{15}) und Diterpenes(C_{20}). Alle Terpene sind aus C_5 Isoprenyl-Einheiten aufgebaut, deren Kondensation zu Geranyldiphosphat, Farnesyldiphosphat und Geranylgeranyldiphosphat von verschiedenen Prenyltransferasen katalysiert wird. Die Verbindungen werden von Terpenesynthasen (TPS) in verschiedenste Mono-, Sesqui- und Diterpene zyklisiert.

Ein Merkmal von Koniferen ist das Vorhandensein von Harzsäuren, welche in speziellen Harzkanälen im Phloem des Stamms und in Nadeln gelagert werden. Die oxidierte Form der Diterpene wird bei Beschädigungen an Holz und Rinde freigesetzt. Das Baumharz umschließt eindringende Organismen und verschließt die Wunde. Die Oxidation von Diterpenen zu Harzsäuren erfolgt in drei Schritten, welche von Cytochrom P450 Monooxygenasen (CYP) aus der CYP720B Unterfamilie katalysiert werden.

Die vorliegende Master Thesis beschäftigt sich mit CYP720B Enzymen aus der Gemeinen Fichte (*Picea abies*). Die Bäume wurden zunächst mit Methyljasmonat (MeJA) behandelt, um eine Korrelation zwischen einem erhöhten Terpengehalt im Vergleich zur Kontrolle und der Expression von CYP720B Gen-Transkripten zu finden. Des Weiteren wurde der Expressionsgrad der Enzyme in den verschiedenen Organen Nadel, Wurzel und Stamm bestimmt. CYP720B1, CYP720B11 und CYP720B4 wurden hauptsächlich in Nadeln, Wurzeln sowie in Nadeln und Stamm exprimiert.

Zur Gewinnung zusätzlicher Substrate für Enzymaktivitätstests mit CYP720B1 und CYP720B4 wurden die Levopimaradien/Abietadien sowie die Isopimaradien Terpenesynthasen (TPS) *Pa*TPS-LAS und *Pa*TPS-ISO in *Escherichia* coli (*E. coli*) heterolog exprimiert. Das Enzym CYP720B1 zeigte sich in den Tests als inaktiv, wohingegen CYP720B4 Aktivität aufwies. CYP720B4 ist ein multifunktionelles, mehrere Substrate verwendendes Enzym. Es ist fähig, Abietadien, Dehydroabietadien und Dehydroabietadienol als Substrat zu nutzen und die Reaktion zur Synthese der jeweiligen Harzsäuren und des Aldehyd-Intermediats zu katalysieren.

Durch dieses Projekt konnte ein tieferes Verständnis über die Expressionsgrade von Cytochrom P450 Monooxygenasen in den unterschiedlichen Organen der Gemeinen Fichte sowie essentielle Erkenntnisse zu ihrer Rolle in der Harzbiosynthese gewonnen werden. Mit den in dieser Arbeit etablierten Methoden ist es nun möglich, CYP720B4 weiter zu charakterisieren, so dass auch die weiteren in die Harzbiosynthese involvierten Cytochrom P450 somit untersucht werden können.

1. Introduction

Conifer trees are gymnosperms of the order *Pinales*, which separated from the angiosperms more than 300 million years ago and from *Pinus* over 100 million years ago. In their long life, the sessile trees have to deal with a multitude of biotic and abiotic stresses. Abiotic factors include climatic conditions as well as soil physical, chemical and moisture characteristics. Biotic stresses include soil fauna, mobile pests and pathogens. During evolution, they adapted their defense and developed different mechanisms for protection.

One way is the constitutive and inducible chemical protection, mainly phenolic and terpenoid compounds [1, 2]. Constitutively, spruce stores copious amounts of resin terpenoids, which are kept in specialized resin ducts in the phloem of the stem and in needles [3-5].

Keeling and Bohlmann showed in 2006, that there is a positive correlation between terpenoid concentrations and the resistance of *Picea glauca* to white pine weevil attacks, the main herbivore [6]. If there is a mechanical damage, insect herbivory or fungal inoculation, the induced defense is activated. The tree alters the developmental program of xylem mother cells to induce de novo formation of traumatic resin ducts in xylem (Figure 1.1), polyphenolic parenchyma cells in secondary phloem and terpenoid biosynthesis. Terpenoid accumulation in xylem and volatile emission are also increased [7]. The same reactions can be observed by treating plants with methyl jasmonate (MeJA), which was used in this project. Methyl jasmonate is an octadecanoic phytohormone, which is involved in signaling mechanical wounding, herbivory, oviposition or fungal inoculation. In research for plant defense, it is often used for topical application as non-destructive mimic of wounding or insect herbivory by stem-boring insects and microbial pathogens [3, 6, 8, 9].

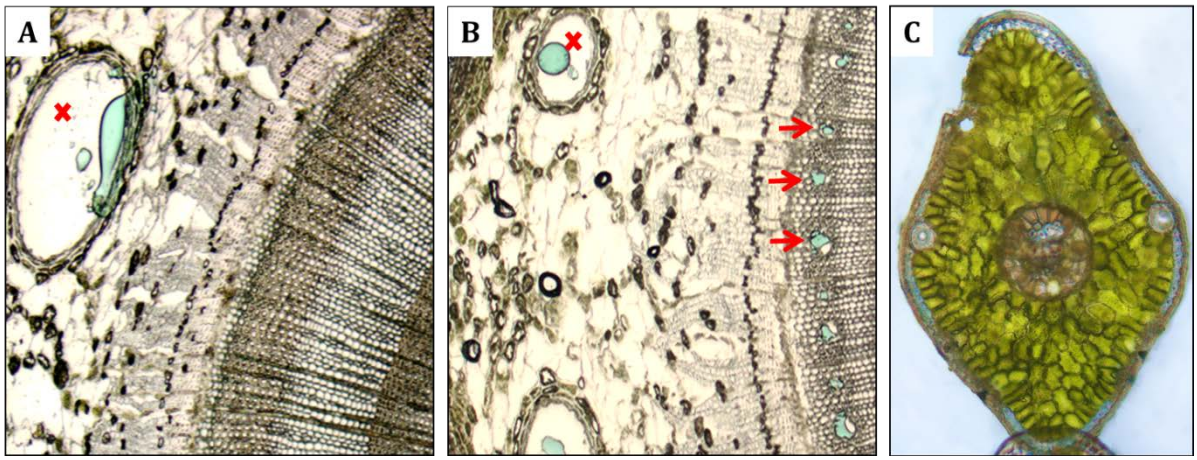

Figure 1.1: Cross sections of Norway Spruce stem and needle. A: Untreated stem control without resin ducts in the constitutive xylem but large constitutive resin duct in the cortex (red crosses). **B**: Stem two months after treatment with circular arranged, fully formed traumatic ducts (red arrows) in the newly developed xylem[3]. **C**: Needle without resin ducts [10].

Terpenes are synthesized from C_5 isopentenoid building blocks, forming the largest class of secondary metabolites. They are divided into hemiterpenes (C_5), monoterpenes (C_{10}), sesquiterpenes (C_{15}) diterpenes (C_{20}), sesterterpenes (C_{25}), triterpenes (C_{30}) and polyterpenes (>C_{40}). Terpenes can be found across the whole plant kingdom with more than 43,000 known structures. Due to the long existing opinion that terpenes as secondary metabolites are not responsible for plant growth, in the 1970s scientist like Hemingway, Hart or Porter started to search for their role in nature [11-15]. Terpenoids have evolved over millions of years for chemical communication in interactions between plants and competitors, beneficial insects, herbivores or microbial pathogens. In addition, the compounds serve to repel, kill, inhibit and also reduce the success of invading species in plant-herbivore and plant-pathogen interactions [16, 17].

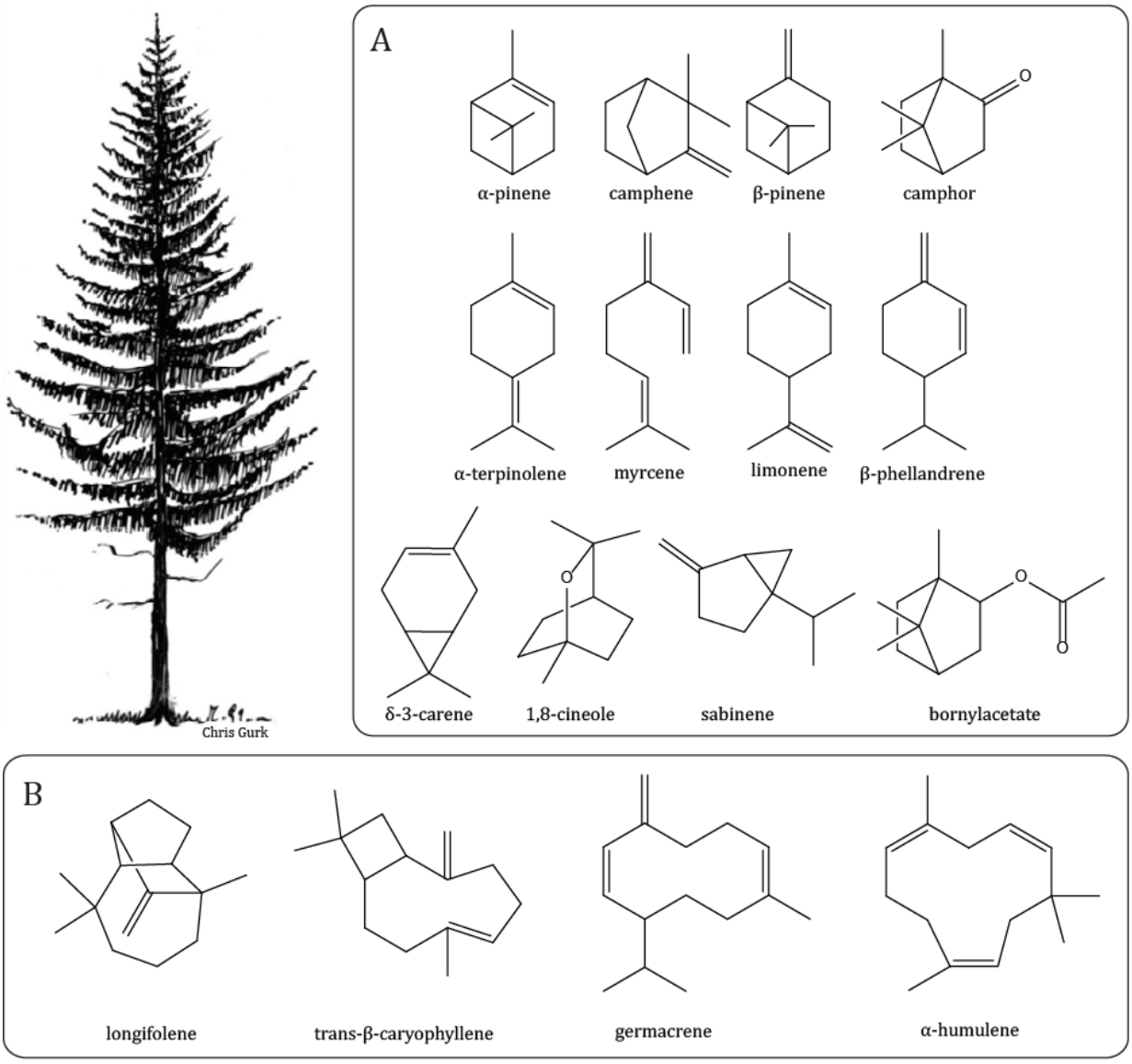

Figure 1.2: Monoterpenes and sesquiterpenes present in Norway Spruce. Silhouette of *Picea abies* (Norway Spruce). **A**: The 12 common monoterpenoids consist of two C_5-units forming a $C_{10}H_{16}$ skeleton. Exeptions were camphor, 1,8-cineole and bornylacetate, which also contain oxygen. 1,8-cineol and bornylacetate also differ by their number of hydrogen and carbon atoms. **B**: Sesquiterpenes consist of three C_5-units with the molecular formula $C_{15}H_{24}$. There are ony four compounds relevant in *P. abies*.

Monoterpenes and DRAs are present in the trees about equal amounts, together more than 98% of resin terpenoids in stems, whereas sesquiterpenes accumulate in smaller quantities. Figure 1.2 shows the twelve monoterpenes and four sesquiterpenes, which are usually present in Norway Spruce [3, 8, 18, 19]. Mono- and sesquiterpenoids are volatile compounds, whereby their emission is influenced by light, temperature and biotic stress factors like pathogens and herbivores. Due to their volatility, they are able to attract pollinators, repel herbivores from a greater distance or attract predators and parasites of arthropod herbivores. Additionally, the volatiles can influence insect communication by interfering with insect development as hormone analogues or pheromone precursors [20].

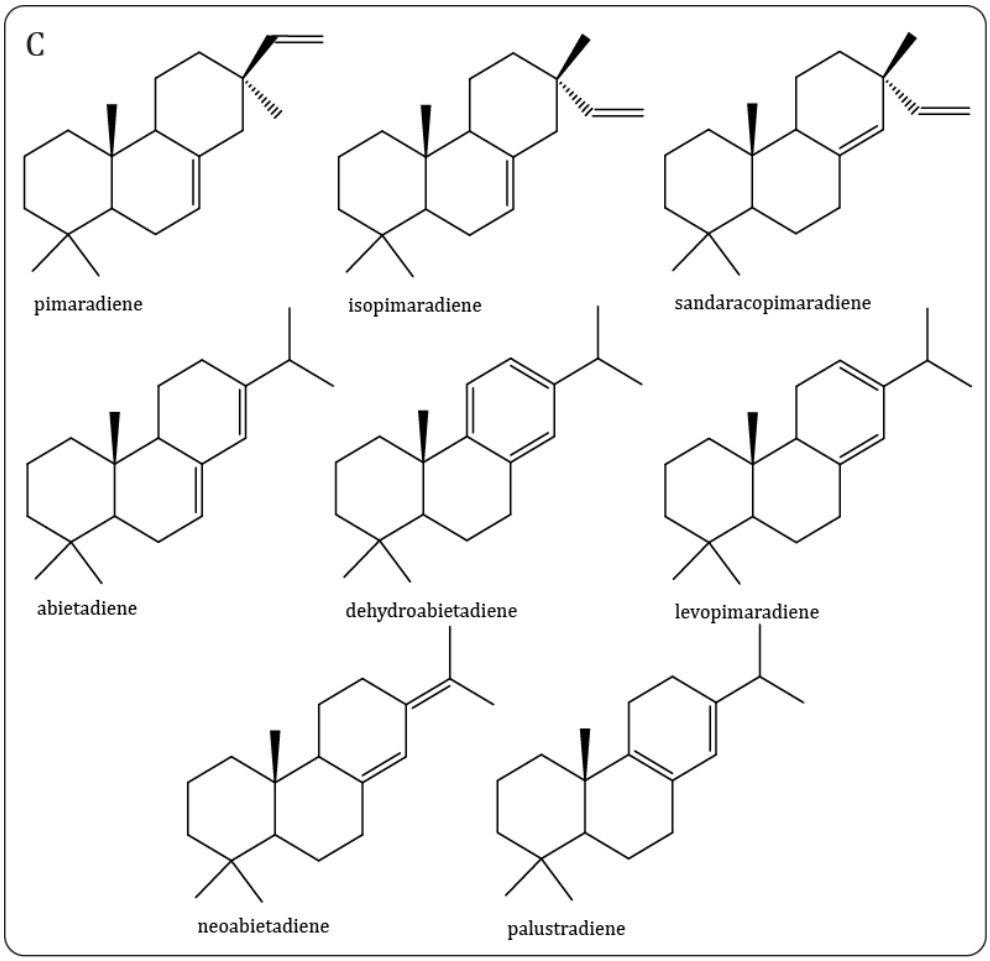

Figure 1.3: Diterpene olefins present in Norway Spruce. The olefins were formed by four isoprene units building up a $C_{20}H_{32}$ skeleton. They were catalyzed by two terpene synthases, TPS-LAS and TPS-ISO. Pimaradiene, isopimaradiene and sandaracopimaradiene differ in their structure with an end standing double bond from diterpenes of abietane-type by TPS-LAS enzyme.

1.1 Diterpene resin acids

Diterpenoids numbers about 12,000 identified metabolites. In contrast to the volatile nature of mono- and sesquiterpenoids, diterpene olefins are non-volatile, viscous substances, which are in conifers predominantly present in their oxidized form as diterpene resin acids (DRA). Due to their oxidation, the compounds became more polar and obtain an adhesive consistence. Diterpene resin acids are tricyclic carboxylic acids built of 20 carbon atoms with several skeletal types. They differentiate into double-bond isomers, diastereoisomers, and additional functionalization. The common diterpene resin acid mixture contains almost equal amounts of palustric acid, levopimaric acid, abietic acid, neoabietic acid and only small amounts of sandaracopimaric acid and pimaric acid [6, 21].

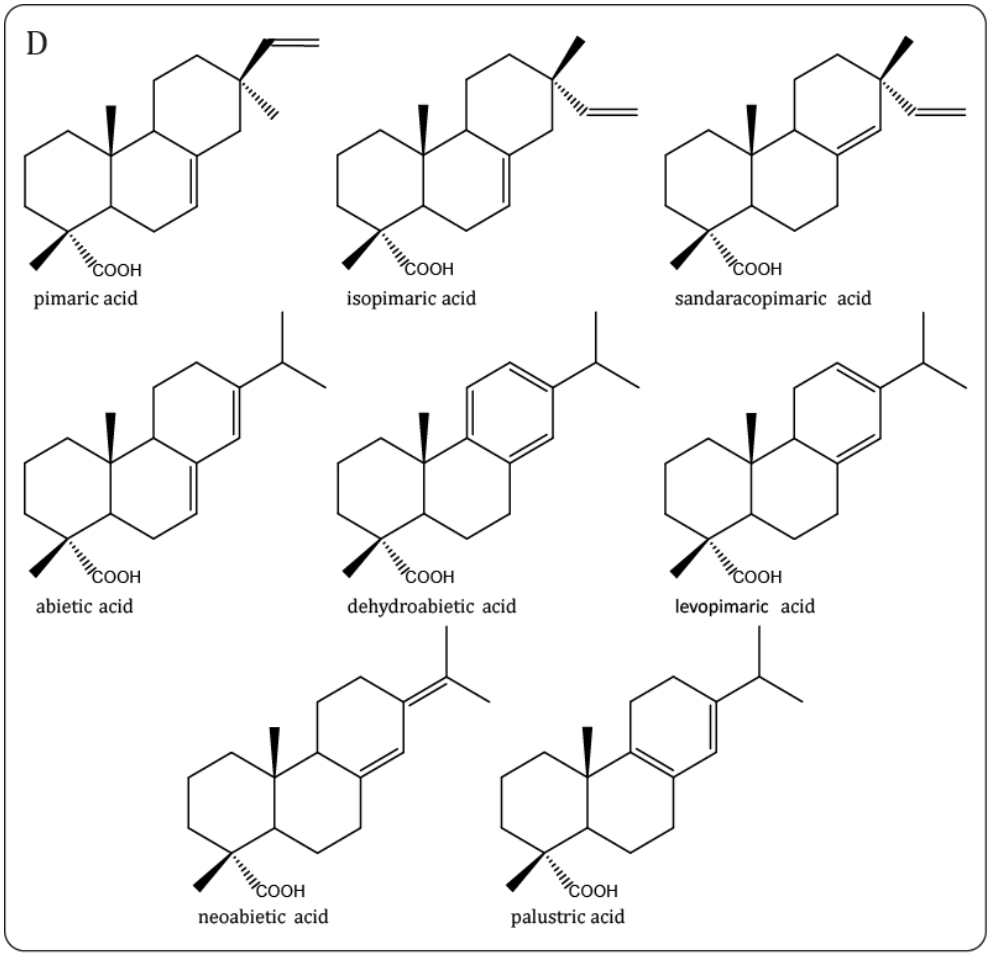

Figure 1.4: Diterpene resin acids present in Norway Spruce. Via an oxidation at the C18-atom, the acid is formed out of the diterpene olefins. Thus this modification, the compounds become more polar and obtain their adhesive consistence. If the monoterpenes evaporate over time, the resin acids crystalize and form a compact barrier against invading organisms.

If there is a wounding site, a monoterpene and DRA mixture is released from the resin ducts. By the releasing pressure, the resin flow is able to push the insect outside the tissue or to capture them (see Figure 1.5, C). The monoterpenes, which enable the fluidity of the mixture, evaporate over time. Exposed to the air, the remaining diterpene resin acids start to crystalize. It seals the wound and cleans the site from microorganisms to build a compact barrier against invading organisms [6, 9, 22].

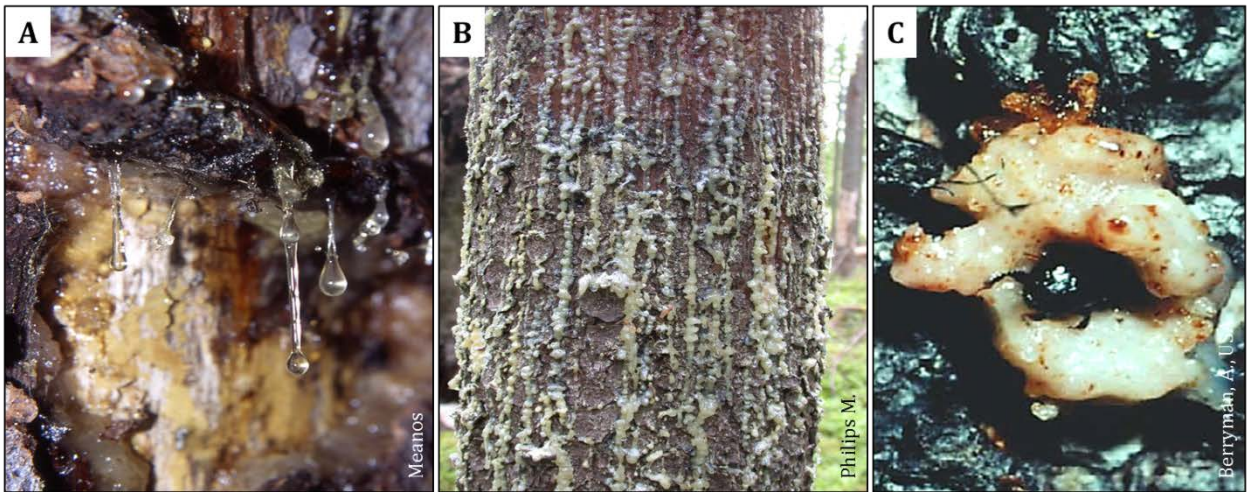

Figure 1.5: Escaped resin flows on bark surfaces. A: Freshly escaped, still fluid resin at open wounding site. **B**: Stem fully covered by hardened resin flows. **C**: Sealed wounding site with resin plug including captured insect.

1.2 Biosynthesis

Isopentenyl-5-diphosphate (IDP) and dimethylallyl-diphosphate (DMADP) are the C_5 units building up the terpenes. Both derive from two possible pathways and can get interconverted by isoprenyl diphosphate isomerase (IDI). The pathways are independent, but also able to do metabolic cross-talk and substrate exchange [23, 24]. First described was MEV- or MVA-pathway (mevalonic acid), starting with condensation of two acetyl-Coenzyme A molecules. At the end of the pathway, mevalonate is decarboxylated into IDP. This pathway primarily exists in eukaryotic cytosol and endoplasmic reticulum [3]. The second possible pathway is the MEP-pathway (methylerythritol phosphate), which is mainly found in prokaryotes and plant plastids. The pathway starts with condensation of glyceraldehyde-3-phosphate and pyruvate. Here, main product is DMADP [11, 17]. In plants, the dominating pathway for farnesyl diphosphate (FDP) is the MEV-pathway, whereas the MEP-pathway produces geranyl diphosphate (GDP) and geranylgeranyl diphosphate (GGDP). A general overview is drawn in Figure 1.6 [7, 23].

