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Characterization of the Prenyltransferase Gene Family in *Populus trichocarpa*

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Abbreviations

IPP	Isopentenyl diphosphate
DMAPP	DiMethylAllyl diphosphate
GPP	Geranyl diphosphate
FPP	Farnesyl diphosphate
GGPP	GeranylGeranyl diphosphate
GPPS	Geranyl diphosphate Synthase
FPPS	Farnesyl diphosphate Synthase
GGPPS	GeranylGeranyl diphosphate Synthase
LSU	Large SubUnit
SSUI	Small SubUnit class I
SSUII	Small SubUnit class II
Mg^{2+}	Magnesium ion
Mn^{2+}	Manganese ion
BLAST	Basic Local Alignment Search Tool
MUSCLE	MUltiple Sequence Comparison by Log-Expectation
P. trichocarpa	Populus trichocarpa
E. coli	Escherichia coli

Abstract

Populus trichocarpa is a model plant for studying plant-insect interactions on molecular and ecological level. After herbivore feeding poplar emits a specific volatile blend comprising mainly of terpenes. The prenyltransferases are forming the terpene precursors (geranyl-, farnesyl- and geranylgeranyl diphosphate) and are branch point enzymes in the terpene biosynthetic pathway. Seventeen putative prenyltransferase genes were identified in Populus trichocarpa and nine of them were heterologously expressed in E. coli and functionally characterized. It was shown, that the interaction of the large subunits with different small subunits influences the geranyl diphosphate production. One farnesyl diphosphate synthase was identified and a novel mechanism of farnesyl diphosphate production after herbivore attack is hypothesized. Furthermore, the influence of different cofactors on the activity of some prenyltransferases was examined and the expression patterns of prenyltransferase genes in undamaged control leaves and herbivore damaged leaves are shown.

Zusammenfassung

Poplulus trichocarpa ist ein Modell Organismus für die Untersuchung der Interaktionen von Pflanzen mit Insekten auf molekularer und auf ökologischer Ebene. Nach Verletzung durch Herbivoren gibt die Pappel ein Duftspektrum ab welches hauptsächlich aus Terpenen besteht. Prenyltransferasen stellen die Vorstufen der Terpene her, das Geranyl diphosphat, das Farnesyl diphosphat und das Geranylgeranyl diphosphat. Somit sind die Prenyltransferasen Schlüsselenzyme in der Terpenbiosynthese. Siebzehn Prenyltransferasen wurden in der Pappel identifiziert und neun wurden erfolgreich heterolog in E. coli exprimiert und funktionell charakterisiert. Es wurde gezeigt, dass die Interaktion der großen Untereinheiten mit verschiedenen kleinen Untereinheiten Geranyl diphosphat Produktion beeinflusst. Eine die Farnesyl diphosphat Synthase wurde identifiziert und ein neuer Mechanismus für die Farnesyl diphosphat Produktion nach Herbivoren Fraß wird vorgeschlagen. Außerdem wurde die Auswirkung verschiedener Co-Faktoren auf die Aktivität ausgewählter Prenyltransferasen untersucht und die Expression der Prenyltransferasen Gene in unbeschädigten und von Herbivoren angefressenen Blättern wurde untersucht.

1. Introduction

1.1 Populus trichocarpa as a Model Plant

Poplar trees are widely distributed around the northern hemisphere. They are cultivated as raw material for the wood, paper and energy producing industry. In addition to their use in wood industry, poplar gained attention in plant physiological and molecular biological science. The western balsam poplar *Populus trichocarpa* (*P. trichocarpa*) serves as a model organism since it was the first tree with a sequenced genome (Tuskan et al., 2006). Further advantage of *P. trichocarpa* as a model organism is the fast growth and the possibility to amplify one genotype via stem cuttings. The sequenced genome allows insights into the molecular mechanisms of the secondary metabolism which seem to be responsible for the defense of the plant against herbivores and pathogens. The data gained by investigating *P. trichocarpa* will increase our knowledge about plant-insect interactions and elucidate molecular differences between woody and herbaceous plants.