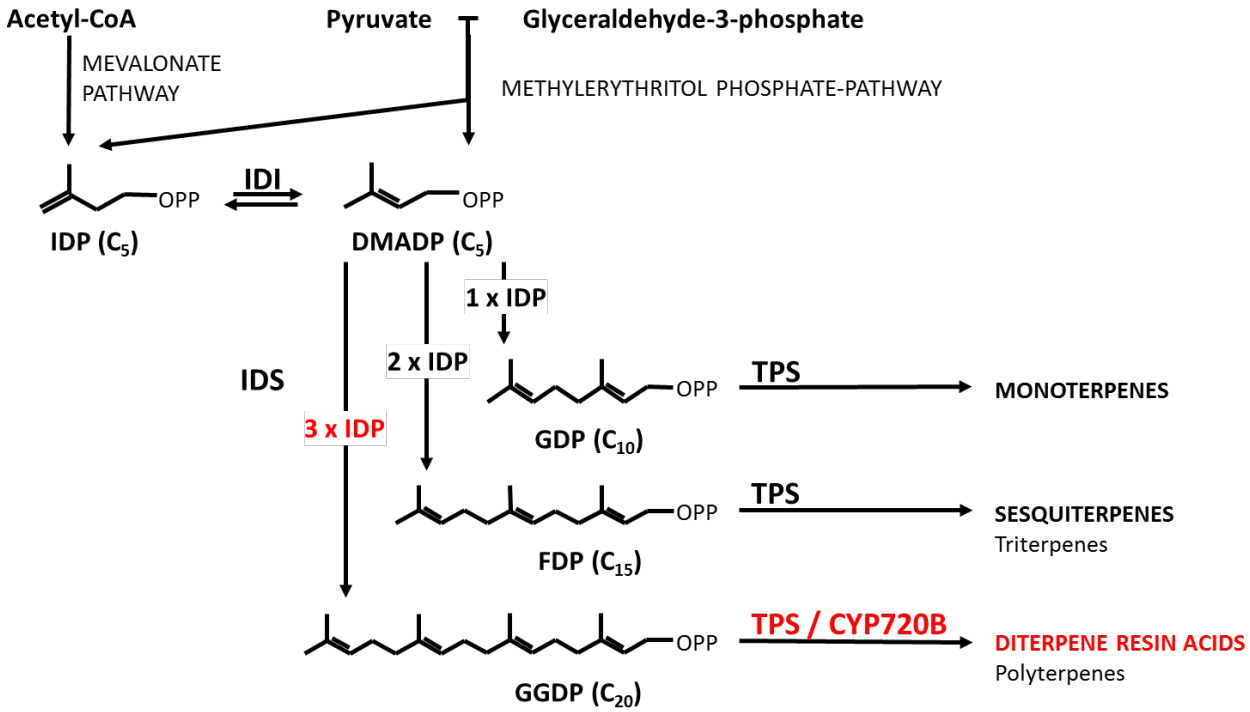

Figure 1.6: Overview of terpenoid pathways. Both, MEP and MEV pathway were resulting in cytosolic Isopentenyl-5-diphosphate (IDP) and dimethylallyl-diphosphate (DMADP). They build up geranyl diphosphate (GDP), farnesyl diphosphate (FDP) and geranylgeranyl diphosphate (GGDP), the precursors for terpene synthases.

Therefore DMADP is undergoing condensation reactions with several IDP molecules, catalyzed by isoprenyl diphosphate synthases (IDS), also called prenyl transferases. Geranyl diphosphate synthases (GDPS), farnesyl diphosphate synthases (FDPS) and geranylgeranyl diphosphate synthases (GGDPS) are using one, two and three units of isoprenyl diphosphate, respectively, to form GDP, FDP and GGDP. Due to their central position in the pathway, IDS have regulatory function

in terpene biosynthesis. After overexpression, there is an accumulation of geranylgeranyl fatty acid esters, which decrease the growth and survival of the herbivores. GDP is the linearized version of monoterpenes. Sesquiterpenes and sterols, primary metabolites, are cyclized from FDP, whereas diterpenes, gibberellins, carotenoids, ubiquinones and phytols derive from GGDP [25-27].

1.2.1 Terpene synthases

The linear GDP, FDP and GGDP molecules are further modified by terpene synthases (TPS). They are grouped into *mono*-, *sesqui*- and *di*TPS, depending on their substrate specialization to GDP, FDP and GGDP, respectively. Like IDS, they are also induced upon MeJA treatment or herbivory. Considering together, all land plants *mono*TPS form approximately 3,800 monoterpenes. The universal intermediate α-terpinyl, formed out of GDP, can be transformed to different monoterpenes via deprotonation, cyclization, hydride shift or water capture [8]. About 13,000 sesquiterpenoids are converted from FDP over 6(R/S)-bisabolyl cation intermediate by *sesqui*TPS [19, 28, 29]. *Mono*- and *sesqui*TPS can catalyze either single or multi products, whereat in general *sesqui*TPS are also even able to use GDP as substrate.

*Mono*TPS typically have a size of 600-650 amino acids, whereas *sesqui*TPS are 50-70 amino acids shorter by the absence of the N-terminal transit peptide for plastid targeting. *Di*TPS are usually around 210 amino acids longer than *mono*TPS [23]. All TPS utilize an electrophilic reaction mechanism, assisted by divalent metal ion Mg^{2+}/Mn^{2+} cofactors [4].

11,600 diterpenoids are synthesized from the acyclic biosynthetic precursor GGDP by *di*TPS. One mechanism is the macrocyclization, analogous to MT and ST to receive macrocyclic diterpenes like taxadiene. By generation of (+)-copalyl diphosphate, tricyclic and tetracyclic diterpenoids like kaurene (gibberellins) and abietadiene (terpenoids) are formed, which is shown in Figure 1.7 [5, 17, 19, 22]. Differences occur in size and shape of the active sites [1, 30]. In 2008, Keeling *et al.* found two diterpene synthases in *P. abies*, which are called isopimaradiene synthase *Pa*TPS-ISO and levopimaradiene/abietadiene synthase *Pa*TPS-LAS [16]. *Pa*TPS-ISO catalyzes isopimaradiene and only small amounts of sandaracopimaradiene, whereas *Pa*TPS-LAS is producing a mixture of abietadiene, levopimaradiene, neoabietadiene, dehydroabietadiene and palustradiene. Starting from GGDP, there is a diphosphate ionization-initiated cyclization of (+)-copalyl diphosphate to isopimar-15-en-8-yl carbocation intermediate. *Pa*TPS-ISO is catalyzing a deprotonation, which leads to isopimaradiene or sandaracopimaradiene. *Pa*TPS-LAS instead catalyzes an intramolecular proton transfer and a 1,2-methyl migration, which results in an abieta-8(14)-en-13-yl carbocation. The carbocation takes up a water molecule to form the instable 13-hydroxy-8(14)-abietene, which converts to abietadiene, levopimaradiene, neoabietadiene and palustradiene [9, 16, 31].

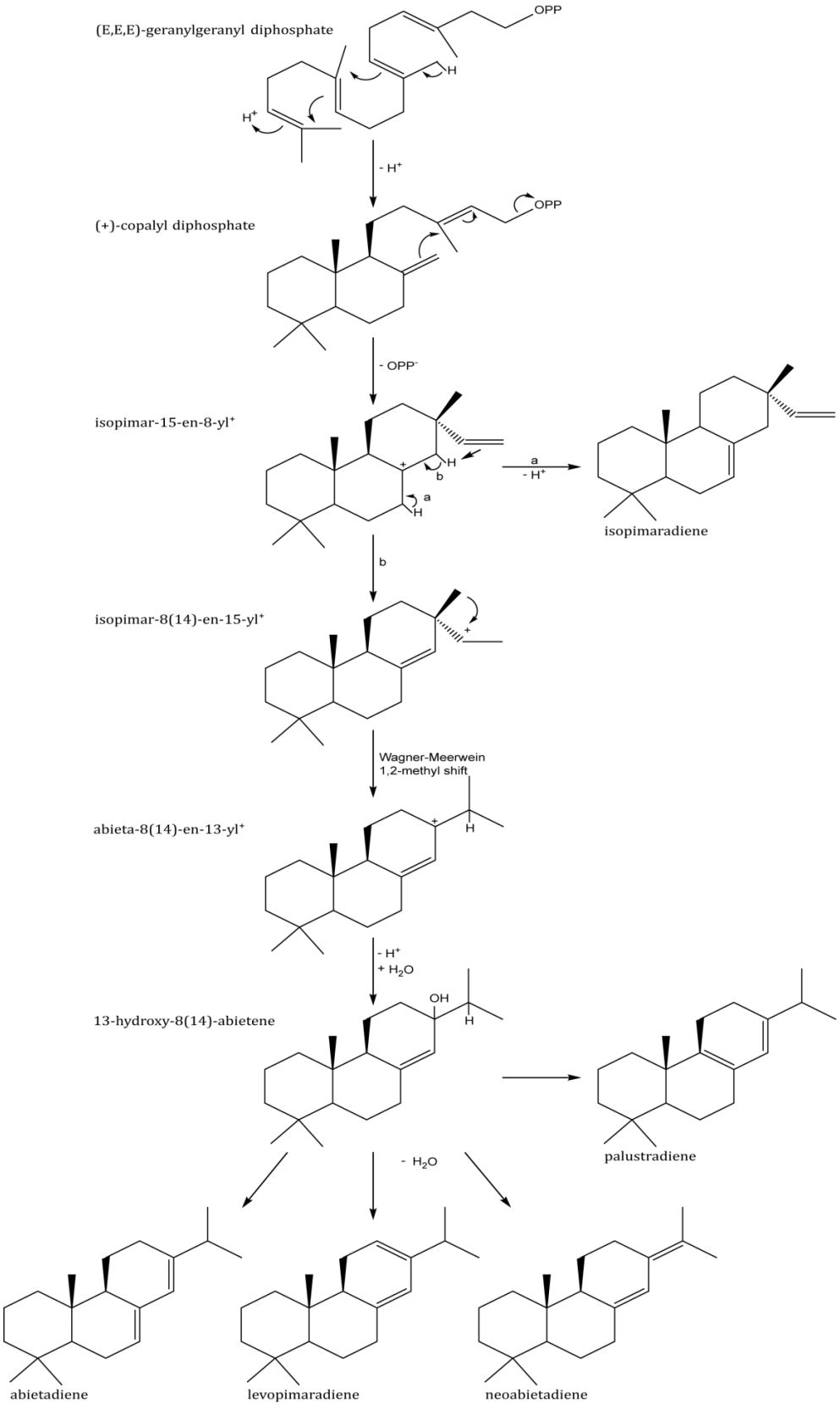

Figure 1.7: Scheme of diterpene olefin biosynthesis by *Pa*TPS-LAS and *Pa*TPS-ISO.The proposed mechanism starts by the formation of (+)-copalyl diphosphate from (E,E,E)-geranygeranyl diphosphate (GGDP). After the cleavage of diphosphate, the proton substraction (**a**) leads to isopimaradiene and sandaracopimaradiene in *Pa*TPS-ISO. Route (**b**) in *Pa*TPS-LAS includes a Wagner-Meerwein 1,2-methyl shift and incorporation of a water molecule. This results in an unstable 13-hydroxy-8(14)-abietene carbocation, which converts by water susbtraction and double bond formation into abietadiene, levopimaradiene, neopimaradiene and palustradiene.

1.2.2 Cytochrome P450

The last step in diterpene biosynthesis is the conversion of diterpene olefins into diterpene resin acids. This reaction is catalyzed by Cytochrome P450 monooxygenases. They belong to the oldest existing plant P450 superfamily containing over 18,000 versatile enzymes. In plants they are mostly membrane-associated, anchored in the endoplasmatic reticulum via their N-terminal membrane domain, but there are also some solved in mitochondria and chloroplasts [21]. Cytochrom P450s oxidize and finally form all phytohormones and biopolymer subunits like lignin, pigments, flavors or defense compounds. About 97% of 46,000 known plant terpenoids are oxygenated at one or more carbon positions. Next to a structural diversity, the oxygenation increases their polarity and a decrease in volatility [12]. In nature, P450 enzymes catalyze most of the rate-limiting and completely irreversible reactions like regio-specific and stereospecific oxygenations or oxidations, for example [12]

- dealkylation
- deamination
- decarboxylation
- C–C cleavage
- ring expansion, ring opening, ring migration, ring coupling
- dehydration

Catalysis is performed by an electron consuming, heme-dependent mechanism, where two consecutive electrons were timed delivered to the heme-iron to form a ferrous ion and later on ferrous-oxy by binding O_2 [32]. Classical eukaryotic P450s either interact with NAD(P)H-dependent diflavin enzyme Cytochrome P450 reductase for microsomal P450s or with flavoprotein adrenodoxin reductase and the iron-sulfur protein adrenodoxin, if it is a mitochondrial P450 [6].

1.2.3 CYP720B family

The CYP720Bs are a subfamily of the CYP85 family, specialized in diterpene resin acid biosynthesis by catalyzing a three-step oxidation of diterpene olefins [28]. Today 62 different CYP720B sequences of different conifers have been discovered; five from *Picea abies*. Due to gene duplication, retention of multiple gene copies, subfunctionalization and neofunctionalization, a large gene family was formed. The metabolism of DRAs share high similarities with the biosynthesis of *ent*-kaurenoic acid, a gibberellic acid intermediate. Unlike enzymes involved in general phytohormone metabolism, some CYP720Bs widened their substrate range. They are able to accept structurally related diterpene olefins, alcohols or aldehydes, as shown in Figure 1.8 [22, 28].

The catalyzed oxidation at the C-18 atom in ring A by P450s leads to corresponding diterpene alcohols, aldehydes or acids. CYP720B1 in *Pinus taeda* has been the first detected and characterized enzyme of the conifer-specific CYP720B group. It is a multifunctional and multisubstrate enzyme, able to use diterpene olefins, alcohols and aldehydes as substrate and to form the consecutive resin acid. Also CYP720B4 from *Picea sitchensis* is a multifunctional Cytochrome P450, able to convert the diterpene olefins, alcohols and aldehydes, respectively. Whereas *Pt*CYP720B1 is mostly active using abietadienol and abietadienal, *Ps*CYP720B4 prefers dehydroabietadiene to form dehydroabietadienol and dehydroabietic acid [5, 33].

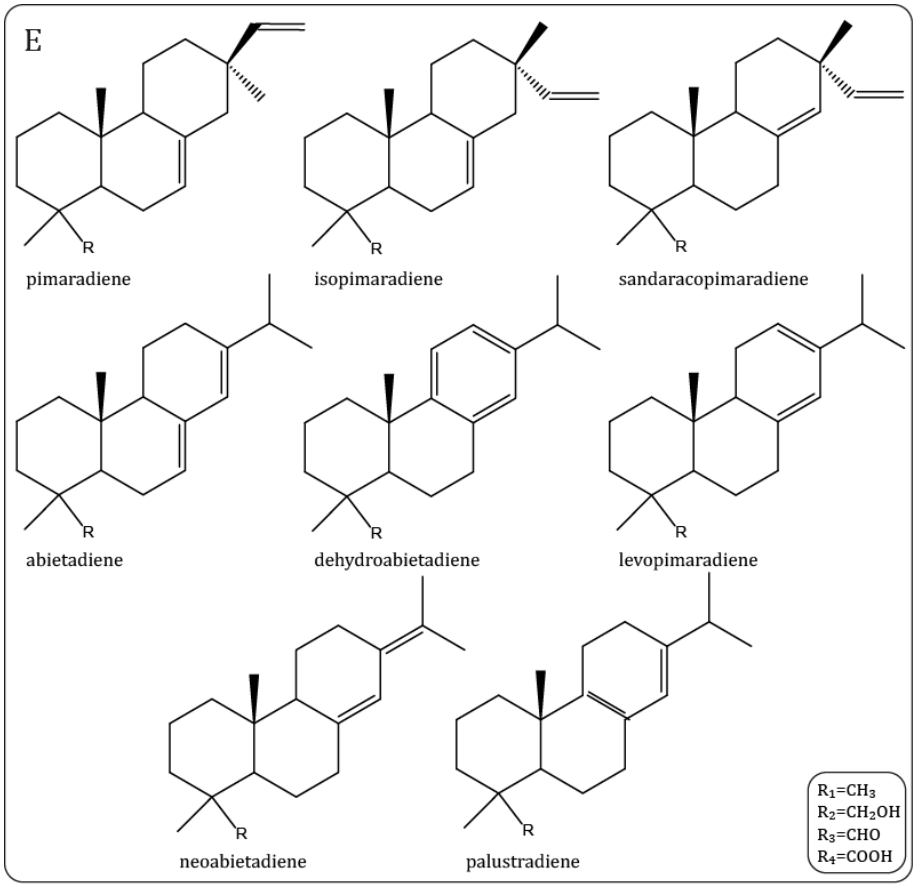

Figure 1.8: Overview about the different diterpenes present in Norway Spruce. Simplest diterpenes are diterpene olefins (**R_1**). Via an oxidation at C18-atom, the corresponding alcohols (**R_2**), aldehydes (**R_3**) and resin acids (**R_4**) are formed. The alcohols and aldehydes are intermediates during resin acid biosynthesis in CYP720Bs.

1.3 Aim of the study

The Norway Spruce is the most abundant conifer species in northern and central Europe. Due to the fast growth and the properties of the timber, the trees became economically important in forestry. To protect themselves of herbivory and pathogens, conifers developed a wide range of defense mechanism utilizing terpenes. Recent studies focused on the role of terpenes in plant defense and the biosynthesis of monoterpenes, sesquiterpenes and diterpene olefins. Since diterpenes are mainly present as resin acids, also the oxidation mechanism were studied. The responsible Cytochrome P450 monooxygenases of the CYP720B subfamily are found to catalyze a three-step oxidation from the olefin into resin acid but also to convert the resulting intermediates. Beside their multifunctionality, CYP720Bs are able to use the olefins, alcohols and aldehydes of various diterpene isomers.

To consider if the previous results also fit for the Cytochrome P450s found in Norway Spruce, this study aims to characterize these enzymes. In samples with induced defense the Cytochrome P450 candidates CYP720B1, B4, B6 and B11 are screened for metabolic and transcriptional changes of expression among different plant tissues. The expression levels of the CYP720B-encoding genes are analyzed towards methyl jasmonate treatment. The enzymes shall be amplified, cloned and heterologous expressed and characterized regarding their activity towards various substrates.

2. Materials and Methods

2.1 Materials

2.1.1 Chemicals

Name	Manufacturer
Dithiothreitol DTT	Carl Roth GmbH, Karlsruhe
Oligo(dT)$_{20}$	ThermoFisher Scientific, Waltham, USA
1 kb Plus DNA Ladder	ThermoFisher Scientific, Waltham, USA
1,9-Decadiene	Sigma-Aldrich Co., St. Louis, USA
10x Fast Digest Buffer	ThermoFisher Scientific, Waltham, USA
Ade hemisulfate, Arg, Asp, Cys, His, Ile, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val, Ura	Carl Roth GmbH, Karlsruhe & Duchefa Biochemie B.V, Haarlem, NL & Sigma-Aldrich Co., St. Louis, USA
Agar-Agar, bacteriological	Carl Roth GmbH, Karlsruhe
Agarose	Carl Roth GmbH, Karlsruhe
Albumin Fraction V, ≥99.8%, powdered	Carl Roth GmbH, Karlsruhe
Ammonium carbonate $(NH_4)_2CO_3$	Sigma-Aldrich Co., St. Louis, USA
Aurintricarboxylic acid ATA	Sigma-Aldrich Co., St. Louis, USA
Bacto peptone	Becton Dickinson, Franklin Lakes, USA
Carbenicillin	Carl Roth GmbH, Karlsruhe
Casein hydrolysate	Carl Roth GmbH, Karlsruhe
Chloramphenicol	Sigma-Aldrich Co., St. Louis, USA
Chloroform/Isoamyl alcohol (24:1)	Sigma-Aldrich Co., St. Louis, USA
D(+)-Galactose, ≥98%	Carl Roth GmbH, Karlsruhe
D(+)-Glucose, p.a.	Carl Roth GmbH, Karlsruhe
D(+)-Sorbitol, ≥98%	Carl Roth GmbH, Karlsruhe
12,14-Dichlorodehydroabietic acid	CanSyn, Toronto, CAN
Dichloromethane	Carl Roth GmbH, Karlsruhe
Diethyl ether, 99.5%	Acros Organics, Waltham, USA
EDTA, ≥99% p.a.	Carl Roth GmbH, Karlsruhe
Ethanol absolute	VWR International GmbH, Darmstadt
Ethyl acetate	Carl Roth GmbH, Karlsruhe
Glycerin, ≥98%	Carl Roth GmbH, Karlsruhe
IPTG, ≥99%	Carl Roth GmbH, Karlsruhe
Isopropanol, ≥99.8%	Carl Roth GmbH, Karlsruhe
Lithium chloride LiCl, ≥99% p.a.	Carl Roth GmbH, Karlsruhe
Methyl jasmonate MeJA, 95%	Sigma-Aldrich Co., St. Louis, USA
Midori Green Advance DNA Stain	Nippon Genetics Europe, Düren
NADPH	Biomol GmbH, Hamburg
n-Hexane	Merck, KGaA, Darmstadt
Phenol/Chloroform/Isoamyl alcohol (25:24:1)	Carl Roth GmbH, Karlsruhe

Polyvinylpolypyrrolidone PVPP	AppliChem GmbH, Darmstadt
Polyvinylpyrrolidone PVP 360 000	Sigma-Aldrich Co., St. Louis, USA
Potassium acetate KAc, ≥99%	Sigma-Aldrich Co., St. Louis, USA
Potassium chloride, ≥99.5% p.a.	Carl Roth GmbH, Karlsruhe
Sodium chloride, ≥99.8%	Carl Roth GmbH, Karlsruhe
Sodium dodecyl sulfate SDS, ≥99%	Carl Roth GmbH, Karlsruhe
Sodium sulfate	Carl Roth GmbH, Karlsruhe
Spectinomycin, 50 mg·mL^{-1}	Duchefa Biochemie B.V, Haarlem, NL
Tert-butyl methyl ether TBME, 99.9%	Acros Organics, Waltham, USA
TMSH	Macherey-Nagel, Düren
TRIS, ≥99.9%	Carl Roth GmbH, Karlsruhe
Yeast extract, powdered	Carl Roth GmbH, Karlsruhe
Yeast nitrogen base	Sigma-Aldrich Co., St. Louis, USA
β-Mercaptoethanol	Sigma-Aldrich Co., St. Louis, USA

2.1.2 Enzymes & kits

Name	**Manufacturer**
BigDye™ Terminator v1.1 Cycle Sequencing Kit	ThermoFisher Scientific, Waltham, USA
DNAse I, RNAse free	Quiagen N.V., Hilden
DyeEx 2.0 Spin Kit	Quiagen N.V., Hilden
GoTaq® G2 DNA Polymerase	Promega, Madison, USA
Invisorb® Spin Plasmid Mini Two	STRATEC Biomedical AG, Birkenfeld
InviTrap® Spin Plant RNA Mini Kit	STRATEC Biomedical AG, Birkenfeld
Not l FD restriction enzyme	ThermoFisher Scientific, Waltham, USA
pESC-Leu Vector	Agilent Technologies, Santa Clara, USA
Q5® High-Fidelity DNA Polymerase	New England Biolabs, Ipswich, USA
Sac l FD restriction enzyme	ThermoFisher Scientific, Waltham, USA
SuperScript™ III Reverse Transcriptase	ThermoFisher Scientific, Waltham, USA
SYBR® Green Supermix	Bio-Rad Laboratories, Hercules, USA
T4 DNA Ligase	New England Biolabs, Ipswich, USA
Zymoclean™ Gel DNA Recovery Kit	Zymo Research, Irvine, USA

2.1.3 Organisms

Name	Manufacturer
One Shot® TOP 10 chemically competent *E. coli*	ThermoFisher Scientific, Waltham, USA
OverExpress™ C41(DE3) chemically competent *E. coli*	Sigma-Aldrich Co., St. Louis, USA
Saccharomyces cerevisiae WAT11	

2.1.4 Instruments

Name	Manufacturer
ABI Prism Genetic Analyzer 3130xl	Applied Biosystems, ThermoFisher Scientific, Waltham, USA
Autoclave	HP Medizintechnik GmbH, Oberschleißheim
Balance BP310S	Sartorius AG, Göttingen
Centrifuge 5810 & 5417R	Eppendorf AG, Hamburg
Centrifuge Avanti™ J25	Beckman Coulter, Brea, USA
GC System 6890 Series	Agilent Technologies, Santa Clara, USA
Gel electrophorese chamber Mupid-21	Eurogentec, Lüttich, BEL
Gel Imaging System	GeneGenius
Heatblock Dri Block DB-2D	Techne, Staffordshire, UK
Incubator B6120	Heraeus, Hanau
Mass Selective Detector 5973 N*etwork*	Agilent Technologies, Santa Clara, USA
Micro balance XP26	Mettler Toledo GmbH, Columbus, USA
NanoDrop2000c Spectrophometer	ThermoFisher Scientific, Waltham, USA
pH meter 526	WTW, Dinslaken
Real-Time System CFX Connect	Bio-Rad Laboratories, Hercules, USA
Rotary evaporator R-114	BÜCHI Labortechnik AG, Flawi, CH
Shaker Innova 4230	New Brunswick Scientific, Edison, USA
Shaker MaxQ 8000	ThermoFisher Scientific, Waltham, USA
Sterile bench Herasafe HSP12	Heraeus, Hanau
Thermocycler Biometra Tpersonal	Biometra GmbH, Göttingen
Ultracentrifuge Optima L-90K	Beckman Coulter, Brea, USA
Vortex-Genie 2	Scientific Industries, Bohemia, USA
Water bath 1002	GFL mbH, Burgwedel
Water bath 215 Isotemp	Fisher Scientific, Hampton, USA
Zebron ZB-5 capillary GC column	Phenomenex Inc., Torrance, USA

2.1.5 Buffer & stock solutions

Name	Ingredients
$(NH_4)_2CO_3$ solution (pH 8.0)	0.1 M $(NH_4)_2CO_3$
ATA solution (pH 8.0)	0.1 M ATA
Lithium chloride solution	8 M LiCl
MeJA solution	1 mM methyl jasmonate 0.05% (v/v) Tween20
NADPH solution	90 mM NADPH
Phosphate buffer (pH 7.4)	0.1 M K_2HPO_4, KH_2PO_4
KAc solution (pH 4.8)	3 M KAc
RNA extraction buffer	50 mM TRIS-HCl (pH 8.0) 300 mM NaCl 5 mM EDTA 20 g·L^{-1} SDS 5 g·L^{-1} PVP (MW 360 000)
SC minimal medium (Ø Leu)	6.7 g·L^{-1} yeast nitrogen base 100 mg·L^{-1} Ade, Arg, Cys, Lys, Thr, Trp, Ura 50 mg·L^{-1} Asp, His, Ile, Met, Phe, Pro, Ser, Tyr, Val 20 g·L^{-1} glucose
TB medium	24 g·L^{-1} casein hydrolysate 12 g·L^{-1} yeast extract 0.4% (v/v) glycerin 0.231 g·L^{-1} KH_2PO_4 1.254 g·L^{-1} K_2HPO_4
TEG buffer	50 mM TRIS-HCl (pH 7.5) 1 mM EDTA 30% (v/v) glycerin
TEK buffer	50 mM TRIS-HCl (pH 7.5) 1 mM EDTA 100 mM KCl
Terpene extraction solution	50 g·L^{-1} 1,9-decadiene 47.3 g·L^{-1} dichlorodehydroabietic acid TBME
TES buffer	50 mM TRIS-HCl (pH 7.5) 1 mM EDTA 600 mM sorbitol 10 g·L^{-1} albumin 0.12‰ (v/v) β-mercaptoethanol (freshly added)
YPGA medium	10 g·L^{-1} yeast extract 20 g·L^{-1} bacto peptone 74 mg·L^{-1} adenine-hemisulfate 20 g·L^{-1} glucose/galactose

2.1.6 Oligonucleotides

All Primers used are listed with their melting temperature in the table below. The oligonucleotides have been synthesized by Eurofins Genomics. By adding the indicated amount of ddH_2O, 100 µM stock solutions were prepared. For all reactions, dilutions of the stocks with a final concentration of 10 µM were used.

Name	Sequence (5'-3')	T_m /°C
Primer for qRT-PCR analysis		
PaUbi*for*12/16	GTTGATTTTTGCTGGCAAGC	55,3
PaUbi*rev* 12/16	CACCTCTCAGACGAAGTAC	56,7
PaTub*for*12/16	CGTTACCTGCTGCCTGAG	58,2
PaTub*rev* 12/16	GCTCTGTATTGCTGTGAACC	57,3
CypB1*qRT*for*	AAGCAGACAATCTGCCACCTGG	62,1
CypB1*qRT*rev*	CCTTCTCTCCCGTTCCTCGAT	61,8
QRTCyp3UTR*for*	CCATGTAATACCTATGGGCATG	58,4
QRTCyp3UTR*rev*	GCATTGCACTAACACATCATGAG	58,9
QRTCyp4ORF*for*	ATGGCGCCCATGGCAGAG	60,5
QRTCyp4ORF*rev*	GAACCTCCTTATTATTCATTTTTGGGC	60,4
QRTCyp5ORF*for*	GGCAGATCAAATATCATTAGTGC	57,1
QRTCyp5ORF*rev*	CCTCCTGATTCTCTTCATTATTAC	57,6
CypB6*qRT*for*	GGAGTAATAAGGAGGTCCATCTG	60,6
CypB6*qRT*rev*	GTATCTTTTCTGTCTGTCCTCGAC	61,0
CypB11*qRT*for*	GGAAAGCCCATCTGCCACC	61,0
CypB11*qRT*rev*	ACCTCTTCCTTCGATCTTCAACG	60,6
Primer for Q5 PCR		
CYPB1_NotI	AAGCGGCCGCAATGGCAGACCAAATAACTCTAGTGTTG	72,7
CYPB11_SacI	TTGAGCTCTCATTGCAGAGGATGGAGGCG	69,5
Primer for Colony PCR and Sequencing Reaction		
Gal10*for*	GGTGGTAATGCCATGTAATATG	56.5
Gal10*rev*	GGCAAGGTAGACAAGCCGACAAC	64.2

2.2 Methods

2.2.1 Plant material, MeJA treatment and sample collection

In this project, the used Norway Spruce (*Picea abies*) saplings were of identical genotype like clone 3369-Schongau (Samenklenge und Pflanzgarten Laufen). The saplings were cultivated outside until they were three years old. Four individual saplings served as control and six saplings were treated by applying a fine spray of 1 L of 1 mM MeJA-solution. Samples were collected 2 and 6 days after spraying, respectively. For harvest, side branches and stem from actual year were cut at interwhorl to previous year's growth. Also roots were sampled and immediately flash frozen in liquid nitrogen before stored at -80°C. In sample preparation, needles were separated from the side branch and stem, respectively. The samples were homogenized to a fine powder using mortar and pestle while cooling in liquid nitrogen. Because sampling time point was in autumn, it has not been possible to separate the stem into bark and wooden tissue.

2.2.2 Terpene extraction

The following protocol is modified after the publication from Lewinsohn *et al.* [34].
100 mg of homogenized tissue were placed in a pre-weighted, cooled 4 mL screw neck glass vial. About 1 mL of the TBME solution (see paragraph 2.1.5) was added to the sample and vortexed for 5-10 sec. After 30 min incubation at room temperate, the vials were weighted again to confirm that all samples contain about the same amount of TBME. Afterwards, the vials were shook for 20 hours in a horizontal shaker at room temperature.
To every sample 0.4 mL 0.1 M ammonia carbonate solution (see paragraph 2.1.5) was added and vortexed for 10 sec. The upper ether phase was transferred into a new, 1.5 mL HPLC-vial with a spatula tip of submitted anhydrous sodium sulfate to get rid of water. An aliquot of 0.4 mL of each sample was transferred into new, 1.5 mL screw neck vials, 50 µL of TMSH added for methylation of the diterpene resin acids and kept for another 2 hours at room temperature. The remaining sample volume was transferred into an additional 1.5 mL screw neck vial for mono- and sesquiterpene analysis. All extracts were stored at -20°C.

2.2.3 GC-MS for qualification and quantification of terpenes

The extracts prepared in paragraph 2.2.2 were analyzed for mono- and sesquiterpenes and diterpene resin acids by GC-MS. GC-MS spectra were recorded on a Agilent 6890 Series GC System, coupled to a Agilent 5973 N*etwork* Mass Selective Detector and equipped with a Zebron ZB-5 capillary GC column (30 m × 0.25 mm × 0.25 µm; Phenomenex) as stationary phase. The analysis was performed by the related Agilent GC/MSD ChemStation Program and the terpenoids were identified by retention times and mass spectra with those of authentic standards.

Helium served as carrier gas at a flow rate of 1 mL/min. Samples were auto-injected (1 µL) either in splitless mode (diterpene resin acids) or split mode (50.1:1; mono- and sesquiterpenes). The separation was done with different heating protocols seen in Table 2.1 and Table 2.2.

Table 2.1: Conditions of the GC-MS oven for diterpene resin acid analysis.

Flow rate: 1.0 mL/min, splitless, heater temperature: 270°C

	°C/min	Next °C	Hold	Runtime
Initial		150	3:00	03:00 min
Ramp 1	3.50	280	4:00	44:14 min
Post		150	0:00	44:14 min

Table 2.2: Conditions of the GC-MS oven for mono- and sesquiterpene analysis.

Flow rate: 1.0 mL/min, split (50.1:1), heater temperature: 270°C

	°C/min	Next °C	Hold	Runtime
Initial		40	3:00 min	03:00 min
Ramp 1	5.00	80	0:00 min	11:00 min
Ramp 2	5.00	200	0:00 min	35:00 min
Ramp 3	60.00	280	4:00 min	40:33 min
Post		40	0:00 min	40:33 min

2.2.4 RNA extraction

RNA was extracted by a procedure modified after Cathala *et al.* [35].

All prepared solutions listed in paragraph 2.1.5 needed to be autoclaved. For the extraction approximately 500 mg of frozen, homogenized tissue powder was transferred to a 50 mL tube. 10 mL extraction buffer, 50 µL 0.1 M ATA and 10 µL β-mercaptoethanol were added and mixed well. The mixture was incubated at 65°C in a water bath for 10 min with occasional, gentle mixing and centrifuged at 14,000 g for 15 min at room temperature. The supernatant was transferred into a new tube and 0.7 mL 3 M KAc solution added. Everything was mixed well and placed on ice for 30 min. Afterwards the sample was centrifuged at 10,000 g for 10 min at 4°C. The supernatant was transferred into a new tube and 2.4 mL of phenol/chloroform-mixture (25:24:1) added. The mixture was mixed well and centrifuged at 16,000 g for 10 min at 4°C. This step was repeated until no whitish interface could be seen. The supernatant was transferred to a new tube and extracted once with 2 mL of chloroform:isoamyl alcohol (24:1) and centrifuged again at 16,000 g for 10 min at 4°C.

100 mg Polyvinylpolypyrrolidone was added to a new tube and the water phase added. The mixture was incubated in a water bath at 78°C with occasional mixing. After cooling to room temperature, 1 volume of chloroform:isoamyl alcohol (24:1) and 5 mL cooled water (4°C) were added. The emulsion was mixed and centrifuged at 10,000 g for 10 min at 4°C. The upper water phase was transferred into a new tube and 1 volume of isopropanol added. After gentle mixing the nucleotides were precipitated at - 20°C over night.

The next day, the sample was centrifuged at 10,000 g and 4°C for 10 min. The supernatant was discarded and the pellet washed once with 5 mL >70% ethanol. Another centrifugation step at 10,000 g and 4°C for 10 min followed and the pellet was dried again. The pellet was resuspended in 0.9 mL ddH_2O at room temperature and the insoluble material pelleted with a centrifugation at 10,000 g for 5 min at 4°C. As much supernatant as possible was transferred to a microcentrifuge tube and the volume adjusted to 0.9 mL with ddH_2O. 0.3 mL 8 M LiCl was added and the sample let to precipitate over night at 4°C.

After precipitation, another centrifugation step at 14,000 g for 10 min at 4°C followed. The supernatant was discarded and 0.75 mL 70% ethanol added to the pellet. The mixture was further used to continue with step 5.) of the InviTrap® Spin Plant RNA Mini Kit protocol. Step 6.) of the manufacturer protocol was modified while just washed with 300 µL R1-Buffer. After centrifugation at 10,000 rpm for 1 min at 4°C a mix of 70 µL RDD-Buffer and 10 µL DNAse was given directly to the column. After incubation time of 15 min at room temperature the sample was centrifuged at 10,000 rpm for 30 sec at 4°C. Another washing step followed with 300 µL and centrifugation at 10,000 rpm for 1 min at 4°C. Step 7.) and 8.) were done like manufacturer protocol before the RNA was eluted with 35 µL ddH_2O at 70°C.

2.2.5 cDNA synthesis

To build up a cDNA library, synthesis was made with SuperScript™ III Reverse Transcriptase (Invitrogen). The amount of RNA used was adjusted to a final concentration of 500 ng·µL^{-1}. The mixture of RNA, Oligo(dT)$_{20}$, dNTP Mix (10 mM) and ddH_2O was filled up to 14 µL and heated to 65°C for 5 min in the thermocycler and sat on ice for 5 min. 6 µL of a master mix containing 5x First-Strand Buffer, 0.1 M DTT and reverse transcriptase was given to the solution and incubated for 60 min at 50°C. The reaction was stopped by heat inactivation to 75°C for 15 min.

Table 2.3: Composition of the cDNA synthesis reaction.

	Volume
Oligo(dT)$_{20}$	1 µL
dNTP Mix (10 mM)	1 µL
RNA (500 ng·µL^{-1})	x µL
ddH_2O	14-x µL
5x First-Strand Buffer	4 µL
0.1 M DTT	1 µL
Reverse Transcriptase	1 µL

2.2.6 PCR techniques

The amplification of cDNA or plasmids was usually carried out in a Biometra Tpersonal Thermocycler. The used oligonucleotides depended on the gene of interest (GOI), accordingly to paragraph 2.1.6.

2.2.6.1 *Quantitative real-time polymerase chain reaction (qPCR)*

The expression levels of the genes of interest (GOI) were analyzed via qPCR. For this, each sample was determined in technical triplicates and three non-template controls for each gene. qPCR was carried out in a Bio-Rad CFX Connect Real-Time System, using SYBR® Green Supermix and analyzed by Bio-Rad CFX Manager (Version 3.1).
Reactions with non-discrete melting curves or divergent Cq-values were excluded from analysis.
The data analysis was quantified using the $2^{\Delta\Delta CP}$ method described by Livak *et al.* [36, 37]. The expression levels were calculated relatively to the reference gene Ubiquitin based on the real-time PCR efficiency (E) and the crossing point difference (ΔCP) of the unknown sample versus a control (see equation below). For the comparison, time point 0 was set as the control. Expression values of the biological replicates were averaged and standard error was calculated.

$$\text{ratio} = \frac{(E_{GOI})^{\Delta CP_{GOI}\,(\text{control}-\text{sample})}}{(E_{Ref})^{\Delta CP_{Ref}\,(\text{control}-\text{sample})}}$$

Table 2.4: Composition of the qPCR reaction.

	Volume
SYBR® Green Supermix	10 µL
Primer *forward* (10 µM)	0.7 µL
Primer *reverse* (10 µM)	0.7 µL
cDNA	0.6 µL
ddH_2O	8 µL

Table 2.5: Thermocycler conditions of the qPCR reaction.

Phase	Runtime	Temperature	
Initial denaturation	3:00 min	95°C	
Denaturation of double strands	0:10 min	95°C	50 cycles
Annealing, Elongation	0:20 min	60°C	
Final denaturation	2:00 min	95°C	
	0:05 min	60°C	
Melt curve (0.5°C/5 sec)	5:50 min	95°C	

2.2.6.2 *Q5 PCR*

A PCR with the Q5 High-Fidelity DNA Polymerase system was made to increase the amount of the desired product and prepare the gene for insertion into the pESC-Leu vector. For this, the primers were constructed with additional sequence parts of NotI and SacI restriction sites.

Table 2.6: Composition of the Q5 PCR reaction.

	Volume
5x Q5 Reaction Buffer	5 µL
dNTP Mix (10 mM)	0.5 µL
Primer *forward* (10 µM)	1.25 µL
Primer *reverse* (10 µM)	1.25 µL
Q5 High-Fidelity DNA Polymerase	0.25 µL
DNA template	1.5 µL
ddH_2O	15.25 µL

Table 2.7: Thermocycler conditions of the Q5 PCR reaction.

Phase	Runtime	Temperature	
Initial denaturation	2:00 min	98°C	
Denaturation of double strands	0:40 min	98°C	35 cycles
Annealing	0:40 min	63°C	
Elongation	2:00 min	72°C	
Final Elongation	5:00 min	72°C	

2.2.6.3 *Colony PCR*

To verify if the *E. coli* colonies contained the expression vector with the inserted gene, single colonies were picked via a sterile pipette tip. The amplification was conducted in a GoTaq® G2 DNA Polymerase system with a total reaction volume of 25 mL.

Table 2.8: Composition of the colony PCR reaction .

	Volume
5x Green GoTaq® G2 Reaction Buffer	5 µL
dNTP Mix (10 mM)	0.5 µL
Primer *forward* (10 µM)	0.5 µL
Primer *reverse* (10 µM)	0.5 µL
GoTaq® G2 DNA Polymerase	0.125 µL
ddH_2O	18.4 µL

Table 2.9: Thermocycler conditions of the colony PCR reaction.

Phase	**Runtime**	**Temperature**	
Initial denaturation	3:00 min	95°C	
Denaturation of double strands	0:30 min	95°C	35 cycles
Annealing	0:40 min	55°C	
Elongation	1:00 min	72°C	
Final Elongation	10:00 min	72°C	

2.2.6.4 *Sequencing*

Sequencing was made after the method from Sanger *et al.* [38] with fluorescent marked dideoxynucleotides. For this the BigDye™ Terminator v1.1 Cycle Sequencing Kit was used. Differences in pipetting scheme for batches with colony PCR products and plasmids, prepared in 2.2.6.3 and 2.2.13 respectively, are shown in Table 2.10 and Table 2.11. 200 ng·µL^{-1} of plasmid DNA were used and the solution adjusted with ddH_2O to a final volume of 10 µL.

Purification of the reaction solutions was done subsequently the DyeEx 2.0 Spin Kit, following the manufacturer protocol. The sequencing itself was performed in the ABI PRISM Genetic Analyzer 3130xl and the data analysis was made by SeqMan Pro 13 Software (DNASTAR® Lasergene, Madison, USA).

Table 2.10: Composition of sequencing PCR reaction for colony PCR soution.

	Volume
BigDye	2 µL
Primer *forward* or *reverse* (10 µM)	1 µL
Colony PCR solution	1 µL
ddH_2O	6 µL

Table 2.11: Composition of sequencing PCR reaction for plasmids.

	Volume
BigDye	2 µL
Primer *forward* or *reverse* (10 µM)	1 µL
Plasmid DNA (~200 ng)	x µL
ddH_2O	7-x µL

Table 2.12: Thermocycler conditions of the sequencing PCR products reaction.

Phase	**Runtime**	**Temperature**	
Initial denaturation	5:00 min	96°C	
Denaturation of double strands	0:10 min	96°C	35 cycles
Annealing	0:30 min	55°C	
Elongation	4:00 min	60°C	

2.2.7 Agarose gel electrophoresis

The agarose gel electrophoresis was used to determine if the desired product was amplified and as a preparative method to separate the product, prepared in paragraph 2.2.6.2, from side products and primers.

The electrophoresis was carried out in a Mupid-21 gel electrophorese chamber filled with 0.5 x TRIS-Borate-EDTA buffer (TBE). To the gel (1% agarose (w/v), 0.5x TBE buffer, 6 µL Midori Green Advance DNA Stain per 100 mL gel) a voltage of 100 V was applied for about 15 min. To get an estimate about the fragment size, 6 µL of a 1 kb plus DNA Ladder were used as a size standard. All applied samples were mixed with a 6 x Orange G loading dye in a ratio of 3:1. Midori Green containing amplicons were visualized by the GeneGenius Gel Imaging System at UV light (254 nm).

2.2.8 Gel purification

All PCR products were purified by the Zymoclean™ Gel DNA Recovery Kit according to the manufacturer protocol. For this the quested bands were cut off the gel with a scalpel under UV light. Unlike the manual, the DNA was eluted with 11 µL ddH_2O, prewarmed to 72°C.

2.2.9 Determination of nucleic acid concentration

DNA and RNA concentrations were determined by using a NanoDrop 2000c Spectrophometer. The absorption of 1 µL sample solution was measured at 260 nm and the operating software was calculating the corresponding concentration automatically (1 $A_{260\ nm\ (DNA)}$ = 50 ng·µL^{-1}; 1 $A_{260\ nm\ (RNA)}$ = 40 ng·µL^{-1}). To evaluate the sample purity, also the absorption at 280 nm and 230 nm were measured and the 260/280 and 260/230 ratios were determined.

2.2.10 Double digestion of Q5 PCR Product and pESC-Leu vector

To subclone the gene into the pESC-Leu vector, both vector and PCR product were digested with NotI and SacI FastDigest restriction enzymes. Differences in the pipetting scheme between vector reaction and PCR product digestion are listed in Table 2.13 and Table 2.14, respectively.

Table 2.13: Composition of Q5 PCR product double digestion.
Activity of FastDigest enzymes: 1 µL of FastDigest enzyme cleaves1 µg of substrate DNA in 5 to 15 minutes.

	Volume
10x Fast Digest Buffer	2 µL
NotI FD restriction enzymes	1 µL
SacI FD restriction enzymes	1 µL
Insert DNA	2 µL
ddH_2O	14 µL

Table 2.14: Composition of circular plasmid double digestion.
Activity of FastDigest enzymes: 1 µL of FastDigest enzyme cleaves1 µg of substrate DNA in 5 to 15 minutes.

	Volume
10x Fast Digest Buffer	2 µL
NotI FD restriction enzymes	1 µL
SacI FD restriction enzymes	1 µL
Vector DNA	2 µL
ddH_2O	14 µL

Table 2.15: Thermocycler conditions of the double digestion with NotI and SacI.

Phase	Runtime	Temperature
Digestion	60:00 min	37°C
Heat inactivation	5:00 min	80°C

2.2.11 Ligation

The insertion of the gene into the vector was done with a T4 DNA Ligase. To the pipetting scheme shown in Table 2.16, the insert and vector were added in a ratio of 3:1 and filled up with ddH_2O to a total reaction volume of 20 µL.

Table 2.16: Composition of the ligation reaction.

	Volume
10x T4 DNA Ligase Buffer	2 µL
T4 DNA Ligase	1 µL
ddH_2O	< 17 µL

Table 2.17: Thermocycler conditions of the ligation reaction.