1.2 Plant Defense Mechanisms

Plants have evolved a variety of defense mechanisms to protect themselves against a horde of enemies. Despite this variety, defense mechanisms can be divided into two kinds: direct and indirect defense.

Direct defense includes all traits that directly affect the herbivore. These comprise both constitutive and inducible direct defense mechanisms. These may be mechanical barriers such as the cuticle, or toxic metabolites like alkaloids stored in plant cells. Plant toxins like alkaloids, terpenes and phenolics poison generalist herbivores and force specialists to invest resources in detoxification mechanisms that in turn incur growth and development costs (Kessler and Baldwin, 2002). Direct defense also includes digestibility reducers like trypsin and chymotrypsin, protease inhibitors which inhibit the digestive enzymes in the insect gut, or polyphenol oxidases which decrease the nutritive value of the plant by cross-linking proteins (Kessler and Baldwin, 2002).

Constitutive defenses are always present in a constant amount, whereas inducible direct defenses are triggered or upregulated by elicitors. These elicitors for example occur in the spit of caterpillars. When caterpillars feed on the plant, the elicitors come into contact with the plant cells and specific receptors and the induced defense mechanisms are enabled as a response.

Indirect defense may comprise volatiles or other traits which attract enemies of herbivores, and is illustrated in Figure 1.1. Here, the plant produces a blend of highly volatile compounds to attract natural enemies of the herbivore (McCormick et al., 2012). The volatile blend varies between species, however, it is mostly dominated by three different classes of chemicals. First, the green leaf volatiles consisting of C₆-alcohols and C₆-aldehydes, second, the shikimate derived compounds like methyl salicylate and indole, which are emitted only after herbivore damage, and third, the terpenes. Mono- and sesquiterpenes often play a role in the attraction of predators and parasitoids. The emitted volatile compounds can have other defensive effects as well, like repelling ovipositors and warning neighboring plants (Kessler and Baldwin, 2002).

Poplar, as a tree species, has a relatively long lifespan for a plant. Therefore it is important for the plants to defend themselves against a huge variety of herbivores and pathogens. Poplar developed a range of different defense mechanisms: on the one hand the direct defenses, both constitutive and inducible; and on the other hand the indirect defenses. As direct constitutive defenses, the phenolic glycosides are most important, they deter generalist herbivores (Lindroth and Peterson, 1988) but may stimulate the feeding (Kolehmainen et al., 1995) and oviposition (Orians et al., 1997) of specialized herbivores. Some of those specialist herbivores can even sequester plant-derived phenolic glycosides (Boeckler et al., 2011). They are amongst the most abundant secondary metabolites produced by poplar (Donaldson et al., 2006).

After herbivore feeding, the transcriptom in poplar leaves changes drastically, increasing the prevalence of enzymes with activities against leaf-eating insects (Bradshaw Jr et al., 1991). Among the most prominently upregulated transcripts are the Kunitz protease inhibitor genes, active against many insect and mammalian proteases (Bradshaw Jr et al., 1991). They can inhibit digestive processes in herbivore guts. Not only does the abundance of protease inhibitors increase after herbivore damage, but the abundance of tannins and polyphenol oxidases as well (Constabel and Lindroth, 2010). Tannins have a deterring and toxic influence on leaf-eating herbivores whereas the polyphenol oxidases oxidate some phenolics and cross-link other phenolics and proteins, which results in browning of the plant (Constabel et al., 2000). These traits are thought to form the induced direct defense of poplar (Constabel and Lindroth, 2010).

P. trichocarpa constitutively emits a specific volatile blend (Danner et al., 2011). In response to herbivore damage, the volatile blend changes, increasing the number and amount of emitted volatiles. The main compounds released after herbivore damage are monoand sesquiterpenes (Danner et al., 2011) as well as green leaf volatiles, which are commonly given off after damage (Walters, 2011). Since the terpenes specifically emitted after herbivore damage are the main compounds of poplar, this work focuses their biosynthesis. Figure 1.1 shows a poplar tree emitting the volatile induced monoterpene (E)- β -ocimene and the sesquiterpene (E,E)- α -farnesene as examples for ingredients of the highly diverse volatile blend emitted after herbivore feeding. As an example for a poplar-feeding herbivore, a hornworm is shown. The emitted volatile blend attracts an ichneumoid wasp, which is a parasitoid, laying its eggs into the caterpillar.