Phase	Runtime	Temperature
Ligation	16:00 h	16°C
Heat inactivation	15:00 min	65°C

2.2.12 Transformation of ligation product into *E. coli*

For transformation of the ligation product prepared in paragraph 2.2.11 into One Shot® TOP 10 competent *E. coli*, the cells were thawed on ice and split into 25 µL per tube. The total volume of the ligation mixture was added to the cells and the solution incubated for 30 min on ice. After the heat shock incubation at 42°C in a water bath for 30 sec and the incubation for 2 min on ice, 150 µL of S.O.C medium was added. The solution was shook for 60 min at 37°C and 220 rpm before grown for 18 h at 37°C on LB agar plate with carbenicillin (50 $mg \cdot mL^{-1}$).

2.2.13 Plasmid isolation

2 mL of transformed One Shot® TOP 10 competent *E. coli* overnight culture in liquid LB-medium with carbenicillin (50 mg·mL^{-1}) were used to isolate plasmids after the Invisorb® Spin Plasmid Mini Two Kit protocol. Different to the manual, the elution step was modified to use 35 µL ddH_2O, prewarmed to 72°C.

2.2.14 Catalyzing of substrates in *E.coli*

For doing enzymatic assays and heterologous expression of DRAs in yeast, substrates are needed. For this, competent C41(DE3) *E. coli* was transformed with pIRS and pGG-DEST vectors containing genes for ISO- and LAS-terpene synthases, respectively as well as GGPP-synthase. The system was kindly provided by Reuben Peters and further modified by Andrew O'Donnell [11].
The bacteria were grown in 50 mL TB-medium (spectinomycin/chloramphenicol [50 mg·mL^{-1} / 34 mg·mL^{-1}]). When the cell solution reached an OD_{600} of 0.6-0.8, 5.6 mL of TRIS-HCl buffer (pH 7.5) were added and transferred to 16°C. After one hour substrate expression was induced via 100 µL 0.5 M IPTG. The cells were shook at 16°C and 220 rpm for 3 days.
The batches were extracted twice with 50 mL n-hexane each and the solvent evaporated afterwards by rotary evaporator. The products were resuspended in diethyl ether and analyzed on GC-MS.

Table 2.18: Conditions of the GC-MS oven for substrate analysis.
Flow rate: 54.2 mL/min, splitless, heater temperature: 270°C

	°C/min	Next °C	Hold	Runtime
Initial		80	1:00 min	01:00 min
Ramp 1	10.00	210	0:00 min	14:00 min
Ramp 2	3.00	240	0:00 min	24:00 min
Ramp 3	60.00	300	5:00 min	30:00 min
Post		40	0:00 min	30:00 min

2.2.15 Heterologous expression of CYP720B in yeast

2.2.15.1 *Preparation of competent cells*

Saccharomyces cerevisiae WAT11 cells with chromosomally integrated *Arabidopsis thaliana* NADPH-dependent P450 reductase [39], were handled according to the *S. c.* EasyComp™ Transformation Kit manual.

2.2.15.2 *Heterologous expression of PaCYP720Bs*

The transformation of the competent yeast cells with *Pa*CYP720B1 and *Pa*CYP720B4 was done following the *S. c.* EasyComp™ Transformation Kit manual instructions. The yeast growth and microsome preparation was modified after a previously described method [40]. As negative control for following experiments, an empty vector control was made. For selection, transformed cells were grown on SC-minimal agar plates without leucine for 3 days.

A single colony was picked and transferred into SC-minimal medium (without leucine) and shook over night at 28°C, 180 rpm. After 18 hours, 100 mL YPGA (Glucose) medium was inoculated with 1 unit OD_{600} of the starter culture and incubated again at 28°C, 180 rpm for 33 hours. The cell suspension was centrifuged at 16°C, 5,000 g for 5 min, washed twice with ddH_2O and resuspended in 100 mL YPGA (Galactose). The cells were further incubated at 25°C and 160 rpm.

2.2.15.3 *Microsome preparation*

After 14 hours the cultures were centrifuged at 4°C, 7, 500 g for 10 min to discard the medium. The pellet was resuspended in 30 mL TEK buffer. After another centrifugation step the pellet was resuspended in 2 mL TES buffer and transferred into a 50 mL tube. Glass beads were added and everything shook 5 times 1 min, with a 1 min break on ice in between. The beads were washed 4 times with 5 mL each, the solution transferred into a new 50 mL tube and centrifuged at 4°C, 7, 500 g for 10 min. The supernatant was put into ultracentrifuge tubes and centrifuged at 4°C, 32,000 rpm for 90 min. Afterwards, the supernatant was discarded and the pellet washed with 2.5 mL both TES and TEG buffer. 2 mL TEG were used to resuspend the pellet and transfer into a Potter-Elvehjem tissue homogenizer, where it got homogenized. Aliquots of 150 µL were flash-frozen in liquid nitrogen and stored at - 80°C.

2.2.16 Enzyme assay

To test the enzyme activity, *in vitro* assays were run. They were set up with different potential substrates (see paragraph 2.2.16.1) at a concentration of 100 mM, the substrate extractions of Iso- and LAS-TPS respectively, prepared in 2.2.14 as well as diethyl ether for solvent control. Empty pESC-Leu vector was used for negative control. As a second control, protein solution was boiled for 10 min prior to assay. This was made to be sure that the products were generated by enzymes and not caused due to contamination.

Table 2.19: Composition of the enzyme assay reaction.

	Volume
Enzyme	150 µL
Phosphate buffer (0.1 M, pH 7.4)	450 µL
NADPH solution (90 mM)	10 µL
Substrate	2 µL
ddH_2O	288 µL

The reaction was shook at 30°C and 100 rpm horizontally for different time periods e.g. 1 h, 1.5 h and over night and different temperatures (28/30°C). After stopping the assays, 2 µL 100 mM 12,14-dichlorodehydroabietic acid was added to the assay. To extract the products, the reaction solution was extracted 3 times with 1 mL ethyl acetate each and transferred into a 4 mL glass vial. After evaporating the solvent, the products were resuspended in 100 µL TBME and methylated with 5 µL TMSH, before analyzing them on the GC-MS with the same conditions listed in Table 2.1.

2.2.16.1 *Possible substrates*

Literature gave presumption, that CYP720Bs were able to convert not only diterpene olefins but also diterpene alcohols and aldehydes as graphed in Figure 1.8. Followed possible substrates were listed. Since the price to order the compounds in general and especially for small quantities is high, Dr. Yoko Nakamura is synthesizing the substrates. At the actual time point, not all of them have been synthesized yet, so that only few of them could get used in the enzyme assays described in 2.2.16.

Substrate	**Used for assays**	**comments**
Diterpene olefins		
Abietadiene	Yes	Produced in 2.2.14; provided by Dr. Yoko Nakamura
Dehydroabietadiene	Yes	Produced in 2.2.14; provided by Dr. Yoko Nakamura
Isopimaradiene	Yes	Produced in 2.2.14; in synthesis by Dr. Yoko Nakamura
Levopimaradiene	Yes	Produced in 2.2.14; in synthesis by Dr. Yoko Nakamura
Neoabietadiene	Yes	Produced in 2.2.14; provided by Dr. Yoko Nakamura
Palustradiene	Yes	Produced in 2.2.14; in synthesis by Dr. Yoko Nakamura
Pimaradiene	No	In synthesis by Dr. Yoko Nakamura
Sandaracopimaradiene	No	In synthesis by Dr. Yoko Nakamura
Diterpene alcohols		
Abietadienol	Yes	Provided by Dr. Yoko Nakamura
Dehydroabietadienol	Yes	Provided by Dr. Yoko Nakamura
Isopimaradienol	No	In synthesis by Dr. Yoko Nakamura
Levopimaradienol	No	In synthesis by Dr. Yoko Nakamura
Neoabietadienol	No	In synthesis by Dr. Yoko Nakamura
Palustradienol	No	In synthesis by Dr. Yoko Nakamura
Pimaradienol	No	In synthesis by Dr. Yoko Nakamura
Sandaracopimaradienol	No	In synthesis by Dr. Yoko Nakamura
Diterpene aldehydes		
Abietadienal	No	No synthesis planned yet
Dehydroabietadienal	Yes	No synthesis planned yet
Isopimaradienal	No	No synthesis planned yet
Levopimaradienal	No	No synthesis planned yet
Neoabietadienal	No	No synthesis planned yet
Palustradienal	No	No synthesis planned yet
Pimaradienal	No	No synthesis planned yet
Sandaracopimaradienal	No	No synthesis planned yet

3. Results

3.1 Terpene analysis

After terpene extraction, the samples were analyzed on GC-MS. An exemplary GC-MS total ion chromatogram is shown in Figure S 1. The individual terpenoid concentrations (mg/g fresh weight) were calculated. The integrated peak area of the sample was divided by the internal standard to get the relative quantities. These values were normalized by dividing of the used amount of fresh tissue. To get a wide overview, mono- and sesquiterpenes as well as diterpene resin acids were evaluated.

In total, diterpene resin acids were the most prominent terpenoids in the examined plants. Monoterpenes were the second most abundant terpenoids, in spite of the concentrations being low (Figure 3.1 & Figure 3.2). Even terpene content is mostly highest in stem, there is to say that in this experiment the stem tissue includes the wooden part and bark tissue. If there would have been the possibility to separate both tissues, the real induction in the bark tissue would have been greater than the obtained concentrations.

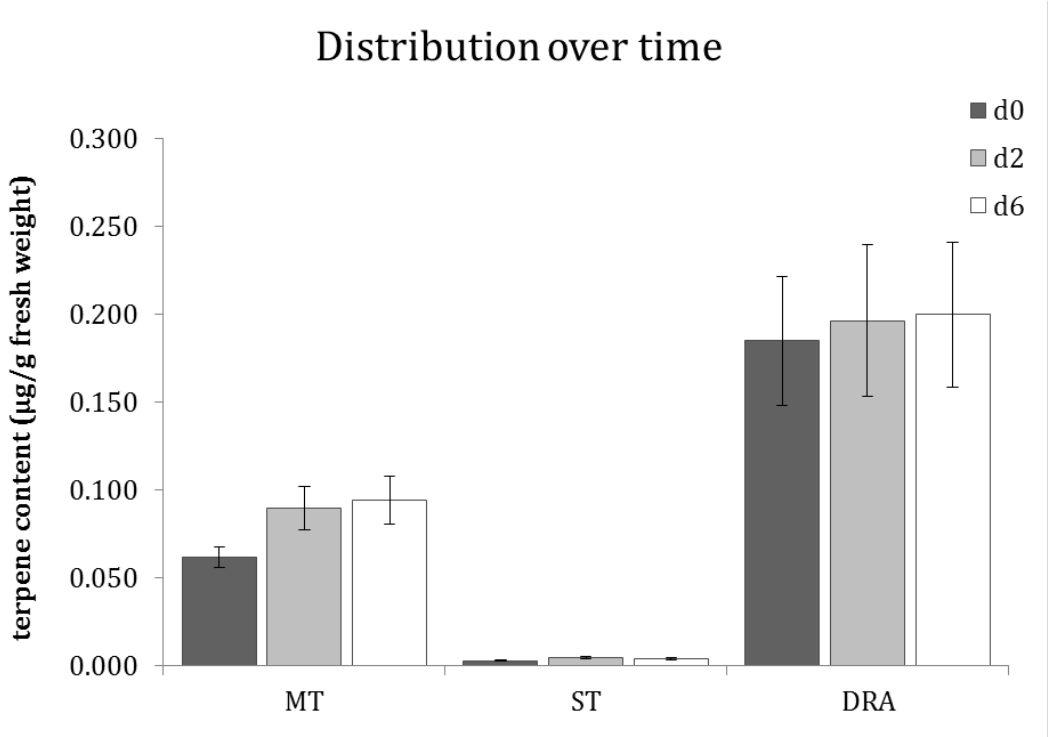

Figure 3.1: Total content of terpenes over time. Plottted are the averaged contents of all tissues (g/ g fresh weigh) for monoterpenes (**MT**), sesquiterpenenes (**ST**) and diterpene resin acids (**DRA**) over the experimental time period ± standard error. **Black**: control samples without treatment at day 0; **grey**: samples two days after treatment; **white**: samples six days after treatment.

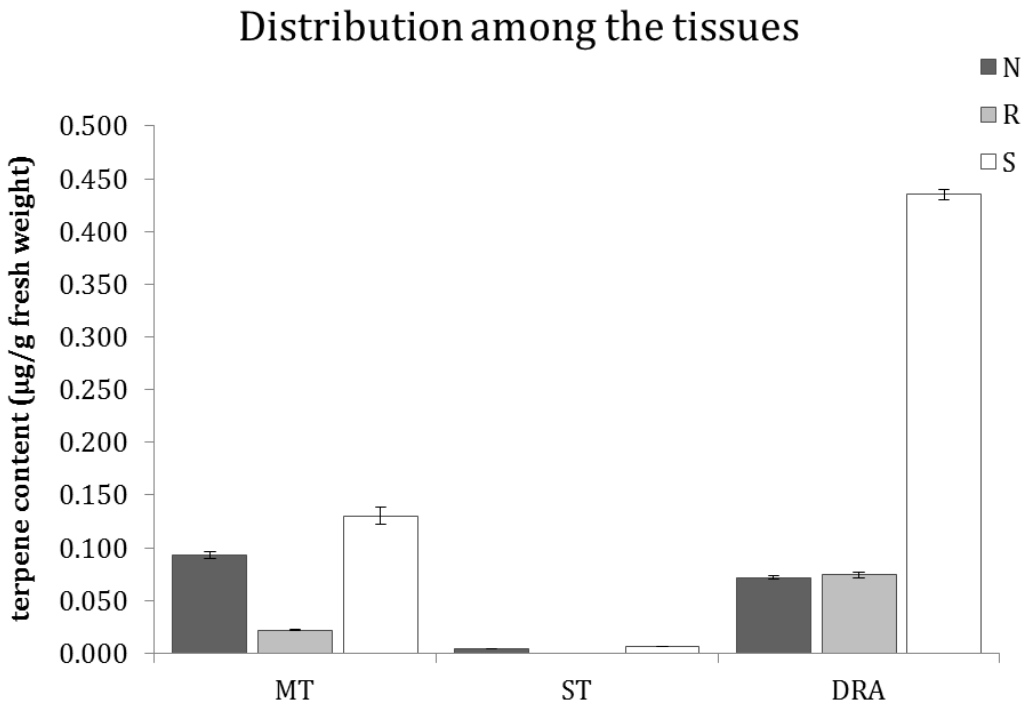

Figure 3.2: Total content of terpenes among different tissues. Plottted are the averaged contents of all time points (g/ g fresh weigh) for monoterpenes (**MT**), sesquiterpenenes (**ST**) and diterpene resin acids (**DRA**) in the different tissues ± standard error. **Black**: needle tissue (**N**); **grey**: root tissue (R); **white**: stem tissue (**S**).

For monoterpenes, eleven substances were analyzed. Within the plants, the total monoterpene content increased after the MeJA treatment. Detailed to the different tissues stem, needles and roots is shown in Figure 3.3. Their content was almost doubled in stem and just increased less in needles. In roots, the amount even decreased. From the analysis, most abundant monoterpenes were limonene, followed by β-pinene and α-pinene.

Camphene was also present with about half concentration than limonene. Sabinene and α-terpinolene showed the least expression in the saplings. Tricyclene, myrcene, camphor and bornylacetate were present in about the same concentration range.

In stem, β-pinene proved to be the most abundant monoterpenoid. Unlike limonene, the second substance, two days after the treatment it was more abundant than after day six. Also relatively high amounts showed α-pinene and δ-3-carene. The other monoterpenes were not or just in low concentrations present.

Concentrations of sabinene, δ-3-carene and α-α-terpinolene were exclusively found in stem tissue. A different distribution was found in needles. Here, camphene was most expressed during the experimental period. The concentrations of limonene and α pinene were almost the same, but unlike camphene, they increased over time. Camphene reached the limit after two days and then declined. However, the final concentration was higher than it was in the control at the beginning. Tricyclene, camphor and bornylacetate were not detectable in stem or roots but only in needles. The components did not show high concentrations as camphene, neither did β-pinene or myrcene.

In the roots, only four of the eleven monoterpenes were detectable. Camphene and limonene were expressed in about equal amounts. β-pinene and α-pinene were present in higher concentrations. The other components were not present.

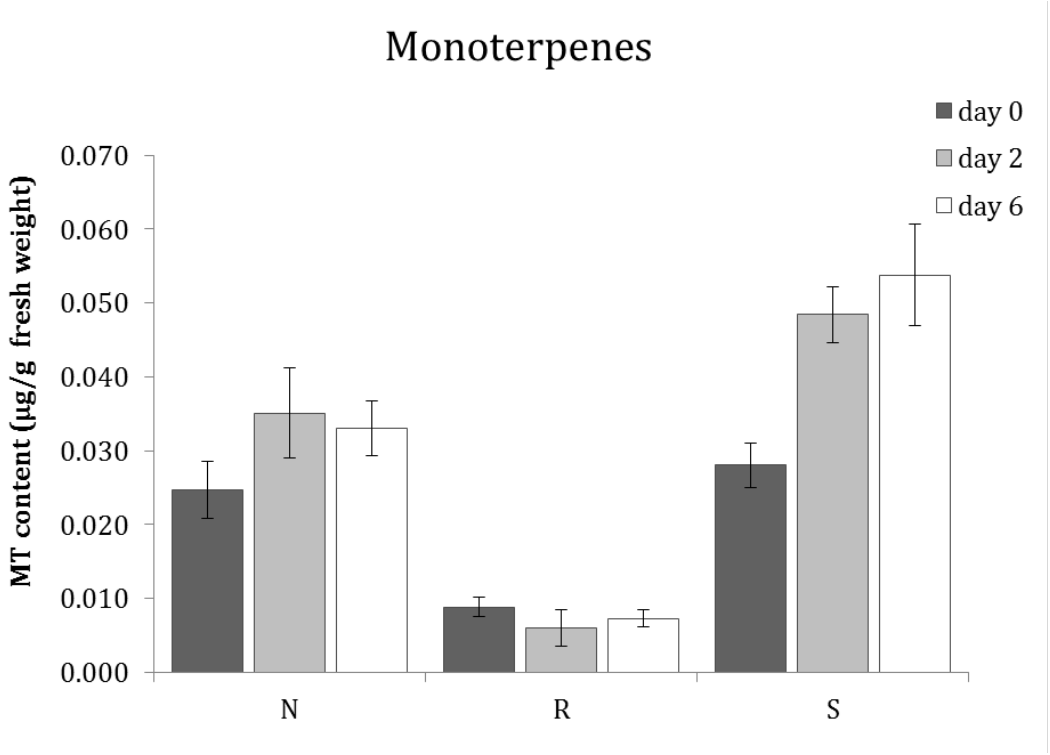

Figure 3.3: Content of monoterpenes for different time points and tissues. Plottted are the averaged monoterpene contents (g/ g fresh weigh) ± standard error from biological replicates among the different tissues needles (**N**), roots (**R**) and stem (**S**). **Black**: control samples without treatment at day 0; **grey**: samples two days after treatment; **white**: samples six days after treatment.

There were the four sesquiterpenes longifolene, trans-β-caryophyllene, α-humulene and germacrene analyzed. However, their total content increased over the time but reached their maximum on the second day after treatment. The compounds were not detected in roots, as charted in Figure 3.4. In the stem and needle tissue, sesquiterpenes showed the same pattern. Generally their concentration increased, but they had a higher concentration on day two than at the end of the experiment. In needles, α-humulene was three times higher than germacrene and trans-β-caryophyllene two times higher than germacrene. In stem, trans-β-caryophyllene had the highest concentration. It was about eight times more expressed than α-humulene and germacrene, and about 3 times more than longifolene. Longifolene was not present in needles. However, it had a great increase in until after the second day of treatment. In controls, germacrene was the second most leading compound in stem. However, the concentration was declined at the second time point about the 8-fold. Eventually it became the least present component after α humulene, which increased over the entire period. Longifolene and trans-β-caryophyllene again showed the pattern of the concentration limit after two days.

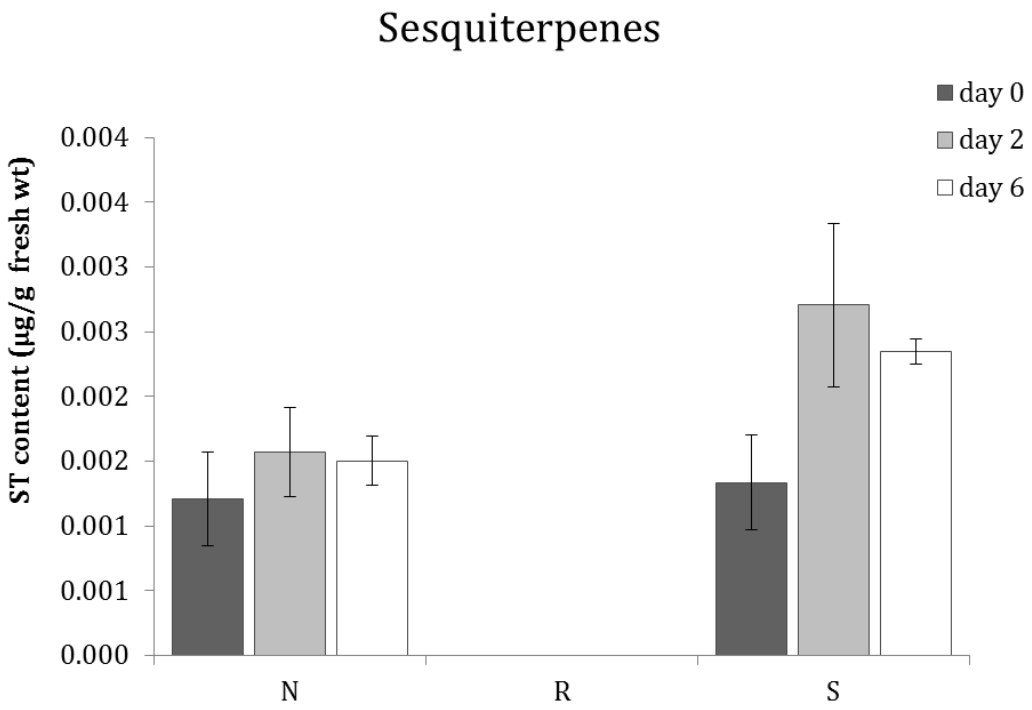

Figure 3.4: Content of sesquiterpenes for different time points and tissues. Plottted are the averaged sesquiterpene contents (g/ g fresh weigh) ± standard error from biological replicates among the different tissues needles (**N**), roots (**R**) and stem (**S**). **Black**: control samples without treatment at day 0; **grey**: samples two days after treatment; **white**: samples six days after treatment.

The total content of diterpene resin acids was raised in the plant over time (Figure 3.5). In the stem tissue, the concentration reached the limit two days after the treatment and declined afterwards. Still, the final concentration was higher than at the beginning. Consistent increase of the total content was observed in needles. In the roots, the total amount first decreased and then increased two days afterwards. But still the final concentration was lower than before treatment.

In general, DRAs were most present in stem tissue. Roots and needles only contained low amounts, of about 1/6 of the content present in stem tissue. In all, levopimaric acid was the most common DRA in the analyzed plants, followed by neoabietic acid and abietic acid. Sandaracopimaric acid was the least present compound, being 2.5 times lower than isopimaric acid and almost 6-fold lower than levopimaric acid.

In roots, levopimaric acid was the most abundant resin acid, up to 5-8 times higher than the other acids. Independent of the total amount of the single compounds, all DRAs declined in their concentration on the second day. The increase at day six yielded in lower concentrations than before treatment. Even if levopimaric acid was present in roots, there was a higher amount of levopimaric acid in stem than in the other tissues. In stem, the concentration of levopimaric acid almost halved two days after treatment and only increased little until the sixth day. Same pattern but less dramatic development was shown by levopimaric acid in root tissue, whereas the amount in needles was greater at the end of the experiment.

Dehydroabietic acid was the most present resin acid in needles. The compound increased over the time, but reached its maximum two days after treatment. Although dehydroabietic acid constantly increased over time in stem, it was just the second lowest DRA before sandaracopimaric acid. In stem tissue, neoabietic acid was the most abundant compound, followed by equal amounts of levopimaric acid and abietic acid. Considering the different time points, neoabietic was most present after two days. Abietic acid had the greatest amount at the sixth day. Neoabietic increased until the second day and decreased almost back to the level of the controls. It was hardly abundant in roots and still lower in needles. Abietic acid increased in stem tissue over time. It showed the same distribution to the different tissues than levopimaric acid, neoabietic acid, isopimaric acid and sandaracopimaric acid. All these resin acids were most present in stem; with a further preference for roots over needles. Isopimaric acid and sandaracopimaric acid increased in the stem and needle tissue over the time.

Summarized to the different time points, the total amount of sandaracopimaric acid, isopimaric acid, dehydroabietic acid and abietic acid in the tissues increased over time with the exceptions of levopimaric acid and neoabietic acid. Levopimaric acid decreased after two days and increased slightly until the last day. In contrast, neoabietic acid increased after the treatment and lowered in concentration, below the value of the control at the end.

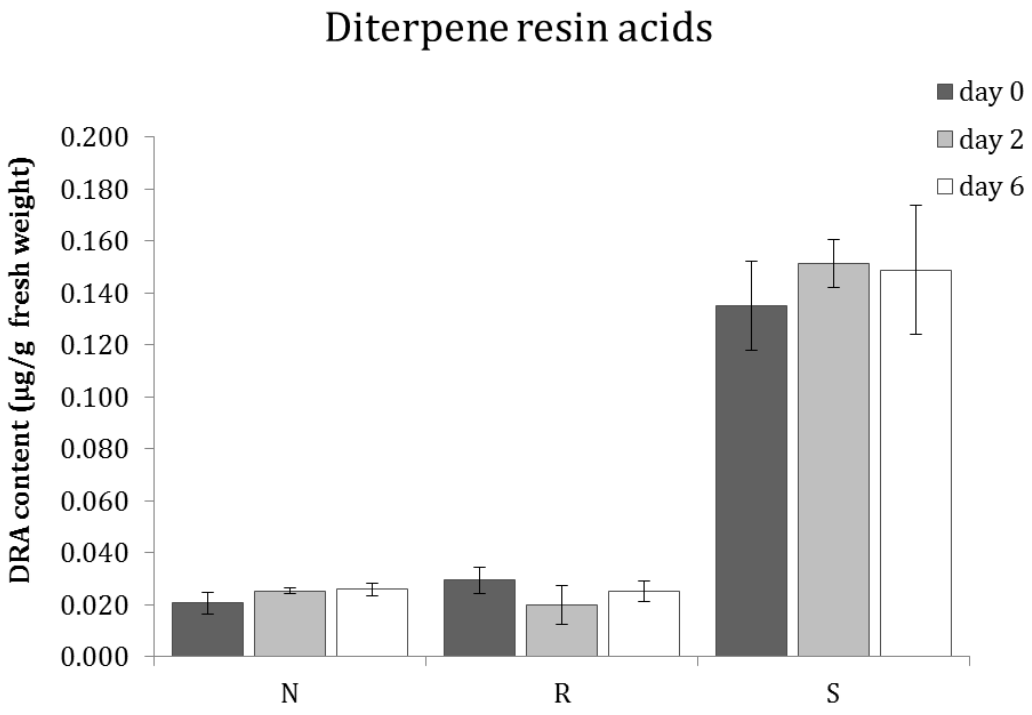

Figure 3.5: Content of diterpene resin acids for different time points and tissues. Plotted are the averaged DRA contents (g/ g fresh weigh) ± standard error from biological replicates among the different tissues needles (**N**), roots (**R**) and stem (**S**). **Black**: control samples without treatment at day 0; **grey**: samples two days after treatment; **white**: samples six days after treatment.

3.2 Expression levels of CYP720Bs

Expression levels of the quested CYP720Bs were relatively quantified using the $2^{\Delta\Delta CP}$ method [36]. In general, the total expression level over the experimental time period was lowest in roots. The highest transcript levels were found in stem tissue.

In root tissue, CYP720B11 had the highest expression. It increased two days after treatment and declined almost to zero after day six. Second most abundant in roots was CYP720B1, which did not change after day two, but decreased on the sixth day with more than half. CYP720B4 and CYP720B6 also declined on the sixth day after treatment (Figure 3.6). CYP720B4 was highly expressed in stem tissue with a 17-fold increase observed after two days (Figure 3.7). The transcript level decreased after six days. This occurred for all of the analyzed P450s in stem. CYP720B1 and B4 were shown a 5-fold and 4-fold decline, respectively.

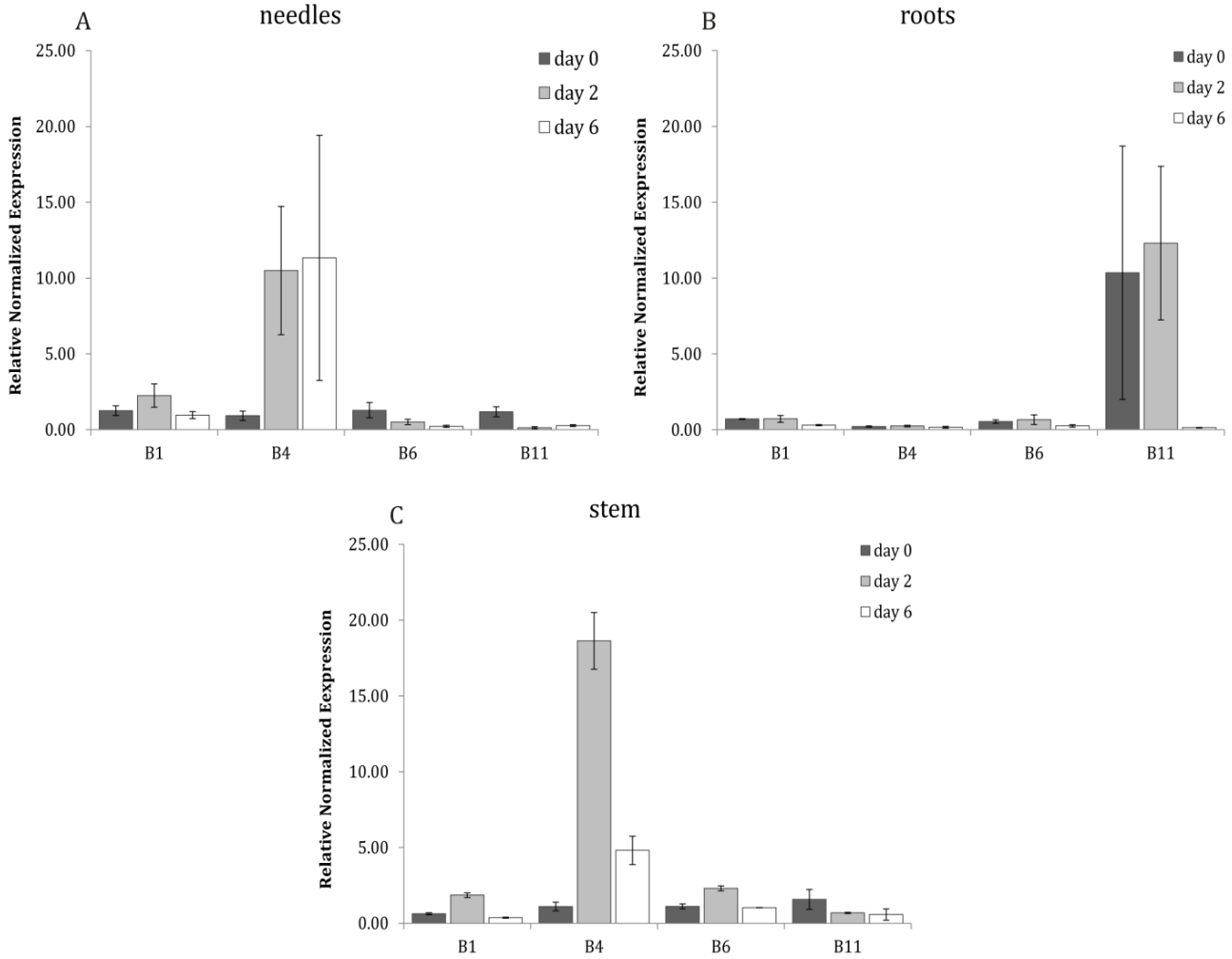

Figure 3.6: Normalized relative expression of four different CYP720Bs in different tissues. Plotted are the normalized relative expression levels of four genes encoding CYP720B1, B4, B6 and B11 ± standard error. The data show their distribution in needles (**A**), roots (**B**) and stem tissue (**C**) and their changes over the experimental period. **Black**: control samples without treatment at day 0; **grey**: samples two days after treatment; **white**: samples six days after treatment.

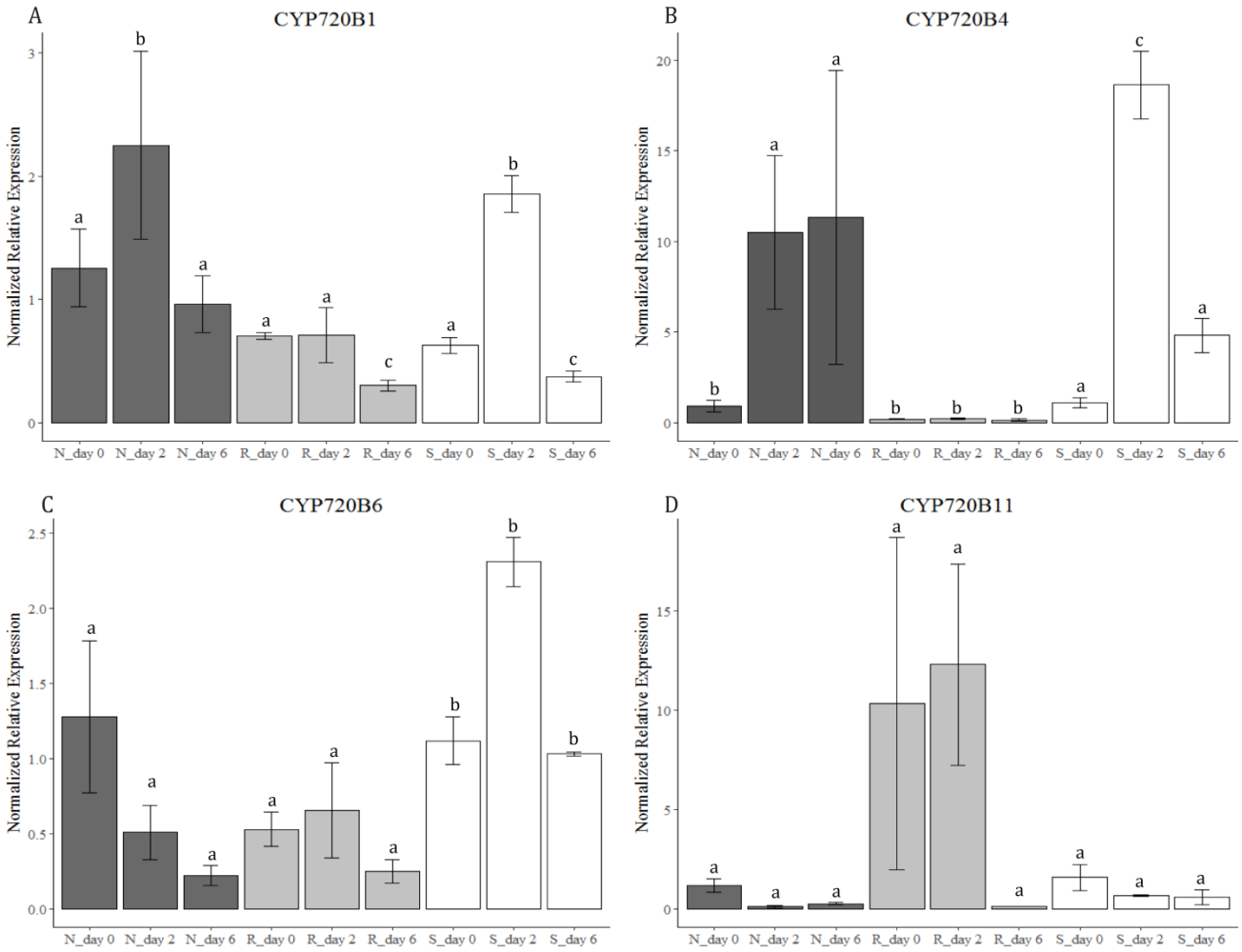

Figure 3.7: Normalized relative expression of four different CYP720Bs over time. Plotted are the normalized relative expression levels of four genes encoding CYP720B1 (**A**), B4 (**B**), B6 (**C**) and B11 (**D**) ± standard error for different time points (day 0, day 2, day 6) among the different tissues needles (**N**), roots (**R**) and stem (**S**). Statistical differences were indicated by small letters (**a**-**c**).

In needles, CYP720B4 was the most present gene again. The transcript level increased during the whole time period and was about 11 times higher than the time before treatment. Next to CYP720B4, CYP720B1 had the highest expression in needles, but the transcript level increased until two days after treatment and declined afterwards. Mostly, the gene transcript levels were low except of CYP720B4 in needles and stem as well as CYP720B1 in roots.

For B4 in needles and B11 in roots there is a great standard error. In case of B4, the sample variability within the biological replicates conditioned the great error. Since the replicate number was only n=3, a greater natural variability leads to more divergent data.

The variability of B11 is caused to a cDNA sample containing less cDNA than the other samples. While the other gene expression levels seem not to be influenced by this, it caused a great standard error to B11.

Consequential, some Cq values generated by qPCR were above 34 and the values of non-template controls, respectively. These values were recommended as upper limit to avoid false-positive results due to primer dimerization. Considering this, the message of the graphs got even more clearly. Then CYP720B1 was the most present enzyme in needles, B4 in stem tissue and B11 the most abundant one in roots.

To proof the amplified PCR products, they were sequenced after Sanger *et al*. All CYP720Bs sequences were about the same length. CYP720B4 had the longest sequence with 1452 basepairs (bp), CYP720B11 1437 bp, CYP720B6 1434 bp and CYP720B1 1428 bp.

3.3 *Pa*TPS-LAS and *Pa*TPS-ISO systems catalyzing substrates

The catalyzed substrates by *Pa*TPS-LAS and *Pa*TPS-ISO systems *E. coli* (paragraph 2.2.14) were shown in Figure 3.8. Both batches resulted in about 200 µL substrate solutions. The reaction yielded high amounts of diterpene olefins, which was demonstrated by the high abundance of the peaks in the chromatograms. Although just 1 µL of the solution was injected into the GC, some of the peaks already reached the column limit and started fronting.

Chromatogram (A) showed product peaks of palustradiene (R_t = 17.50 min), levopimaradiene (R_t = 17.64 min), dehydroabietadiene (R_t = 18.05 min), abietadiene (R_t = 18.63 min) and neoabietadiene (R_t = 19.66 min). Levopimaradiene and abietadiene were the most abundant substrates, whereas palustradiene and dehydroabietadiene were scarcely present.

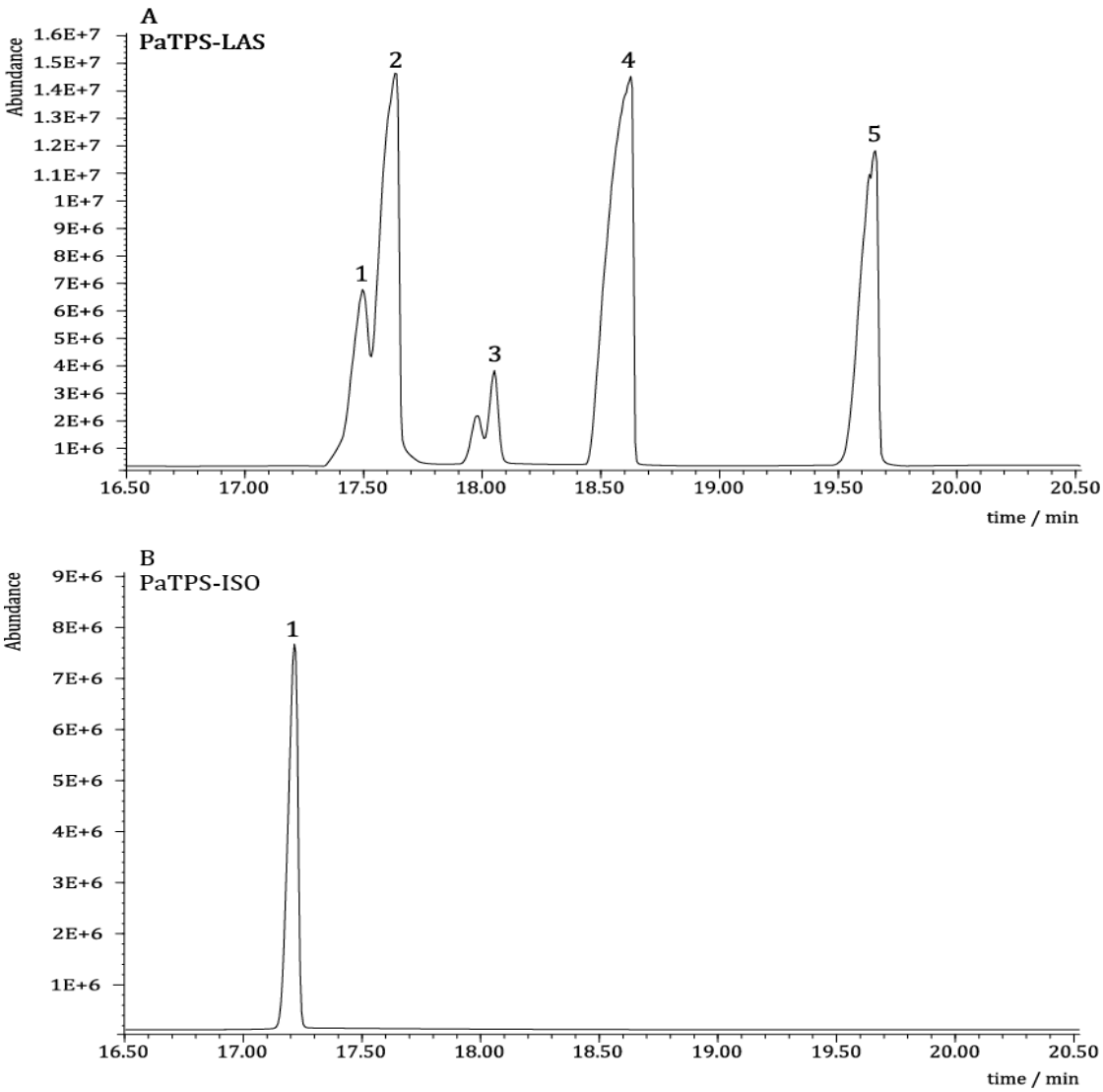

Figure 3.8: GC-MS total ion chromatograms of products formed by *Pa*TPS-LAS and *Pa*TPS-ISO.
Multiple products of *Pa*TPS-LAS (**A**): 1. palustradiene (R_t= 17.50 min), 2. levopimaradiene (R_t= 17.64 min), 3. dehydroabietadiene (R_t = 18.05 min), 4. abietadiene (R_t = 18.63 min), 5. neoabietadiene (R_t = 19.66 min); Single product of *Pa*TPS-ISO (**B**): 1. isopimaradiene (R_t = 17.19 min).

Via the used temperature gradient, it was possible to separate most of the substances clearly with a distance about 0.5-1 min. Dehydroabietadiene was a combined peak with a side product in front without baseline separation. Also palustradiene and levopimaradiene were not fully separated. Because palustradiene peak seemed to be smaller than the levopimaradiene peak, it was difficult to determine the approximate peak area of the single components.

Chromatogram (B) was shown an isopimaradiene peak at R_t = 17.19 min (Peak 1). For *Pa*TPS-ISO, isopimaradiene was the only substance which was catalyzed. In comparison to the peak sizes in chromatogram (A), the isopimaradiene peak had less abundance and a sharper, straighter form.

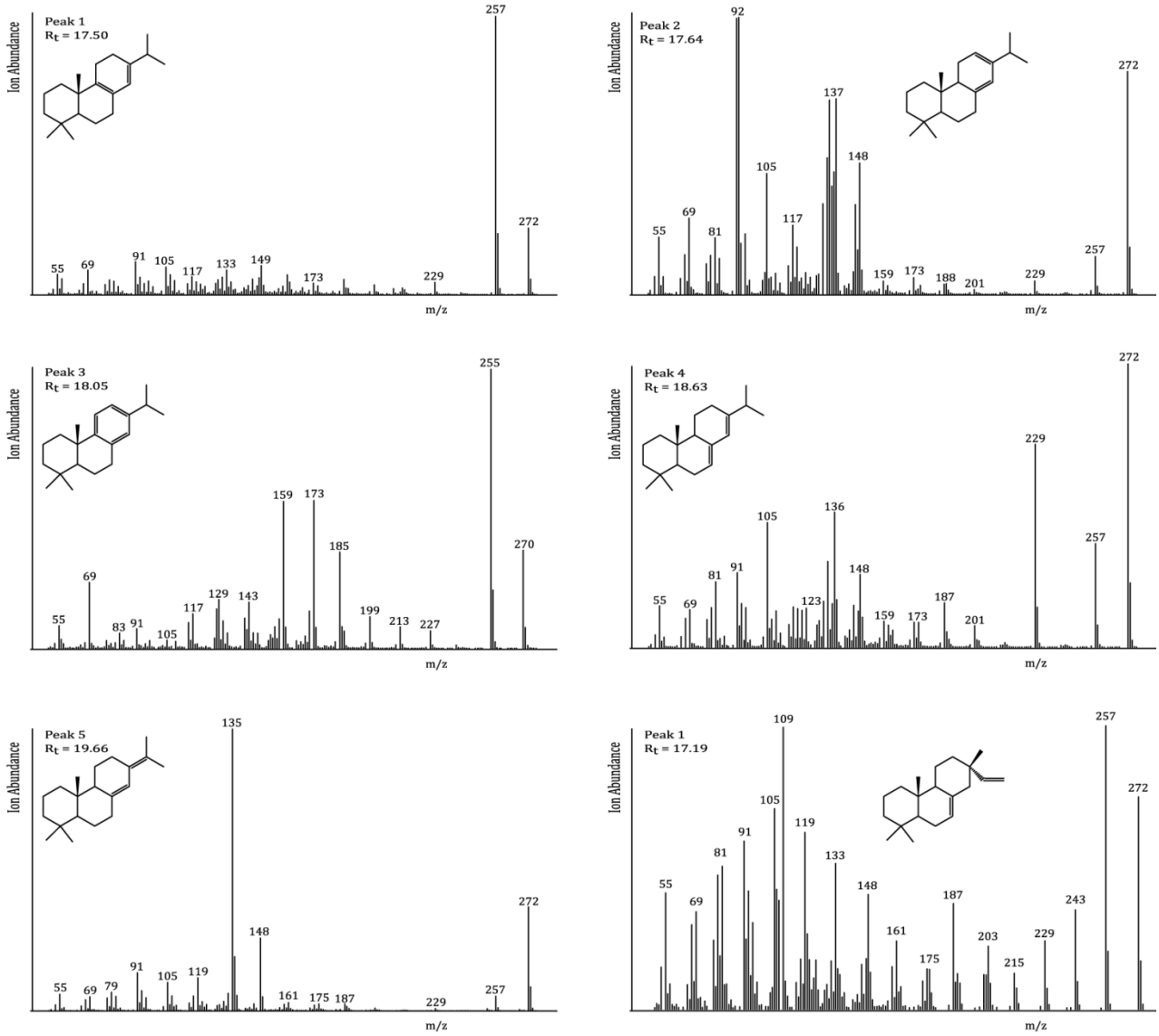

Figure 3.9: GC-MS spectra of products formed by *Pa*TPS-LAS and *Pa*TPS-ISO. Mass spectra were shown for each peak (**1**-**5**;**1**)and the belonging molecule structures from authentic standards and literature [18]. Characteristic for diterpene olefin mass spectra was the base peak with m/z=272. Because of the additional double bond, the molecule mass of dehydroabietiadiene was only m/z=270.