Figure 1.1: Indirect Plant Defense. The plant emits a blend of volatiles after herbivore damage which is mainly consisting of mono- and sesquiterpenes. The emitted volatiles attract parasitoids of the herbivore. With this indirect defense mechanism, the plant reduces herbivore damage. In this picture a poplar tree is shown emitting the volatile induced monoterpene (*E*)- β -ocimene and the sesquiterpene (*E*,*E*)- α -farnesene as examples for ingredients of the highly diverse volatile blend emitted after herbivore feeding. As an example for a poplar-feeding herbivor a hornworm (larva of sphingid) is shown. The emitted volatile blend attracts an ichneumoid wasp, which is a parasitoid, laying its eggs into the caterpillar. Illustration by courtesy of Benjamin Naumann.

1.3 Terpenes and their Metabolism

The terpenes are the most diverse class of natural compounds, containing at least 50,000 different products (Vranova et al., 2012). Terpenes have key roles in almost all basic plant processes, including growth, development, reproduction and defense.

Terpenes are built up of the C₅ isoprene units isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP). Both are products of the mevalonic acid pathway or the glyceraldehyde phosphate/pyruvate pathway. One C₅ unit can be used for the formation of hemiterpenes like prenol. As shown in Figure 1.2, one molecule of IPP condensed to one molecule of DMAPP in a head-to-tail orientation results in the monoterpene precursor geranyl diphosphate (GPP) with ten carbon atoms. For the formation of farnesyl diphosphate (FPP) with 15 carbon atoms, either one unit GPP can be fused with one unit IPP or one unit of DMAPP can be fused with two units of IPP. FPP is the precursor for the biosynthesis of sesqui- and triterpenes. Geranylgeranyl diphosphate (GGPP), the 20-carbon precursor for di- and tetraterpene synthesis, can be produced by adding either three molecules of IPP to one molecule of DMAPP, two molecules of IPP to one molecule of GPP or one molecule of IPP to one molecule of FPP. Those terpene precursors are called prenyl diphosphates. The prenyl diphosphates are formed by prenyltransferases. They condense IPP with either DMAPP, GPP or FPP via head to tail condensation. FPP is formed by FPP synthases (FPPS, EC 2.5.1.10), GPP is formed by GPP synthases (GPPS, EC 2.5.1.1) and GGPP is formed by GGPP synthases (GGPPS, EC 2.5.1.30). The FPPS, GPPS and GGPPS form the class of short-chain prenyltransferases. The C₁₀, C₁₅ and C₂₀ allylic precursors undergo a wide range of cyclizations and rearrangements to produce the parent carbon skeletons of the terpenes (Gershenzon and Kreis, 1999).



Figure 1.2: The Formation of the Terpene Precursors. The prenyl diphosphates geranyl diphosphate (GPP), farnesyl diphosphate (FPP) and geranylgeranyl diphosphat (GGPP) are formed by the fusion of the C_5 units isopentenyl diphosphate and its allylic isomer dimethylallyl diphosphate catalyzed by prenyltransferases. PP indicates a diphosphate moiety.

Terpene biosynthesis is separated into two different compartments of the cell. The synthesis of FPP and the following formation of sesqui- and triterpenes by terpene synthases are located in the cytosol, whereas the production of GPP, GGPP and the terpene synthases utilizing them are located in the plastids (Gershenzon and Kreis, 1999).

1.4 The Prenyltransferases

Prenyltransferases catalyze the chain elongation of allylic diphosphates like DMAPP, GPP and FPP via condensation reactions with IPP to generate linear polymers with defined chain length (Liang et al., 2002). This thesis focuses on the short-chain prenyltransferases. They produce linear terpene precursors from C_5 to C_{20} .