To assign the given peaks to the quested substrates, the mass spectra were compared to literature [18] and available standard solutions. Common to diterpene olefins was the non-fragmented base peak m/z=272. Due to the third double bond, dehydroabietadiene were shown as a molecular mass of m/z=270.

Characteristic for palustradiene was the fragment peak of m/z=257, which was also present in the other compounds. In the levopimaradiene, fragments with the size of m/z=92 and m/z=137 were shown with greatest intensity, whereas dehydroabietadiene (Peak 3) were m/z=159, m/z=173 and m/z=255. Peak 4, abietadiene, was mainly fragmented into molecules with m/z=229 and m/z=257. The greatest intensity peak in neoabietadiene mass spectra was represented with fragment m/z=135. Contrary to neoabietadiene, which seemed to fragmentize less, isopimaradiene showed many fragment peaks with high intensity; m/z=105, m/z=109, m/z=187 and m/z=257, etc.

3.4 Enzyme Assays

The enzyme assays were performed with different substrates listen in paragraph 2.2.16.1. Beside the addition of the single substrates, also mixtures were used to test for preferences in substrate choice. These were abietadiene, dehydroabietadiene and neoabietadiene as well as the LAS substrate (paragraph 2.2.14). The obtained product peak areas were listed in Table 3.1 below.

Table 3.1: Peak areas of catalyzed diterpene resin acids in enzyme assays. Listed are the product peak areas obtained from empty-vector control pESC-Leu, CYP720B1 and CYP720B4 for the different substrates. **-** not detectable; **(-)** identified but amount was below the limit of accurate integration.

		Peak area of corresponding DRA			aldehyde
		pESC-Leu	**CYP720B1**	**CYP720B4**	**CYP720B4**
Abietadiene		-	-	(-)	-
Dehydroabietadiene		-	-	13,297,766	1538781
Isopimaradiene		-	-	-	-
Neoabietadiene		-	-	-	-
Abietadienol		-	-	-	-
Dehydroabietadienol		11,212,717	5,295,321	19,875,101	17,145,032
Abietadiene	mix	-	-	1,521,696	-
Dehydroabietadiene	mix	-	-	12,067,717	587,865
Neoabietadiene	mix	-	-	-	-
Abietadiene	LAS substrate	-	-	639,115	-
Dehydroabietadiene	LAS substrate	-	-	413,407	-
Levopimaradiene	LAS substrate	-	-	-	-
Neoabietadiene	LAS substrate	-	-	-	-
Palustradiene	LAS substrate	-	-	-	-

No products were detected in the empty-vector control and in CYP720B1 except for the assay using abieta- and dehydroabietadienol as substrates. In the boiled controls no products were found. There were also no products detected for isopimaradiene, neoabietadiene, levopimaradiene, palustradiene and abietadienol in CYP720B4. In case of isopimaradiene and abietadienol, the substrates got too much diluted in the assays. Whereas both substances showed high peaks in their substrate solutions, they were not found again in the assay extractions. In extracted ion chromatogram (EIC) for m/z=272 and m/z=288 respectively, the substrate compounds were not detectable any more. In all the assays, none of the diterpene alcohol intermediates was accumulated to detectable levels in the different samples.

The aldehyde dehydroabietadienal was detected when used the single dehydroabietadiene, dehydroabietadiene in the mixture and dehydroabietadienol as substrates (Figure 3.10, also see paragraph 7.5, Figure S 3). No aldehyde was found for the assays performed with LAS substrate.

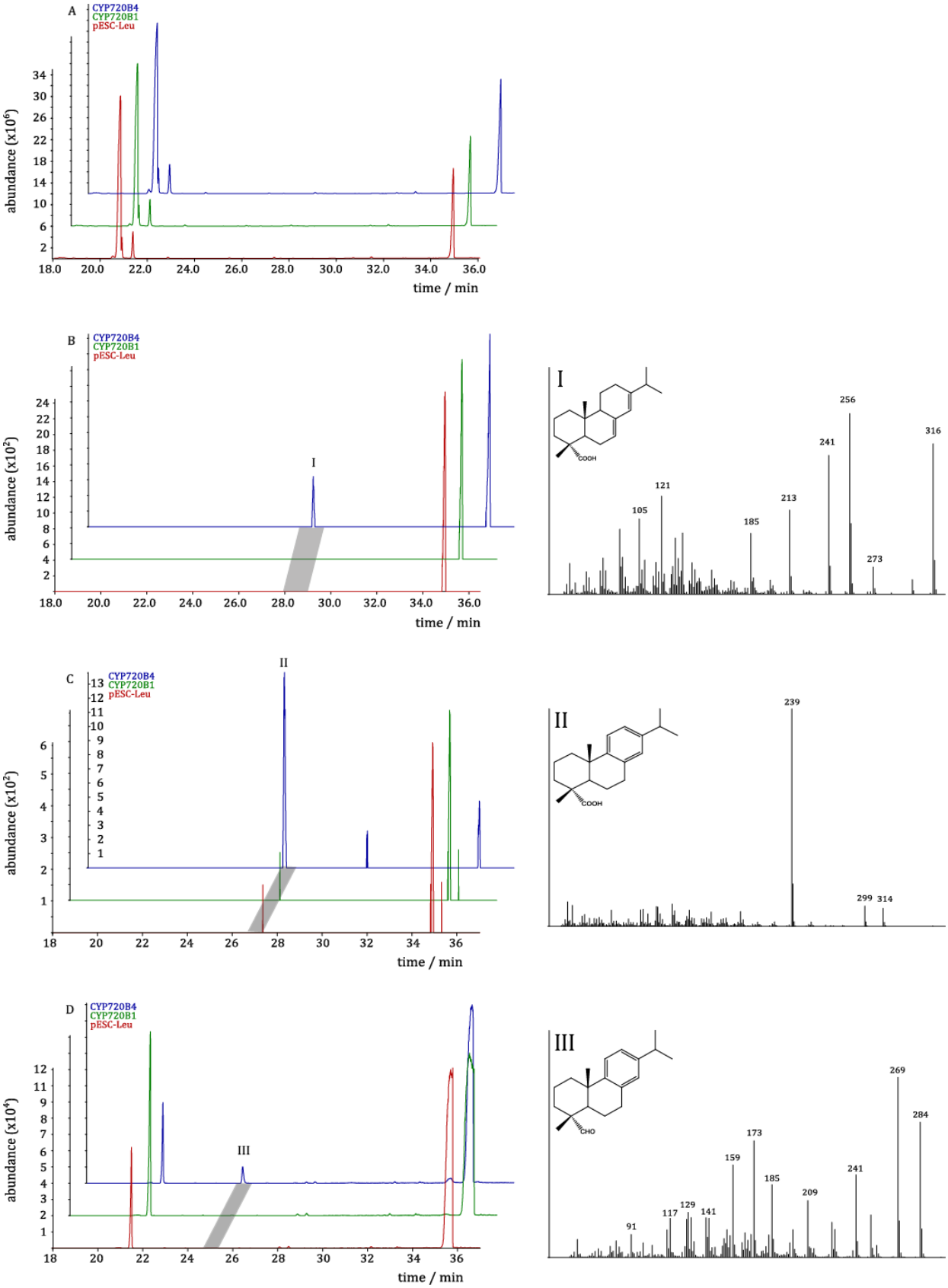

Figure 3.10: GC-MS chromatograms of emzyme assay extractions. (**A**) exemplary TIC of the assay extractions with non-visible product peaks but internal standard (R_t = 35.6 min). IEC for abietic acid at m/z=316 (**B**), dehydroabietic acid at m/z=314 (**C**) and dehydroabietadienal (m/z=269) (**D**). **I-III** show the related mass spectra and molecular structure. **Blue**: CYP720B4; **green**: CYP720B1; **red**: pESC-Leu empty vector control.

CYP720B4 showed activity for abietadiene, dehydroabietadiene and dehydroabietadienol, as proofed in assays using the pure substrates, a mix of abietadiene, dehydroabietadiene and neoabietadiene and the generated LAS substrate in paragraph 2.2.14. In general, the peaks of abietic acid (R_t = 27.7 min), dehydroabietic acid (R_t = 26.7 min) and dehydroabietadienal (R_t = 25.1 min) were small, already close to the background. In EIC the base peaks m/z=316 and m/z=314 of the acids became more obvious and sharper (Figure 3.10). Same took place for m/z=269, the greatest fragment peak of dehydroabietadienal. Due to this small intensity, no peak integration in the assay performed with abietadiene as substrate was possible. The small amount of abietic acid catalyzed was not enough to be graphed as a peak in total ion chromatogram. Nevertheless, in EIC an abietic acid peak was found and could also get verified by mass spectra (Figure 3.10, B).

Abietic acid, dehydroabietic acid and dehydroabietadienal were obtained in different amounts for using the mixture of abietadiene, dehydroabietadiene and neoabietadiene. The area of dehydroabietic acid is 8-fold greater than of abietic acid and more than 20-fold greater than the dehydroabietadienal peak. For LAS substrate only resin acid products were detectable. Abietic acid was the most catalyzed substance, but only 1.5-fold greater than dehydroabietic acid.

The empty-vector control as well as CYP720B1 showed a peak in the m/z=314 EIC at the same retention time as dehydroabietic acid (Figure 3.10, C). However, the mass spectra of these peaks were not accorded to dehydroabietic acid.

Contrary to the other substrates tested, the assay performed with dehydroabietadienol as substrate gave dehydroabietic acid product peaks for pESC-Leu, CYP720B1 and CYP720B4. The greatest peak area was given for CYP720B4, pESC-Leu medium-sized and CYP720B1 with the smallest area, almost 1/4 of CYP720B4. However, only the CYP720B4 sample contained dehydroabietadienal.

Although the added internal standard 12,14-dichlorodehydroabietic acid was not in a linear range to the obtained product peaks, an approximate percentage can be used. In comparison to the standard peak area, dehydroabietadienal showed a greater accumulation in alcohol-assays (0.36%) than for olefin substrates (0.13%).

4. Discussion

4.1 Terpenoid contents increased during treatment

One aim of this master thesis was to see if there is terpenoid accumulation after the treatment with MeJA. Another focus was set on the distribution in the different plant organs needles, roots and stem, which was composed of wood and bark.

Martin *et al.* and Schmidt *et al.* focused in their work at Norway Spruce on needles and traumatic resin ducts in xylem, respectively [3, 9, 25]. Their experiments showed significant changes in terpenoid accumulation after treatment. In 2002, the content of monoterpenes increase in stem 12-fold, the DRAs even 40-fold. Sesquiterpenes showed no change in accumulation. The treated saplings were harvested after two months, when traumatic resin ducts were fully developed. The samples in [9, 25] were already harvested after 25 days. In the work of Martin *et al.* in 2003, mono- and sesquiterpenes increased about 2-fold in needles, whereas the DRA concentration was stayed constant. An increase of 4.4 times in monoterpenes, 13.6 times for sesquiterpenes and 7 times for DRAs was detected from Schmidt *et al.* in 2011.

In this work, it was found that the amounts of all analyzed terpene classes were increased after treatment. Monoterpenes and sesquiterpenes rose in total about 1.5-fold, DRAs just 1.1-fold. Like previously described, in stem tissue, mono- and sesquiterpenes and DRAs showed the highest concentrations. Sesquiterpenes always were present in small concentrations, whereas DRAs were most present in stem and roots. Summarized, the total accumulation of terpenes was lower than reported in literature before.

The formation of traumatic resin ducts in the developing xylem is slow, with a delay of several days. Most likely, the formation is synchronized to the time point when larvae emerged from their eggs [3]. Also the *de novo* formation of terpenoids is delayed. Here, the harvesting took place already six days after treatment, which means that the increase could not be as high as reported in literature. For a second reason, only a 1 mM MeJA solution was used. In 2002, Martin *et al.* tried different concentrations with 1 mM, 10 mM and 100 mM methyl jasmonate. The greatest changes were detected using a 10 mM MeJA solution, however a concentration too high to be physiological to plants.

For monoterpenes, no qualitative change in composition was detected. For particularity, sabinene, δ-3-carene and α-terpinolene were mostly exclusively present in stem tissue. Some saplings had detectable amounts also in needles, but there were not enough biological replicates for statistical analysis. Next to this, tricyclene, camphor and bornylacetate seemed to play a special role in the plant. The compounds were for both, the controls and the treated saplings only detectable in needles. Presumable, they were involved in volatile emission. As a result of this, bornylacetate was

the compound given the characteristic conifer scent. It is possible, that there was higher increase, but compounds were volatilized.

Unlike monoterpenes, there was a composition change in sesquiterpenes. Longifolene increased from least prominent to the second most abundant compound in stem. Meanwhile, germacrene decreased to become least present. Changes in sesquiterpene composition were also recognized in 2003 by Martin and coworkers before, but in volatile emission. There the percentage of monoterpenes declined, whereas sesquiterpenes increased. Additionally, β-farnesene rose more than 100-fold. In this work the volatile emission was not measured, also did not β-farnesene.

Like sesquiterpenes, DRAs showed changes in composition for stem tissue. Levopimaric acid was dropped from most common compound to third most abundant compound. Abietic acid was increased the other way round; from third most abundant to most frequent resin acid. These were different results compared to Schmidt and coworkers, where levopimaric acid was found for highest DRA content after MeJA treatment.

In total, the terpenes were found to be influenced during a short time period after the MeJA treatment. They partially showed significant changes in concentration and composition. Additionally it was found that the roots of Norway Spruce contain terpenes, whose concentrations declined there at the second day. This may indicates redistribution within the plants, due to the fact that the attack was only on stem and needles.

4.2 Expression of CYP720B4 gene was activated by MeJA

During the last years, many groups have been working on terpenes in diverse plants. A wide variety of *mono*-, *sesqui*- and *di*TPS were already identified and characterized [18, 19, 41, 42]. In Norway Spruce these were *Pa*TPS-LAS and *Pa*TPS-ISO, named after their product profile levopimaradiene/abietadiene and isopimaradiene, respectively. Since only conifers contain diterpenes in their oxidized form and just few scientists were working on this topic, many questions were still open.

The first reported DRA-related P450 was *Pt*CYP720B1, published in 2005 by Ro *et al.* [33]. Still, just a few P450s were described and only some of them were fully characterized. Because of this, the project focused on CYP720Bs. The aim was to get information about their distribution among the different organs in conifers, their regulation associated by MeJA treatment, the used substrates and the products.

In this work, the expression analysis by quantitative Real-Time PCR of the selected four CYP720Bs showed differences in expression profiles over time and among the different tissues. CYP720B4 was found in little quantities in roots, whereas CYP720B11 was present in this tissue. CYP720B1 and CYP720B6 in general had a lower expression level than CYP720B4 and CYP720B11. CYP720B1 had the highest expression levels in needles, whereas B6 was most abundant in stem tissue.

In 2017, Celedon *et al.* analyzed the transcriptomes for all genes involved in the MEV and MEP pathway, different TPS and P450s in White Spruce. The transcriptome levels in different tissues were compared before and 8 days after treated with MeJA. Overall, the transcript levels for most of the genes post treatment, also for *Pg*CYP720B4, were reduced [7]. Same expression pattern as shown by Celedon *et al.* were obtained in the present work. CYP720B1, CYP720B6 and CYP720B11 had a lower expression level six days after treatment than the non-treated controls. The exception was CYP720B4, where higher expression levels were found at the last sampling time point.

Hamberger *et al.* in 2011 treated Sitka Spruce with MeJA and got a significant increase of *Ps*CYP720B4 transcript in bark tissue. Though, other CYP720Bs were not shown MeJA-induced changes in transcript levels [5]. The transcript of *Pa*CYP720B4 was like the transcript of *Ps*CYP720B4 also most abundant in stem.

The results of CYP720B4 support the initial hypothesis, where parallel to DRA increase in the tissue, the expression of a Cytochrome P450 should also increase. *Pa*CYP720B4, *Pa*CYP720B6, *Pa*CYP720B11 showed significance among the tissues, whereas *Pa*CYP720B1 was influenced by the treatment. The increased expression of the CYP720Bs after two days and the increase in DRA accumulation seems to support the hypothesis, where an increase in Cytochrome P450 expression

should result in an increased amount of DRAs in the tissue. However, it has to be considered that changes on the genotype take some time until they are noticeable on the phenotype level.

Due to the results from Hamberger et al., where *Ps*CYP720B8 was mostly expressed in young and mature needles and *Ps*CYP720B9 only found in roots, respectively, there was also the expectation to detect some Cytochrome P450 candidates in *P. abies* especially expressed in certain tissues [5].

The fact that CYP720B11 is mainly present in the roots supports the suggestion, that also Norway Spruce has organ-related enzymes. These are probably coordinated to the need of certain DRAs in the respective tissue and the substrate and product profile of the enzyme.

The sequences obtained after the Sanger sequencing were compared with each other and other relevant published CYP720Bs by multiple sequence alignment. *Ps*CYP720B4 (E5FA70_PICSI) belongs to clade III in the CYP720B subfamily. *Pa*CYP720B4 and *Ps*CYP720B4 share about 98.6% similarity, whereas all sequences were 71.6% identical. For many regions the sequence was conserved or differed only by few amino acids.

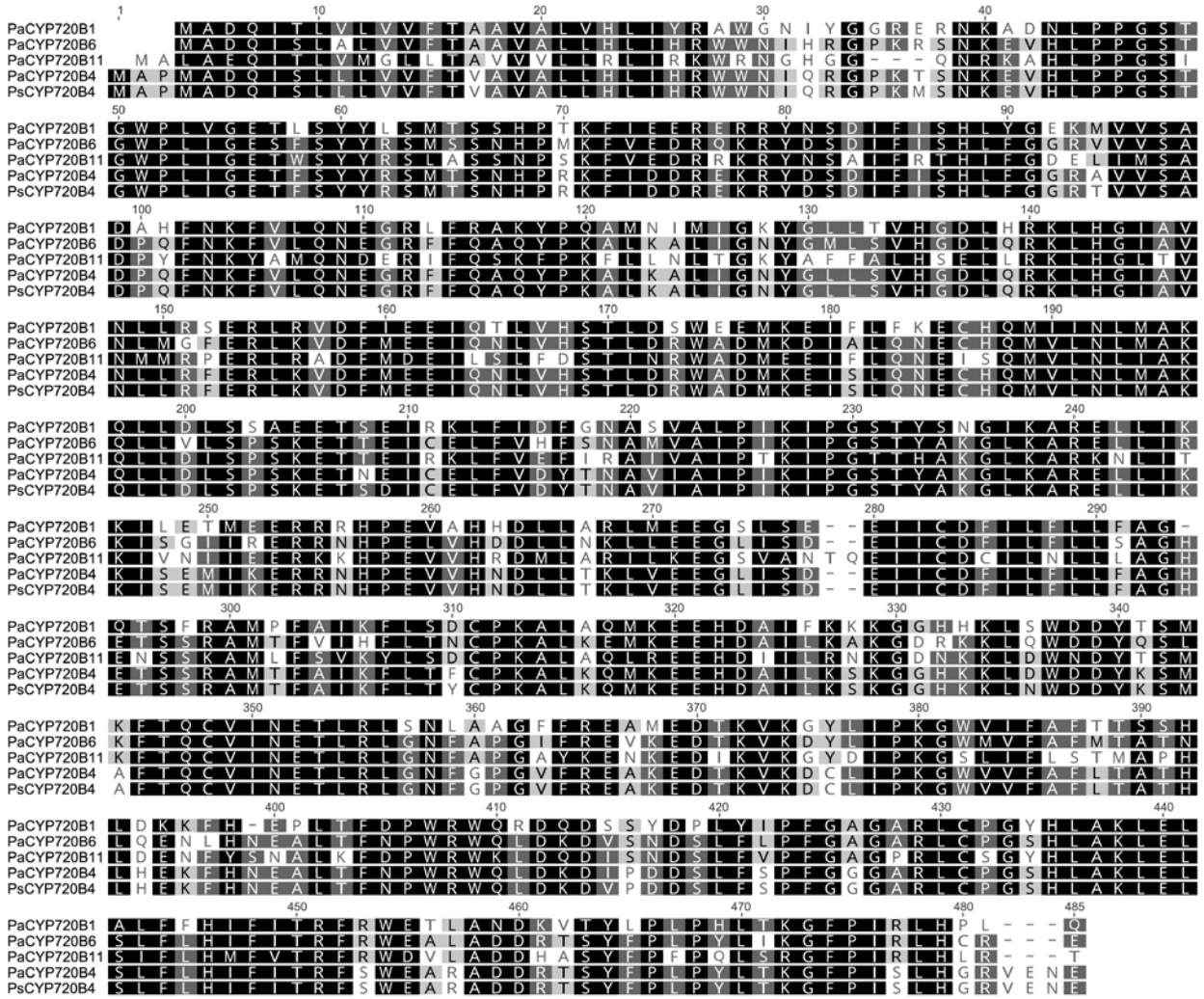

Figure 4.1: Multiple sequence alignment of different CYP720Bs. Alignment of the quested *Pa*CYP720B1, B6, B11, B4 and literature known *Ps*CYP720B4 (E5FA70_PICSI, www.uniprot.org). Sequences share 71.6% pairwise identity and were 84.1% pairwise positive. The similarity between *Pa*CYP720B4 and *Ps*CYP720B4 was about 98,6% pairwise identity.

4.3 *Pa*TPS-LAS and *Pa*TPS-ISO systems yielded in diterpene olefins

The expression of *Pa*TPS-LAS and *Pa*TPS-ISO in *E. coli* yielded in high amounts of the desired diterpene olefins, greater than the concentrations within the stem 6 days after treatment.

Martin *et al.* first reported in 2004 on *Pa*TPS-LAS and *Pa*TPS-ISO substrate expression [18]. It was observed, that the chromatograms of this thesis are similar to their results. The characterization of *Pa*TPS-LAS products was verified in 2011 by Keeling *et al.* [1]. They showed a different product profile than Martin *et al.*, with two additional alcoholic products and also dehydroabietadiene. According to their findings, the differences were caused by conditions and temperatures of the GC-MS injector and column. Keeling *et al.* also incubated *Ps*TPS-LAS and *Ps*TPS-ISO diterpene synthases with GGPP [1].

In this master thesis, the *Pa*TPS-LAS chromatogram showed almost the same amounts of levopimaradiene, abietadiene and neoabietadiene, and additional peak of dehydroabietadiene. Dehydroabietadiene was neither present in the work of Martin *et al.* nor Keeling *et al.* using *Ps*TPS-LAS but detected by Keeling *et al.* working with *Pa*TPS-LAS. In literature, the quantities of the compounds were not similar as shown in this project. For both enzymes, *Pa*TPS-LAS and *Ps*TPS-LAS, Keeling *et al.* found the same profile of quantities. Abietic acid was the main product, followed by levopimaric acid and neoabietic acid. In the work of Martin *et al.*, levopimaric acid was the main product, with fewer amounts of abietic acid and neoabietic acid.

An explanation for the differences in product quantities for *Ps*TPS-LAS was the various product profiles of the two enzymes. Probably, *Ps*TPS-LAS product profile is also depending upon the GC-MS conditions like term of the run or column temperature gradient and the injector temperature, as shown for *Pa*TPS-LAS [1].

Consequently, dehydroabietic acid would not additionally appear in this experiment but was not detectable in the chromatogram of Martin *et al.* Another difference to the chromatograms in literature was the separation between palustradiene and levopimaradiene. In the present work, the peaks were not baseline separated. Due to the high similarity of their double bond positions and the polarity of the rings, it is difficult to separate them by baseline. However, the separation was clearer in [18] and successful in [1] and [31]. Also for dehydroabietadiene was no baseline separation between the relevant peak and the side product peak in front.

In case of *Ps*TPS-ISO, Keeling *et al.* obtained with sandaracopimaradiene a second substance after expression. Sandaracopimaradiene was not catalyzed by *Pa*TPS-ISO in this work and in the experiment done by Martin *et al.* Because it is known that *Pa*TPS-ISO catalyzes sandaracopimaradiene [16], an explanation to the missing sandaracopimaradiene peak was the general low formation rate of the compound catalyzed by the enzyme. Second, less sensitivity of the used GC-MS or rather a too high dilution of the sample could be the reason for non-detecting. In

opposite to the isopimaradiene extraction, too much of the LAS extraction was injected for analysis. Because the stationary phase was overloaded, the peaks of levopimaradiene and abietadiene already started to front. This seems not to be caused by different efficiencies of the *Pa*TPS-LAS and *Pa*TPS-ISO system. Probably isopimaradiene was resuspended in a greater volume of diethyl ether. The greater dilution leaded to lower abundance in the analysis.

To assign the given peaks to a certain compound and to verify the products, the MS spectra of the substances (Figure 3.9) were compared to MS spectra as reported in Martin *et al.* (2004)as well as Lee *et al.* 2001 [43]. Additionally, all substrate peaks were compared to available standards and the mass spectra published by Martin *et al*.

The formation of diterpene olefins in a *Pa*TPS-LAS or *Pa*TPS-ISO system in yeast is a simple method to obtain high yields of the corresponding diterpene olefins. As alternative to a (retro-) synthesis, the system is able to produce contamination-free olefins mixtures, which can be separated by preparative chromatographic methods.

4.4 CYP720B4 is a multifunctional, multisubstrate enzyme

The literature-known *Ps*CYP720B4 from Hamberger *et al.* [5] shares about 98.6% amino acid identity to *Pa*CYP720B4, the new P450 found in Norway Spruce (Figure 4.1). This Cytochrome P450 was described as a multifunctional and multisubstrate enzyme and to be active with in total 24 different compounds. *Pa*CYP720B1 also shares similarity with *Pt*CYP720B1 (Figure S 2), also known as a multifunctional and multisubstrate P450 by Ro *et al.* in 2005 [33]. Here, multifunctional is defined as the possibility to catalyze at least two consecutive oxidation steps [5]. Due to the high similarity, the hypothesis was set that *Pa*CYP720B4 should have a similar substrate and product profile than *Ps*CYP720B4. Same was not expected for *Pa*CYP720B1 and *Pt*CYP720B1 because of the less similarity, but the followed enzymatically tests should at least gave a hint of possible substrate range and product variety.

*Pt*CYP720B1 was able to convert abietadiene, abietadienol, abietadienal, dehydroabietadienol, dehydroabietadienal, isopimaradienol, isopimaradienal and levopimaradienol. These were converted to resin acids in case when the assays were performed with aldehyde as substrates and converted into aldehydes when the alcohol was used as substrate. With neoabietadienol and neoabietadienal the enzyme showed no activity and palustradienates, pimarates and sandaracopimarates were not tested in the publication. The experiments were done in microsomes and directly in yeast cells. In general, there was no difference between the systems. Only abietadiene was not converted into abietic acid in the microsomes. Highest activity was received by dehydroabietadienol, followed by the other alcohols and the aldehydes of the other diterpenes. In this project, *Pa*CYP720B1 showed no activity for any substrate. Ro and coworkers used almost the same expression system with the same pESC-Leu vector but a different yeast strain. Technically *Pa*CYP720B1 should be able to convert the substrates. It is possible that *Pa*CYP720B1 showed activity but products were not catalyzed in a detectable amount. This could be caused by a low concentration of *Pa*CYP720B1 in the microsomes, resulting from the reason that no codon optimization for expression in yeast was done, unlike to *Pt*CYP720B1. Another reason can be that crude microsomes, which were still containing yeast enzymes, were used. Because of a missing FLAG-epitope, it was not possible to get the purified P450 and to perform enzymatically assays with.

As already mentioned, *Ps*CYP720B4 showed activity to the diterpene olefins, alcohols and aldehydes of abietadienate, dehydroabietadienate, neoabietadienate, levopimarate, palustradienate, pimarate, isopimarate and sandaracopimarate. Most products were obtained with dehydroabietadienate, whereby there was a preference for producing the alcohols and aldehydes over the olefins. It was possible for Hamberger and coworkers to stop the reaction after a single oxidation step. Assessing the results, the pathway-intermediates and the postulated mechanism

could get proofed. Thereby, the step from alcohol to aldehyde intermediate seemed to be the most efficient at their reaction conditions. Beside the single step oxidation, they also tested the activity from substrate to the consecutive acid. Here, the last oxidation step from aldehyde to the resin acid was the most active one.

In the present master thesis the main products of dehydroabietadienate diterpenes were found. The assay performed with dehydroabietadiene as substrate was producing the resin acid dehydroabietic acid as well as the aldehyde dehydroabietadienal. Hamberger *et al.* did not show data about the activity to convert the olefin into aldehyde, but in the actual project, the three-step-oxidation to the acid was preferred over the two-step-oxidation till the aldehyde. Comparing the data to literature indicated, that abietadienate should also be converted into the intermediates. Following the idea that *Pa*CYP720B4 prefers to catalyze the acid form, the amount of the corresponding abietadienol was only too low for detection. The amount of abietic acid found in the mixture was already close to the background and for single abietadiene the product was only detected by EIC. For dehydroabietadiene the amount of the aldehyde was about 9-fold (single dehydroabietadiene) and 20-fold (mixture) less than the acid. This would also explain why there was no aldehyde detected for the LAS-substrate. In the assay performed with the mixture as substrate, the used concentration of dehydroabietadiene was only 1/3 of the concentration used for single dehydroabietadiene. However, the peak areas of dehydroabietic acid were about the same size, whereas the aldehyde formation in the single assay is 3 times greater for the mixture. Thus, *Pa*CYP720B4 catalyzed dehydroabietic acid more efficient in the mixture than having only the single substrate. In numbers, the dehydroabietic acid peak in the mixture was only 16% of the dehydroabietadiene size, whereas single dehydroabietadiene was only 6.8%. Although the assay of dehydroabietadienol was contaminated by dehydroabietic acid, the aldehyde is only formed in the CYP720B4 assay. The dehydroabietic acid peak areas indicate, that there was an additionally acid formation aside the contamination. The peak area of dehydroabietadienal is almost about the same size more than the acid peak area. This result conforms to the results from Hamberger, where dehydroabietadienol showed a greater activity to form the aldehyde (100%) than the resin acid (81.1%). Owing to the fact that most of the substrates were not commercial available in general or only in a mixture like palustradiene and levopimaradiene, a broader substrate conversion cannot be excluded. As Ro *et al.* before, also Hamberger *et al.* tested the activity in two different systems. Next to the assay directly on yeast cells, they tested *Ps*CYP720B4 produced in *E. coli* membrane fraction. For both cases, the enzyme was codon optimized before expression. The usage of *E. coli* as expression system is not common for Cytochromes, since they anchore in the endoplasmatic reticulum. To prepare the enzyme for prokaryotes, Hamberger *et al.* exchanged the N-term of *Ps*CYP720B4 by the N-term of bovine CYP17α. Also in laboratory-used yeast strains, the NADPH-dependent Cytochrome P450 reductase of *A. thaliana* is already implemented. This was not the case

for *E. coli*, as such the enzyme was ligated into the bacteria first. As an advantage of the methodology, they used the reductase of *P. sitchensis* to provide a more related system. Because the system of *E. coli* is less complex than the one of *S. cerevisiae*, background activity from *E. coli* genes can be excluded. On the other hand, Ro *et al.* and Hamberger *et al.* did not show great differences comparing both systems, even when they used living yeast cells and no microsomes. Similarly to literature, it cannot be ignored or excluded that solubility of the substrates was influencing the results. Diterpene olefins were less polar than diterpene alcohols and aldehydes. All substrates were solved in diethyl ether, which has a water solubility of 69 $g \cdot L^{-1}$ [44]. There is the chance that not all of the olefin substrate was available for reaction. Adding up to the purity of the substrates, only dehydroabietadienol was contaminated by dehydroabietic acid. Experiments showed that, the different diterpenes start to epimerize and also oxidize after time (personal communication from Dr. Nakamura). The used substrates were stored closed at a temperature of -20°C in organic solvent (diethyl ether), creating little chance for spontaneous transformation. It is however not unfounded that the diterpenes start oxidizing into their different intermediate forms, even in the assay extractions. Prior to the fact that most assays showed no products, the concentration of NADPH was modified to check the influence. Moreover, the assays were also prepared with an old and new batch of NADPH to verify that the substance is not already fully oxidized. Both modifications did not lead to different results. Also no changes in the results showed the use of different temperatures at 28°C and 30°C. If the product concentration has been higher then probably there would be a difference which is not detectable yet. It could be that there was also no difference found among the incubation period. The shortest incubation time was 60 minutes, the longest about 15 hours. This was done to be sure to get products in detectable amounts, related to the general less production. Comparing the product peak areas, no difference can be reported. So the reaction is finished before one hour and the products are not further converted or degraded. There will be a change if the incubation time is shortened. Hamberger *et al.* run their assays only for 10 and 20 minutes to see differences in product abundance. In summary, *Pa*CYP720B1 was found to be inactive in this project. Further work needs to be done to obtain a working recombinant enzyme and to do characterization. Still it is possible that CYP720B1 is active with other, not in this thesis used substrates. The enzymatically activity of CYP720B4 showed, that the used expression system in yeast and the established enzyme assays can be used for Cytochrome P450 characterization. For *Pa*CYP720B4, the hypothesis of a multifunctional, multisubstrate enzyme was confirmed. It showed activity with abietadiene, dehydroabietadiene and dehydroabietadienol, converting them into the corresponding resin acid (abietic acid, dehydroabietic acid) and the aldehyde (dehydroabietadienal). For further characterization, especially for the diterpene intermediates products, further experiments need to be done. The missing diterpene intermediate substrates should be used to get a full overview of the possible oxidation steps of *Pa*CYP720B4.

5. Conclusion

Diterpene resin acids play an important role in the chemical defense of conifers against herbivores and pathogenic pests. The terpenes are constitutively stored together with mono- and sesquiterpenes in resin ducts. During an attack they are released to prevent the affected site from invading organisms. Caused by divergent factors, a *de novo* formation of traumatic resin ducts and terpene synthesis as well as terpene accumulation can be induced. The three-step oxidation of diterpene olefins to diterpene resin acids is catalyzed by Cytochrome P450 Monooxygenases of the 720B subfamily. Whereas for other conifer species some CYP720Bs are already characterized, there is no assayed enzyme for Norway Spruce yet.

In the present master thesis, *Picea abies* saplings were treated with methyl jasmonate to induce their defense mechanisms. The monoterpenes, sesquiterpenes and diterpene resin acids were found to be influenced methyl jasmonate within a short time period of 2-6 days after the treatment, leading in concentration and composition changes. In addition, the plant organs contained different amounts and combinations of terpenes. Also the roots of the plants contain secondary defense metabolites, which however declined over the experimental time period. The terpene amount and distribution used as basic, the CYP720B candidates in *P. abies* were examined about their distribution in the plant organs, their possibility to get induced by methyl jasmonate and to a correlation between diterpene resin acid accumulation and CYP720B transcript activation. It turned out, that CYP720B1 was mostly present in needles, CYP720B11 in roots and CYP720B4 most abundant in needles and stem. The increased expression of the CYP720Bs after two days and the increase in DRA accumulation seems to support the hypothesis, where increased Cytochrome P450 expression levels should result in increased amounts of diterpene resin acids. Both outcomes manifest the suggestion, that even certain CYP720B enzymes are responsible for the formation of defined diterpene resin acids. The CYP720B1 and B4 enzymes were further used for characterization. Therefore the enzymes were tested for different substrates to analyze their substrate specificity and the produced resin acids. CYP720B1 was found to be inactive for all in this project tested substrates. On the contrary, CYP720B4 showed activity to different substrates. CYP720B4 is not only able to catalyze reactions starting with diterpene olefins but also with the alcohol intermediate. Additionally the enzyme catalyzed the formation of diterpene aldehyde intermediates and diterpene resin acids and was shown to be a multifunctional and multisubstrate Cytochrome P450.

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7. Supplement

7.1 Data of terpene contents in *Picea abies*

Table S 1: Terpenoid content for different time points and tissues. The Table shows averaged sesquiterpenes, monoterpenes and DRAs content ± standard error in µg/g fresh weight. The terpenoids were extracted with TBME and analyzed on GC-MS (see paragraph 2.2.2& 2.2.3). Terpenes were grouped by a significant level($p < 0.05$) and declared with small letters. For non-declared compounds not sufficient biological replicates were available for mixed effect model statistics.

	needles						roots						stem					
	day 0		day 2		day 6		day 0		day 2		day 6		day 0		day 2		day 6	
α-pinene	5.29E-03 ±	7.78E-04 (a)	7.51E-03 ±	1.35E-03 (b)	6.98E-03 ±	6.37E-04 (b)	4.46E-03 ±	6.85E-04 (a)	3.11E-03 ±	1.27E-03 (a)	3.76E-03 ±	5.59E-04 (a)	4.58E-03 ±	4.53E-04 (a)	8.45E-03 ±	6.61E-04 (b)	8.58E-03 ±	3.94E-04 (b)
α-terpinolene	2.16E-05 ±	2.16E-05	2.32E-05 ±	2.32E-05	2.90E-05 ±	2.90E-05	n.d.		n.d.		n.d.		1.68E-04 ±	1.66E-05	2.36E-04 ±	8.99E-05	3.50E-04 ±	1.77E-04
β-pinene	7.75E-04 ±	1.53E-04 (a)	9.76E-04 ±	1.56E-04 (a)	1.05E-03 ±	9.63E-05 (a)	2.15E-03 ±	3.76E-04 (a)	1.36E-03 ±	7.10E-04 (b)	1.41E-03 ±	1.93E-04 (b)	1.00E-02 ±	1.37E-03 (c)	2.01E-02 ±	1.71E-03 (d)	1.86E-02 ±	1.09E-03 (d)
bornylacetate	2.34E-03 ±	4.16E-04	2.73E-03 ±	3.76E-04	3.13E-03 ±	2.98E-04	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
campehene	7.80E-03 ±	1.17E-03 (a)	1.16E-02 ±	2.13E-03 (a)	1.03E-02 ±	1.32E-03 (a)	9.02E-04 ±	1.33E-04 (b)	7.01E-04 ±	2.01E-04 (b)	1.11E-03 ±	2.55E-04 (b)	3.44E-04 ±	6.70E-05 (c)	2.52E-04 ±	2.82E-05 (c)	2.96E-04 ±	6.89E-06 (c)
camphor	1.10E-03 ±	2.45E-04	2.38E-03 ±	5.12E-04	1.81E-03 ±	6.25E-04	n.d.		n.d.		n.d.		n.d.		n.d.		n.d.	
δ-3-carene	1.70E-05 ±	1.70E-05	n.d.		6.13E-05 ±	6.13E-05	2.49E-05 ±	2.49E-05	n.d.		n.d.		4.54E-03 ±	6.42E-04	5.17E-03 ±	2.74E-04	9.08E-03 ±	3.05E-03
limonene	5.83E-03 ±	1.03E-03 (a)	7.48E-03 ±	1.21E-03 (a)	7.54E-03 ±	8.24E-04 (a)	1.19E-03 ±	1.53E-04 (b)	7.65E-04 ±	2.80E-04 (c)	9.22E-04 ±	1.04E-04 (b)	7.43E-03 ±	6.70E-04 (a)	1.26E-02 ±	9.07E-04 (d)	1.48E-02 ±	2.66E-03 (d)
myrcene	6.63E-04 ±	9.62E-05 (a)	9.13E-04 ±	1.60E-04 (a)	8.65E-04 ±	1.02E-04 (a)	1.14E-04 ±	1.13E-05 (b)	3.72E-05 ±	3.72E-05 (c)	5.65E-05 ±	3.06E-05 (c)	8.24E-04 ±	8.61E-05 (a)	1.36E-03 ±	1.23E-04 (d)	1.68E-03 ±	2.72E-04 (d)
sabinene	4.73E-05 ±	2.74E-05	1.64E-04 ±	2.80E-05	7.12E-05 ±	7.12E-05	n.d.		n.d.		n.d.		1.69E-04 ±	2.45E-05	2.63E-04 ±	1.63E-05	4.23E-04 ±	1.49E-04
tricyclene	8.56E-04 ±	1.32E-04	1.28E-03 ±	2.40E-04	1.15E-03 ±	1.59E-04	n.d.		n.d.		5.65E-05 ±	3.34E-05	n.d.		n.d.		n.d.	
α-humulene	6.30E-04 ±	1.43E-04 (a)	7.33E-04 ±	1.24E-04 (a)	6.88E-04 ±	5.56E-05 (a)	n.d.		n.d.		n.d.		1.87E-04 ±	1.09E-04 (b)	2.34E-04 ±	6.12E-05 (b)	2.35E-04 ±	1.65E-05 (b)
germacrene	1.69E-04 ±	1.10E-04 (a)	2.59E-04 ±	1.30E-04 (a)	2.45E-04 ±	8.35E-05 (a)	n.d.		n.d.		n.d.		3.67E-04 ±	1.44E-04 (ab)	4.68E-05 ±	2.46E-05 (b)	1.05E-04 ±	5.36E-05 (b)
longifolene	1.30E-05 ±	1.30E-05	5.22E-05 ±	2.62E-05	3.91E-05 ±	3.91E-05	n.d.		n.d.		n.d.		1.36E-04 ±	5.01E-05	6.72E-04 ±	1.49E-04	3.99E-04 ±	5.01E-05
trans-β-caryophyllene	3.95E-04 ±	1.10E-04 (a)	5.26E-04 ±	7.40E-05 (a)	5.30E-04 ±	4.36E-05 (a)	n.d.		n.d.		n.d.		6.45E-04 ±	9.41E-05 (a)	1.76E-03 ±	4.06E-04 (b)	1.61E-03 ±	1.09E-04 (b)
abietic acid	1.00E-03 ±	1.60E-04 (a)	9.65E-04 ±	6.88E-05 (a)	1.28E-03 ±	1.99E-04 (a)	2.28E-03 ±	6.19E-04 (b)	1.35E-03 ±	5.82E-04 (b)	1.79E-03 ±	3.70E-04 (b)	2.49E-02 ±	4.16E-03 (c)	3.33E-02 ±	2.26E-03 (c)	4.21E-02 ±	8.79E-03 (c)
dehydroabietic acid	1.18E-02 ±	2.71E-03 (a)	1.71E-02 ±	2.64E-03 (a)	1.49E-02 ±	2.08E-03 (a)	3.09E-03 ±	5.79E-04 (b)	2.16E-03 ±	5.29E-04 (b)	2.87E-03 ±	2.91E-04 (b)	8.21E-03 ±	1.47E-03 (a)	1.19E-02 ±	6.43E-04 (a)	1.41E-02 ±	2.68E-03 (a)
isopimaric acid	6.04E-04 ±	1.05E-04 (a)	8.84E-04 ±	2.59E-05 (a)	8.05E-04 ±	1.67E-04 (a)	2.03E-03 ±	4.02E-04 (b)	1.37E-03 ±	4.15E-04 (b)	1.84E-03 ±	2.77E-04 (b)	1.27E-02 ±	2.15E-03 (c)	2.35E-02 ±	2.54E-03 (d)	2.44E-02 ±	4.08E-03 (d)
levopimaric acid	5.57E-03 ±	1.42E-03 (a)	4.61E-03 ±	1.28E-03 (a)	6.82E-03 ±	2.00E-03 (a)	1.58E-02 ±	2.49E-03 (b)	1.08E-02 ±	4.47E-03 (b)	1.33E-02 ±	2.06E-03 (b)	4.83E-02 ±	4.12E-03 (b)	2.67E-02 ±	1.83E-02 (b)	2.75E-02 ±	1.84E-02 (b)
neoabietic acid	8.18E-04 ±	1.87E-04 (a)	6.33E-04 ±	1.82E-04 (a)	9.48E-04 ±	3.38E-04 (a)	3.20E-03 ±	7.60E-04 (b)	2.03E-03 ±	7.99E-04 (b)	2.60E-03 ±	5.79E-04 (b)	3.62E-02 ±	4.82E-03 (c)	5.04E-02 ±	3.65E-03 (c)	3.40E-02 ±	1.31E-02 (c)
sandaracopimaric acid	7.57E-04 ±	8.23E-05 (a)	1.03E-03 ±	1.57E-04 (a)	1.09E-03 ±	4.57E-05 (a)	2.89E-03 ±	4.68E-04 (b)	2.08E-03 ±	7.36E-04 (b)	2.72E-03 ±	4.28E-04 (b)	4.60E-03 ±	5.60E-04 (c)	5.61E-03 ±	3.33E-04 (d)	6.74E-03 ±	1.03E-03 (d)

Table S 2: Statistical parameters of terpene analysis for different time points and tissues. (Table S 1)
The degrees of freedom of the F-value and L-ratio are given in brackets.

compound	transformation	method	comparison	F / chi^2 / L-ratio	p-value
monoterpenes	-	OneWay ANOVA		0.264 (2)	0.776
sesquiterpenes	-	Kruskal-Wallis test by ranks		1.249 (2)	0.536
diterpenes	log	OneWay ANOVA		0.005 (2)	0.995
α-humulene	-	mixed effect model	tissue	26.076 (5)	< 0.0001
germacrene	-	mixed effect model	interaction	6.916 (6)	0.032
longifolene	-	Kruskal-Wallis test by ranks		7.436 (2)	0.024
trans-β-caryophyllene	-	mixed effect model	interaction	11.030 (6)	0.004
α-pinene	-	mixed effect model	interaction	16.112 (7)	0.003
α-terpinolene	-	Kruskal-Wallis test by ranks		0.656 (2)	0.720
β-pinene	log	mixed effect model	interaction	13.953 (7)	0.007
bornylacetate	-	OneWay ANOVA		1.099 (2)	0.385
camphene	log	mixed effect model	tissue	91.714 (5)	< 0.0001
camphor	-	OneWay ANOVA		2.147 (2)	0.187
δ-3-carene	-	Kruskal-Wallis test by ranks		2.955 (2)	0.228
limonene	log	mixed effect model	interaction	13.763 (7)	0.008
myrcene	log	mixed effect model	interaction	17.470 (7)	0.002
sabinene	-	Kruskal-Wallis test by ranks		7.000 (2)	0.030
		Pairwise Wilcoxon	day 2 - day 0		0.170
		rank sum test	day 6 - day 2		2.000
			day 6 - day 0		0.170
tricyclene	-	OneWay ANOVA		1.676 (2)	0.254
abietic acid	log	mixed effect model	tissue	84.770 (5)	< 0.0001
dehydroabietic acid	log	mixed effect model	tissue	50.947 (5)	< 0.0001
isopimaric acid	log	mixed effect model	interaction	11.937 (7)	0.018
levopimaric acid	log	mixed effect model	tissue	24.059 (5)	< 0.0001
neoabietic acid	log	mixed effect model	tissue	73.596 (5)	< 0.0001
sandaracopimaric acid	-	mixed effect model	interaction	9.703 (7)	0.046

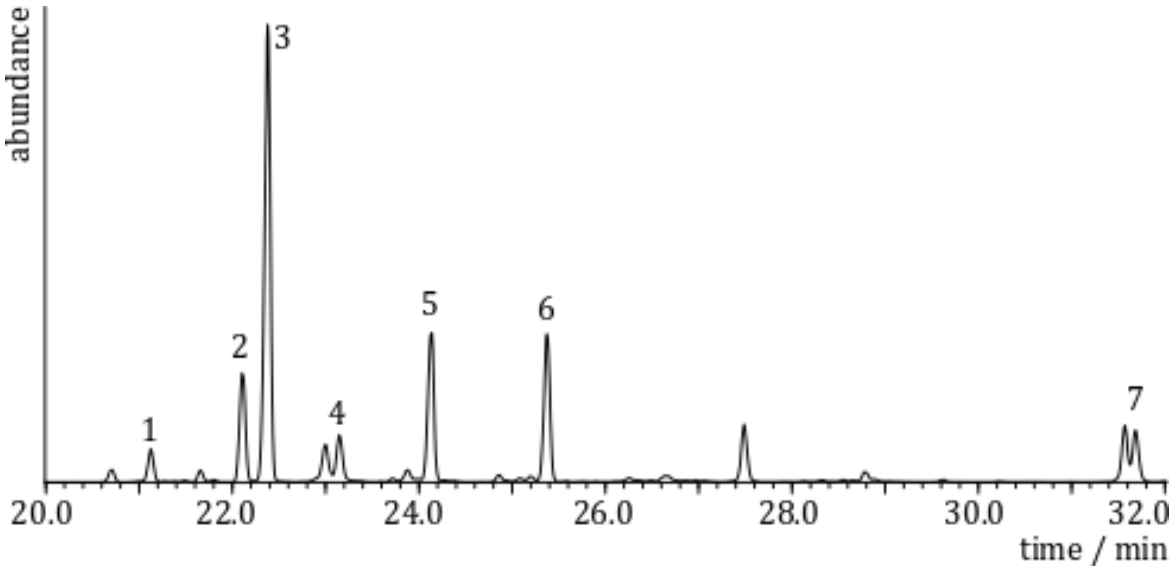

Figure S 1: GC-MS total ion chromatogram of diterpene resin acid extraction. The chromatogram shows exemplary the resin acids sandaracopimaric acid (1), isopimaric acid (2), levopimaric acid (3), dehydroabietic acid (4), abietic acid (5), neoabietic acid (6) and dichlorodehydroabietic acid as standard (7).