1.4.1 Structure and Motifs of Prenyltransferases

The first crystal structure of a prenyltransferase was an avian FPPS reported by Tarshis and co workers (1994). It revealed a structure with 13 α -helices. Ten of these α -helices surround a large central cavity. The size and geometric structure of this pocket is thought to be the critical factor for product specificity with large amino acids at the floor of the pocket blocking further elongation (Liang et al., 2002). Two highly conserved DD(xx)_{2/4}D motifs (with x representing any amino acid) are located on opposite sides of the pocket (Tarshis et al., 1994). These motifs are responsible for substrate and cofactor binding and therefore required for catalytic activity (Nagegowda, 2010). Divalent metal ions such as magnesium (Mg²⁺) or manganese (Mn²⁺) bind to the DD(xx)_{2/4}D motifs that in turn binds the substrate's diphosphate (Dickschat, 2011). Therefore all short-chain prenyltransferases require divalent metal ions as cofactors. Next to the DD(xx)_{2/4}D motifs, the prenyltransferases possess another

Next to the DD(xx)_{2/4}D motifs, the prenyltransferases possess another specific conserved motif, the CxxxC motif (where x can be Ala, Leu, Ile, Val, Gly, Ser or Met) which is required for subunit interaction (Orlova et al., 2009). This motif is further described in the next chapter. The conserved motifs of the different kinds of prenyltransferases are shown in Figure 1.3.



Figure 1.3: Conserved Amino Acid Motifs of Prenyltransferases. A11 prenyltransferases possess significant conserved motifs indicating their activity. For enzymatic activity, two DD(xx)_{2/4}D motifs are required. They are located in the active site cavity and bind cofactors and substrates. The catalytically active farnesyl synthases synthases diphosphate (FPPSs), geranyl diphosphate (GPPSs), geranylgeranyl diphosphate synthases (GGPPSs) as well as large subunits (LSUs) possess two $DD(xx)_{2/4}D$ motifs. The catalytically inactive small subunits (SSUs) possess one $DD(xx)_{2/4}D$ motif at most. For subunit interaction, a CxxxC motif is necessary. All putative LSUs, SSUs and GGPPSs possess one CxxxC motif, except for the class II SSUs, which possess two CxxxC motifs each.

1.4.2 Subunit Structure of Prenyltransferases

Short chain prenyltransferases act as dimers or as tetramers (Chang et al., 2010). All FPPSs and GGPPSs act solely as homodimers. It was suggested, that the subunits of the FPPS interact with each other to form a shared active site in the homodimer structure rather than an independent active site in each subunit (Koyama et al., 2000).

In contrast, the GPPS can be active both as homo- and heterodimers. The heterodimers consist of a catalytically inactive small subunit (SSU) and a large subunit (LSU). New results show that a hetero-tetramer structure consisting of two LSUs and two SSUs can occur as well (Chang et al., 2010). The LSU possesses two $DD(xx)_{2/4}D$ motifs for the catalytic activity. It was shown that there are different types of LSUs. One the one hand prenyltransferases which possess GGPPS activity alone can function as LSUs for GPPS, and on the other hand GGPPS-like proteins which are

inactive alone can function as LSUs (Nagegowda, 2010). Both GGPPS and GGPPS-like proteins functioning as LSUs produce GPP when interacting with the SSUs (Orlova et al., 2009).

Both the LSU and SSU possess a CxxxC motif (where X can be Ala, Leu, Ile, Val, Gly, Ser or Met) (Orlova et al., 2009). This motif is found in all putative LSUs, SSUs and in most GGPPS as well. Most prenyltransferases capable of subunit interaction possess only one CxxxC motif. But a relatively new class of SSUs was found, possessing two CxxxC motifs. On this basis, the SSUs can be divided into two classes:the class I SSU (SSUI), possessing one CxxxC motif, and the class II SSU (SSUI), possessing two CxxxC motifs (Wang and Dixon, 2009). Figure 1.3 shows the conserved amino acid motifs found in the prenyltransferases.

1.4.3 Regulation of Prenyltransferase genes

Terpenes are emitted under certain circumstances and in specific plant tissues. This requires regulation mechanisms for their biosynthesis. Prenyltransferases are branch point enzymes in terpene biosynthesis, as shown by Orlova and coworkers (2009). They tested the overexpression of a snapdragon GPPS SSU gene in tobacco which resulted in increased production of the monoterpenes in tobacco. These data provided evidence that the formation of GPPS activity and the control of GPP flux to monoterpene biosynthesis is accomplished by the regulation of GPPS.SSU gene expression and the recruitment of GGPPS or GGPPS-like proteins. Thus, the regulation of prenyltransferases, next to the regulation of the terpene synthases, is the most important step for controlling the terpene production.

In general, regulation can occur in every step of the expression of a gene. The expression can be influenced by controlling transcription from the DNA, the processing of the mRNA or its transport from the nucleus to the cytoplasm, by regulation on the level of translation or by posttranslational modifications which for example could lead to an accumulation of the protein. The regulation of prenyltransferases mainly occurs on the transcriptional level. This idea is supported by findings in hop, where the expression of the GPPS SSU and LSU are highly correlated with the formation of the monoterpene myrcene (Wang and Dixon, 2009). In spruce, the expression of the bifunctional GPPS and GGPPS enzyme was found to be highest in wood where oleoresin comprising of monoterpenes and diterpenes accumulates (Schmidt et al., 2010). These results indicate that the prenyltransferases are regulated on the transcript level and that their expression regulates the terpene biosynthesis.

1.5 Aim of this Work

Plant prenyltransferases have been studied in a number of species like spruce (Schmidt and Gershenzon, 2008), hop (Wang and Dixon, 2009) and snapdragon (Tholl et al., 2004). But very little about the prenyltransferases from poplar is known so far. The prenyltransferases are branch point enzymes for terpene synthesis and may influence their production by limiting the precursors GPP, FPP and GGPP (Schmidt and Gershenzon, 2007). Since terpenes play a major role in indirect defense, the study of prenyl diphosphate biosynthesis and its regulation can help us to understand this important defense trait. The knocking out of single prenyltransferases could not only elucidate their function in the metabolism but also enlighten the functions of the terpenes and the terpene classes as a whole.

The aim of this thesis was to characterize the family of short chain prenyltransferases in *Populus trichocarpa* to identify candidate genes for the creation of knockout trees. Such trees will provide the opportunity to study the interactions of woody plants and its herbivore on an ecological and molecular level.

2. Materials and methods

All chemicals and solvents were analytical grade and were obtained from Merck (www.merck.de), Roth (www.carlroth.com), Serva (www.serva.de), or Sigma (www.sigmaaldrich.com).

If not stated differently, the kits were used following the manufacturers' instructions.

2.1 Plant and Insect Material

Populus trichocarpa (P. trichocarpa) trees were propagated from monoclonal stem cuttings (clone 625, NW-FVA) and grown under summer conditions in the greenhouse (24 °C, 60 % rel. humidity, 16 h/8 h light cycle) in a 1:1 mixture of sand and soil (Klasmann potting substrate), until they reached about 1 m in height. Fourteen young trees were selected (80-100 cm tall); seven of them were used for the herbivore treatment, the other seven served as untreated control plants. Generalist Gypsy moths (Lymantria *dispar*) were used as herbivores. Egg batches were kindly provided by Melody Keena (USDA Forest Service). After hatching, the caterpillars were reared on an artificial diet (Gypsy moth diet, MP Biomedicals LLC, www.mpbio.com) until they reached the third instar. The caterpillars were starved individually for 24 hours before they were released to the trees. Fifty caterpillars per tree were allowed to feed for 24 hours. The leaf material was then harvested and directly frozen in liquid nitrogen, then grinded and afterwards stored at -80 °C until further use. The preparation and collection of the plant material was carried out by Holger Danner(Danner et al., 2011).