7.2 CYP720B expression levels

Table S 3: Relative normalized expression of CYP720Bs for different time points and tissues.
The Table shows the averaged CYP720B expression levels ± standard error. After statistical mixed effect model, they were grouped by a significant level ($p < 0.05$) and declared with small letters.

		time period		
		day 0	day 2	day 6
B1	needles	1.25E+00 ± 3.16E-01 (a)	2.25E+00 ± 7.64E-01 (b)	9.62E-01 ± 2.30E-01 (a)
	roots	7.06E-01 ± 2.81E-02 (a)	7.11E-01 ± 2.22E-01 (a)	3.02E-01 ± 4.26E-02 (c)
	stem	1.53E+00 ± 7.86E-01 (a)	1.86E+00 ± 1.49E-01 (b)	3.76E-01 ± 4.21E-02 (c)
B4	needles	9.18E-01 ± 3.15E-01 (a)	1.05E+01 ± 4.23E+00 (a)	1.13E+01 ± 8.09E+00 (a)
	roots	2.02E-01 ± 3.78E-02 (b)	2.39E-01 ± 5.43E-02 (b)	1.56E-01 ± 5.80E-02 (b)
	stem	1.11E+00 ± 2.83E-01 (a)	1.86E+01 ± 1.86E+00 (a)	4.82E+00 ± 9.37E-01 (a)
B6	needles	1.28E+00 ± 5.06E-01 (a)	5.09E-01 ± 1.82E-01 (a)	2.23E-01 ± 6.62E-02 (a)
	roots	5.30E-01 ± 1.12E-01 (a)	6.55E-01 ± 3.14E-01 (a)	2.50E-01 ± 7.82E-02 (a)
	stem	1.03E+00 ± 1.39E-01 (b)	2.31E+00 ± 1.63E-01 (b)	1.03E+00 ± 1.54E-02 (b)
B11	needles	1.18E+00 ± 3.31E-01 (a)	1.25E-01 ± 6.36E-02 (a)	2.67E-01 ± 5.69E-02 (a)
	roots	7.09E-01 ± 4.66E-01 (a)	1.23E+01 ± 5.06E+00 (a)	1.30E-01 ± 1.63E-02 (a)
	stem	1.32E+00 ± 5.41E-01 (a)	6.88E-01 ± 4.27E-02 (a)	5.79E-01 ± 3.69E-01 (a)

Table S 4: Statistical parameters of CYP720B gene expression levels. (Table S 3)
The degrees of freedom of the L-ratio are given in brackets.

compound	transformation	method	comparison	L-ratio	p-value
B1	log	mixed effect model	interaction	3.303 (7)	0.509
B4	log	mixed effect model	tissue	19.745 (5)	0.000
B6	log	mixed effect model	tissue	7.606 (5)	0.022
B11	--	mixed effect model	tissue	6.025 (5)	0.049

7.3 Coding sequences of CYP720Bs

7.3.1 *Pa*CYP720B1

Coding DNA sequence, 1428 bp

ATGGCAGACCAAATAACTCTAGTGTTGGTAGTATTCACTGCAGCAGTGGCGCTTGTTCACCTTATCTACAG
AGCTTGGGGAAATATTTATGGCGGAAGAGAAAGAAATAAAGCAGACAATCTGCCACCTGGGTCGACTGGT
TGGCCGCTGGTTGGCGAAACGTTGAGTTATTATCTGTCCATGACCAGCAGTCATCCCACGAAGTTCATCGA
GGAACGGGAGAGAAGGTATAATTCGGACATTTTCATATCTCATTTATATGGAGAGAAGATGGTTGTTTCG
GCAGATGCCCACTTCAACAAGTTTGTGCTGCAAAACGAGGGAAGGCTCTTTCGAGCCAAATACCCACAGGC
TATGAATATTATGATTGGAAAATATGGGTTGCTCACAGTGCATGGCGATCTCCACAGGAAGCTCCATGGA
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ACTCCACTCTGGATAGCTGGGAAGAGATGAAGGAAATTTTTCTTTTTAAAGAGTGTCACCAGATGATCAT
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CATCATGATTTGCTGGCAAGGCTCATGGAAGAAGGTTCCCTGTCAGAGGAAATTATTTGTGATTTTATTC
TATTTTTGCTCTTTGCTGGCCAGACATCCTTCAGAGCCATGCCATTTGCCATCAAGTTTCTCAGTGATTGT
CCCAAAGCATTGGCACAGATGAAGGAAGAGCATGATGCTATATTTAAAAAGAAAGGTGGCCATCACAAAC
TCAGCTGGGATGATTACACATCAATGAAATTTACTCAATGTGTCATAAATGAAACACTACGATTAAGTAA
TTTGGCAGCTGGGTTTTTCAGAGAAGCCATGGAAGATACAAAGGTCAAAGGTTATCTTATTCCTAAAGGA
TGGGTGATTTTTGCATTTACGACGTCTAGCCATTTAGATAAAAAGTTCCATGAACCTCTTACGTTCGATCC
ATGGCGTTGGCAACGAGATCAAGACTCTTCATATGATCCTTTGTACATACCTTTTGGAGCTGGAGCCAGGC
TTTGCCCAGGATATCATCTGGCTAAACTTGAGTTGGCTCTCTTTTTCCACATTTTCATAACCAGATTCAGA
TGGGAAACACTGGCAAATGATAAGGTCACATATCTTCCATTACCTCACTTGACCAAGGGCTTTCCCATTCG
CCTCCATCCTCTGCAATGA

Translated protein sequence, 475 aa, 54.82 kDa

MADQITLVLVVFTAAVALVHLIYRAWGNIYGGRERNKADNLPPGSTGWPLVGETLSYYLSMTSSHPTKFIE
ERERRYNSDIFISHLYGEKMVVSADAHFNKFVLQNEGRLFRAKYPQAMNIMIGKYGLLTVHGDLHRKLHGI
AVNLLRSERLRVDFIEEIQTLVHSTLDSWEEMKEIFLFKECHQMIINLMAKQLLDLSSAEETSEIRKLFIDFG
NASVALPIKIPGSTYSNGIKARELLIKKILETMEERRRHPEVAHHDLLARLMEEGSLSEEIICDFILFLLFAGQT
SFRAMPFAIKFLSDCPKALAQMKEEHDAIFKKKGGHHKLSWDDYTSMKFTQCVINETLRLSNLAAGFFREA
MEDTKVKGYLIPKGWVIFAFTTSSHLDKKFHEPLTFDPWRWQRDQDSSYDPLYIPFGAGARLCPGYHLAKL
ELALFFHIFITRFRWETLANDKVTYLPLPHLTKGFPIRLHPLQ

7.3.2 *Pa*CYP720B4

Coding DNA sequence, 1452 bp

ATGGCGCCCATGGCAGACCAAATATCATTACTGTTGGTGGTGTTCACGGTAGCGGTGGCGCTCCTCCACCT
TATTCACAGGTGGTGGAATATCCAGAGAGGCCCAAAAACGAGTAATAAGGAGGTTCATCTGCCTCCTGGG
TCGACTGGATGGCCGCTTATTGGCGAAACCTTCAGTTATTATCGCTCCATGACCAGCAATCATCCCAGGAA
ATTCATCGACGACAGAGAGAAAAGATATGATTCGGACATTTTCATATCTCATCTATTTGGAGGCCGGGCG
GTTGTATCAGCGGATCCCCAGTTCAACAAGTTTGTTCTACAAAACGAGGGGAGATTCTTTCAAGCCCAATA
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TCCACGGAATAGCTGTGAATTTGCTGAGGTTTGAGAGACTGAAAGTCGATTTCATGGAGGAGATACAGAA
TCTCGTGCACTCCACGTTGGATAGATGGGCAGATATGAAGGAAATTTCTCTGCAGAATGAATGTCACCAG
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Translated protein sequence, 483 aa, 55.42kDa

MAPMADQISLLLVVFTVAVALLHLIHRWWNIQRGPKTSNKEVHLPPGSTGWPLIGETFSYYRSMTSNHPR
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HGIAVNLLRFERLKVDFMEEIQNLVHSTLDRWADMKEISLQNECHQMVLNLMAKQLLDLSPSKETNEICEL
FVDYTNAVIAIPIKIPGSTYAKGLKARELLIKKISEMIKERRNHPEVVHNDLLTKLVEEGLISDEIICDFILFLLF
AGHETSSRAMTFAIKFLTFCPKALKQMKEEHDAILKSKGGHKKLDWDDYKSMAFTQCVINETLRLGNFGP
GVFREAKEDTKVKDCLIPKGWVVFAFLTATHLHEKFHNEALTFNPWRWQLDKDIPDDSLFSPFGGGARLC
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7.3.3 *Pa*CYP720B6

Coding DNA sequence, 1434 bp

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GCGGATCCCCAGTTCAACAAGTTTGTTCTACAAAACGAGGGGAGGTTTTTTCAAGCCCAATACCCAAAGGC
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Translated protein sequence, 477 aa, 54.96 kDa

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VNLMGFERLKVDFMEEIQNLVHSTLDRWADMKDIALQNECHQMVLNLMAKQLLVLSPSKETTEICELFVH
FSNAMVAIPIKIPGSTYAKGLKARELLIRKISGIIRERRNHPELVHDDLLNKLLEEGLISDEIICDFILFLLSAGH
ETSSRAMTFVIHFLTNCPKALKEMKEEHDAILKAKGDRKKLQWDDYQSLKFTQCVINETLRLGNFAPGIFR
EVKEDTKVKDYLIPKGWMVFAFMTATNLQENLHNEALTFNPWRWQLDKDVSNDSLFLPFGAGARLCPGS
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7.3.4 *Pa*CYP720B11

Coding DNA sequence, 1437 bp

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Translated protein sequence, 478 aa, 55.19kDa

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7.4 Multiple sequence alignment

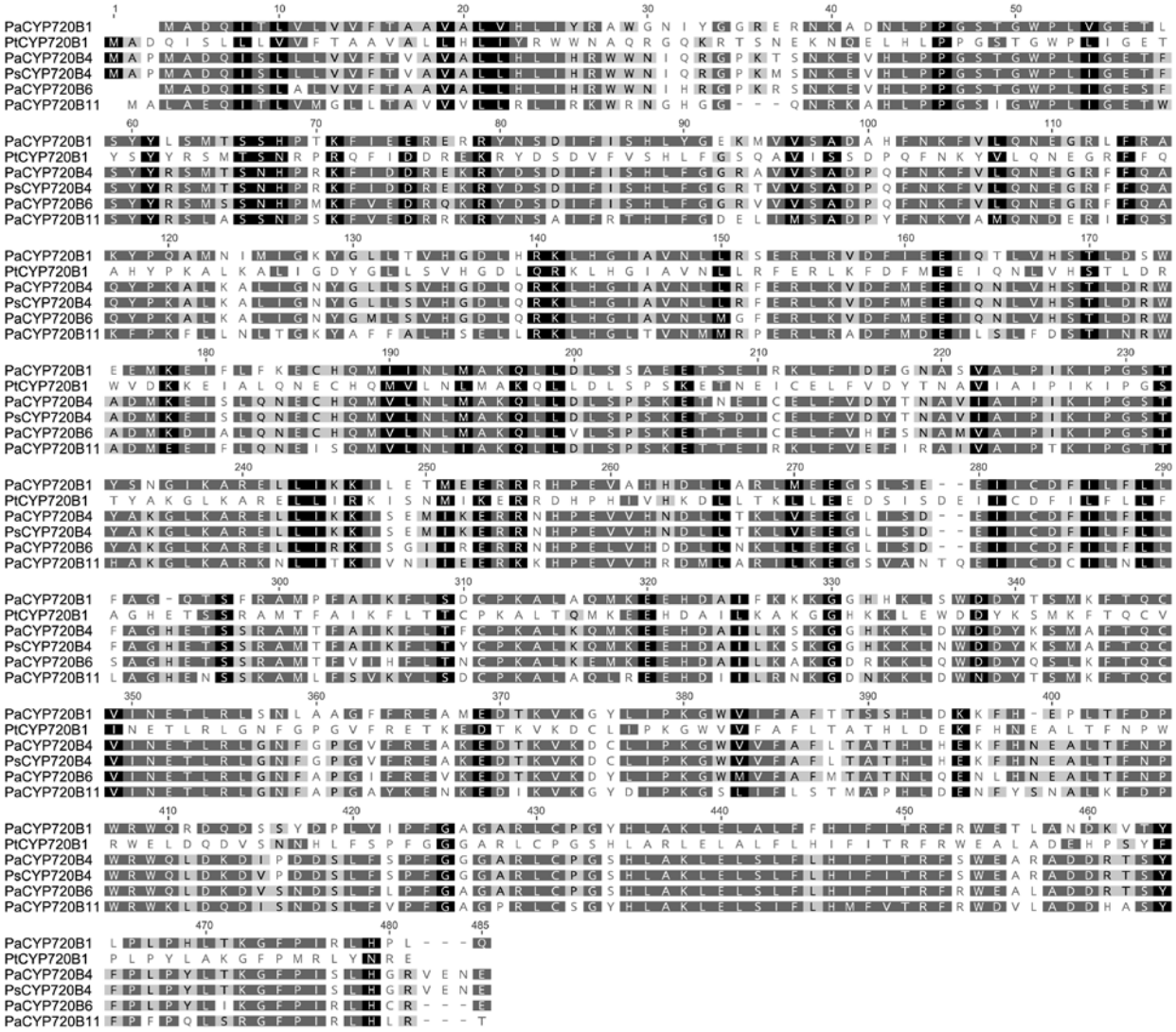

Figure S 2: Multiple sequence alignment of different CYP720Bs. Alignment of the quested *Pa*CYP720B1, B6, B11, B4 and literature known *Ps*CYP720B4 (E5FA70_PICSI, www.uniprot.org) and *Pt*CYP720B1 (C72B1_PINTA, www.uniprot.org). Sequences share 49.9% pairwise identity and were 62.3% pairwise positive. The similarity between *Pa*CYP720B4 and *Ps*CYP720B4 was about 98,6% pairwise identity.

7.5 Data of enzyme assays

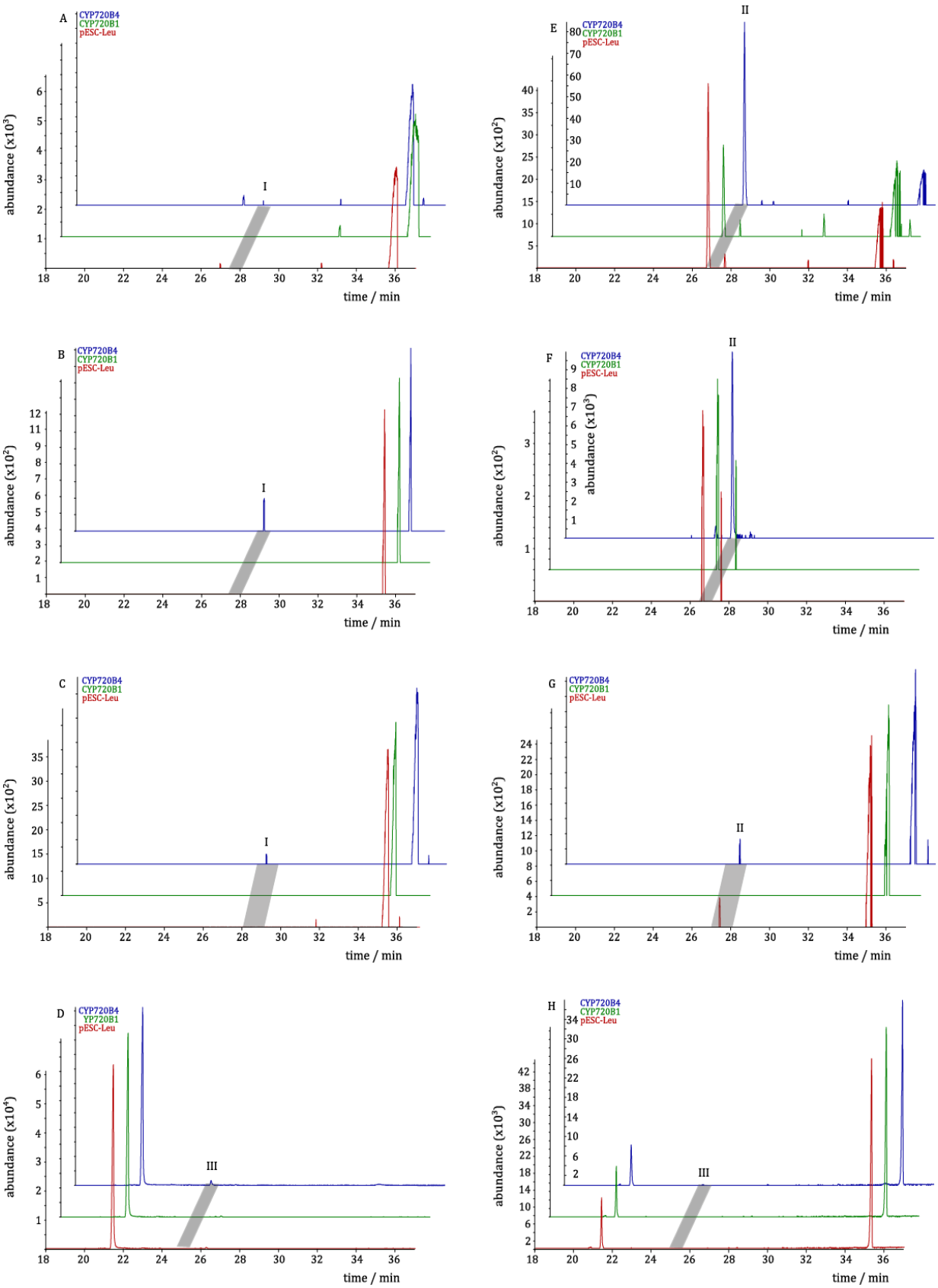

Figure S 3: GC-MS extracted ion chromatograms of enzyme assay extractions. A-C: EIC for abietic acid at m/z=316 in abieta-, dehydroabietadienol, mixture and LAS substrate assay. **E-G**: EIC for dehydroabietic acid at m/z=314 in abieta-, dehydroabietadienol, dehydroabietadiene and LAS substrate assay. **D** & **H**: EIC for dehydroabietadienal at m/z=269 in dehydroabietadiene and mixture assay. **Blue**: CYP720B4; **green**: CYP720B1; **red**: pESC-Leu empty vector control. The mass spectra **I-III** are shown in Figure 3.10.

Table S 5: Peak areas of catalyzed diterpenes in enzyme assays. Listed are the product peak areas obtained from empty-vector control pESC-Leu, CYP720B1 and CYP720B4 for the different substrates. Abietadiene, dehydroabietadiene and neoabietadiene were also used in a mixture to see preferences in substrate choice. The LAS substrate catalyzed in paragraph 2.2.14 was also used as substrate mixture. **–** not detectable; **(-)** identified but amount was below the limit for accurate integration.

	Peak area of corresponding DRA			**Peak area of corresponding alcohol**			**Peak area of corresponding aldehyde**		
	pESC-Leu	**CYP720B1**	**CYP720B4**	**pESC-Leu**	**CYP720B1**	**CYP720B4**	**pESC-Leu**	**CYP720B1**	**CYP720B4**
Abietadiene	-	-	(-)	-	-	-	-	-	-
Dehydroabietadiene	-	-	13,911,287	-	-	-	-	-	1,538,781
Isopimaradiene	-	-	-	-	-	-	-	-	-
Neoabietadiene	-	-	-	-	-	-	-	-	-
Abietadienol	-	-	-	-	-	-	-	-	-
Dehydroabietadienol	11,520,067	5,560,060	20,376,022	-	-	-	-	-	17,145,032
Abietadiene	-	-	1,521,696	-	-	-	-	-	-
Dehydroabietadiene	-	-	12067717	-	-	-	-	-	587,865
Neoabietadiene	-	-	-	-	-	-	-	-	-
Abietadiene	-	-	639,115	-	-	-	-	-	-
Dehydroabietadiene	-	-	413.407	-	-	-	-	-	-
Levopimaradiene	-	-	-	-	-	-	-	-	-
Neoabietadiene	-	-	-	-	-	-	-	-	-
Palustradiene	-	-	-	-	-	-	-	-	-

8. Acknowledgement

At this point I would like to thank Prof. Dr. Jonathan Gershenzon for giving me the opportunity to prepare this master thesis in the Department of Biochemistry at the Max Planck Institute for Chemical Ecology and for the supervision. I also want to thank Dr. Thomas Wichard for the supervision and reading my thesis.

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I want to thank Dr. Michael Reichelt for the introduction and the help in the analytical work and Dr. Yoko Nakamura for synthesizing all the different substrates. Also a thank you to Katrin Luck for patient answering my numerous questions and the useful advices.

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Finally I want to thank my family, my boyfriend and friends, which always believed in me and encouraged me especially in stressful phases of my study.
Thanks that you always have been there for me!

9. Declaration of Authorship

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbstständig und ohne fremde Hilfe verfasst und keine andren Hilfsmittel als angegeben verwendet habe. Insbesondere versichere ich, dass ich alle wörtlichen und sinngemäßen Übernahen aus anderen Werken als solche kenntlich gemacht habe. Die Arbeit wurde noch keiner Kommission zur Prüfung vorgelegt.

Jena, 07. Mai 2018 Bianca Fiedler