2.2 Total RNA Extraction and cDNA Synthesis

Total RNA was extracted from frozen and ground *P. trichocarpa* leaf material using the InviTrap Spin Plant RNA Mini Kit (STRATEC, www.stratec.com). The provided RP lysis buffer was chosen because it is optimized for tissue with high amounts of phenolic compounds such as in poplar. The RNA was bound to the RTA Spin Filter in an ethanolic solution and then eluted with water.

The RNA was analyzed on the Agilent Bioanalyzer 2100 and RNA 6000 Nano Labchip using the Expert software (Agilent version B.02.02.SI258, www.agilent.com) to determine quality, integrity and rRNA ratios. The NanoDrop 2000c (Thermo Fisher Scientific, www.thermofisher.com) was used to quantify the RNA by measuring the absorbance. To remove residual genomic DNA the RNA was treated with DNase I (Fermentas, www.fermentas.de). cDNA was synthesized using SuperScript III First-Strand Synthesis SuperMix (Invitrogen, www.invitrogen.com). The cDNA preparation was made twice and the reactions were pooled afterwards to minimize variations caused by sample preparation.

2.3 Identification and Cloning of Prenyltransferase Genes

2.3.1 Alignment and Construction of an Alignment Tree with all Putative Prenyltransferases of *P. trichocarpa*

Sequences were extracted out of Phytozome (www.phytozome.org) using a BLAST analysis with known prenyltransferases from other plants as references. The names given in the database are listed in the appended Table A.1. The obtained sequences were aligned as amino acid sequences using MUltiple Sequence Comparison by Log-Expectation (Muscle) (Edgar, 2004). The alignment tree was created with MEGA5 using a neighborjoining algorithm, estimating the robustness of the tree with n = 1,000 replications for bootstrapping.

As reference sequences the sequences of the geranyl diphosphate synthase 1 of *Arabidopsis thaliana* (GPPS) with the NCBI accession number NP_001031483.1, the farnesyl pyrophosphate synthase of *Humulus lupulus* (FPPS) with the GenBank accession number BAB40665.1, the geranylgeranyl pyrophosphate synthase 6 of *Arabidopsis thaliana* (GGPPS) with the GenBank accession number BAA23157.1, the geranyl diphosphate synthase large subunit of *Humulus lupulus* (LSU) with the GenBank accession number ACQ90682.1, the geranyl diphosphate synthase small subunit of *Humulus lupulus* (SSUI) with the GenBank accession number ACQ90681.1 and the class II small subunit of *Oryza sativa* (SSUII) with the GenBank accession number EAY87007.1 were applied.

2.3.2 Signal Peptide Prediction

For the signal peptide prediction the programs TargetP, ChloroP (Emanuelsson et al., 2007) and Predotar (Small et al., 2004) were used.

2.3.3 Cloning of the Prenyltransferase Genes

Genes were amplified using the primers listed in the appended table A.2 from cDNA made with herbivore-damaged poplar leaves. PCRs were performed with Phusion High-Fidelity DNA Polymerase (New England Biolabs, www.neb.com) with an annealing temperature of 55 °C. Primers for the expression were designed using the program Primer D'Signer 1.1 (IBA GmbH, www.iba-lifesciences.com).

The PCR products were purified using the QIAquick PCR Purification Kit and afterwards the DNA was digested with the corresponding restriction enzymes, listed in table A.2. The bacterial expression vector pASK-IBA33+ (IBA GmbH, www.iba-lifesciences.com) was cut with *Bsa*I and *Pst*I. The DNA fragments were introduced into the vector by mixing them in a vector:DNA ratio of 1:5, which results in a ratio of 1:2,8 by considering the different sizes. 2 μ L ATP (10 mM) and 0,5 μ L T4-Ligase were added for ligation. The vector contains a 6 x His-tag fused to the C-terminus of the protein which allows the expression of the introduced gene as a His-fusion protein. The vector was introduced into *E. coli* K12 TOP10 cells (Invitrogen) and plate out on LB-agar plates with 50 μ g/mL ampicyllin as antibiotic using the resistance on the pASK-IBA33+ vector.

Colonies were picked from the agar plate and colony PCR was performed using the Go-Taq (Promega, www.promega.com) and the vector specific primers provided by the manufacturer. A band around 1.5 kBp on the agarose gel indicated an insert in the vector.

The clones containing an appropriate insert were inoculated into 5 mL LB medium containing 50 μ g/mL ampicillin (LB-Amp) and incubated at 37 °C over night in a shaker with 200 rpm. Afterwards plasmid mini preparation and sequencing were performed as described below using the vector specific primers (IBA GmbH, www.iba-lifesciences.com).

2.3.4 Sequencing

From the 5 mL overnight cultures plasmid mini preparations were performed using the QUIAGEN Plasmid Mini Kit (Quiagen, www.quiagen.com). The plasmid concentration was measured with the NanoDrop as described above and 150-200 μ g DNA were applied for sequencing.

BigDye v1.1 (Applied Biosystems, www.appliedbiosystems.com), containing a mix of labeled ddNTPs was used for the sequencing PCR with a reaction volume of 10 μ L each. The reaction mix was purified with the DyeEX Spin Kit (Quiagen) and analyzed on the ABI Prism Gen Analysator 3100 (Applied Biosystems). The data were analyzed with the program package DNASTAR (Lasergene, www.dnastar.com).

2.4 Expression and Purification of Prenyltransferases in *E. coli*

2.4.1 Gene Expression in E. coli

The clones with validated sequences were inoculated into a 5 mL LB-Amp starter culture and incubated at 37 °C over night in a shaker at 200 rpm. The starter culture was inoculated in 100 mL LB-Amp and grown at 18 °C to an OD₅₅₀ between 0.4 and 0.6. Expression was induced by adding 10 μ L anhydrotetracyclin which mimics the function of tetracycline, and thus activates the promotor. The induced culture was incubated for 16 hours at 18 °C and 200 rpm to ensure the stability of the prenyltransferases.

2.4.2 Protein Purification with Ni-NTA Spin Columns

The whole purification procedure was performed at 4 °C to assure the highest protein stability. The *E. coli* cell suspension was centrifuged at 5,000 x g for 10 minutes. The cell pellet was resuspended in 3 mL lysis buffer (50 mM Tris-HCl pH=8, 500 mM NaCl, 20 mM imidazol pH=8, 1 % Tween20, 10 % glycerol) and the cells disrupted by sonification with a Bandelin Sonopuls HD 2070 (Bandelin Electronica, www.bandelin.com) for four times for 30 seconds, cycle 2, power 65 %. Cell fragments were removed by centrifugation for 20 minutes at 16,100 x g. The lysate, containing the soluble proteins, was taken and stored at -20 °C. Preliminary assays showed that the prenyltransferases stayed active when frozen with the other soluble *E. coli* proteins in the lysate.

The expressed proteins were purified with Ni-NTA Spin Columns (Quiagen), taking advantage of the 6 x His tag introduced to the protein by the pASK-IBA33+ vector. The column was washed with washing buffer (50 mM Tris-HCl pH=8, 500 mM NaCl, 20 mM imidazol pH=8, 10 % glycerol) and eluted with elution buffer (50 mM Tris-HCl pH=8, 500 mM NaCl, 250 mM imidazol pH=8, 10 % glycerol). Protein concentrations were measured

on the NanoDrop 2000c (Thermo Fisher Scientific) applying the 280 nm method.

In view of the fact that storage at -20 °C could compromise protein stability, the purified proteins were directly used for the assays.

2.5 Functional Characterization on the HPLC/MS

For the enzyme assays, 6 µg purified protein were assayed with 10 mM MgCl₂ and 0.1 mM MnCl₂ as cofactors and 7.5 mM of each substrate (IPP and either DMAPP, GPP or FPP). It was filled up to 100 µL with assay buffer. The assays were incubated for 1 hour at 30 °C, afterwards placed on ice and diluted with 100 µL water. 3 µL of these assays were analyzed on the Agilent 1200 HPLC system (Agilent Technologies) coupled to an API 3200 triple quadrupole mass spectrometer (Applied Biosystems). For separation, a ZORBAX Extended C-18 column (1,8 µm, 50 x 4,6 mm, Agilent Technologies) was used. The mobile phase consisted of 5 mM ammonium bicarbonate in water as solvent A and acetonitrile as solvent B, with the flow rate set at 0,8 mL/min and the column temperature kept at 20 °C. Separation was achieved by using a gradient starting at 0 % B, increasing to 10 % B in 2 min, 64 % B in 12 min, and 100 % B in 2 min (1-min hold), followed by a change to 0 % B in 1 min (5-min hold) before the next injection. The injection volume for samples was 3 µL. The mass spectrometer was used in negative electrospray ionization mode. Optimal settings were determined using standards. Ion source gas 1 and gas 2 were set at 60 and 70 psi, respectively, with a temperature of 700 °C. Curtain gas was set at 30 psi, and collision gas was set at 7 psi, with all gases being nitrogen (Nagel et al., 2012).

2.6 SDS-PAGE and Western Blot

2.6.1 Sodium Dodecyl Dulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

For the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) a 12 % polyacrylamid gel was casted containing 8 mL separation gel buffer (181.5 g/l Tris pH 8.8), 12.8 mL acrylamide/bisacrylamide 30:0.8 %, 10.7 mL water, 320 μ L 10 % SDS, 250 μ L 10 % APS and 25 μ L TEMED for the separation gel and 3.75 mL stacking gel buffer (60.5 g/l Tris pH 6.8 HCl), 2.25 mL acrylamide/bisacrylamide30:0.8%, 7.5 mL water, 150 μ L 10 % SDS, 50 μ L 10 % APS and 15 μ L TEMED for the stacking gel.

The samples were mixed with 2 volumes 3 x loading buffer (50 mM Tris-HCl pH 6,8, 2 % SDS, 10 % glycerol, 1 % β -mercaptoethanol, 12,5 mM EDTA and 0,02 % bromphenol blue) and incubated at 95 °C for 5-10 minutes. 10 μ L of each sample and 5 μ L PageRuler Prestained Protein Ladder (Fermentas, www.fermentas.de) were load on the gel and run with 1 % SDS running buffer (3.03 g Tris, 14.4 g glycine and 1 g SDS solved in 1 L water) for 15 minutes with 90 V to center the dye front and 90 to 120 minutes with 120 V till the dye front reached the end of the gel.

The gels were afterwards used for either coomassie stain or western blot.

2.6.2 Coomassie Stain

The gels were incubated overnight in coomassie staining solution containing 1 g/L Coomassie Brilliant Blue R-250, 225 mL/L methanol and 35 mL/L acetic acid. Afterwards they were destained for at least one day in 35 mL/L acetic acid with 25 mL/L methanol in distilled water.

2.6.3 Western Blot

The SDS gel was assembled with pre-wetted Optitran BA S-85 Reinforced NC nitrocellulose membrane (Whatman, www.whatman.com) and 3MM Chr Blotting Papers (Whatman) and blotted with transfer buffer (3.02 g Tris, 14.4 g glycine, 200 mL methanol filled to 1 L with water) in a Mini Protean II gel chamber with Mini Trans-Blot Module from connected to a Bio-Rad Power Pac 300 (all from Bio-Rad Laboratories, www.bio-rad.com) over night with 22 V at 4 °C. The membrane was blocked in 5 % skim milk in TTBS (tween-Tris-buffered-saline, 20 mM Tris pH 7.5, 150 mM NaCl, 0.1 % tween-20) for 2 h at room temperature. Afterwards the membrane was incubated with the primary anti-HIS antibody (Fermentas) 1:1,000 in 5 % TTBS milk. The secondary antibody was an anti mouse antibody containing an alkaline phosphatase 1:500 in 5 % TTBS milk. The blot was washed with alkaline phosphatase buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM MgCl₂) and developed in alkaline phosphatase buffer containing 34 µL 50 mg/mL BCIP (5-bromo-4-chloro-3-indolyl-phosphate, Roche Diagnostics GmbH, www.roche.de) and 34 µL 100 mg/mL NBT (4-Nitro blue tetrazolium chloride, Roche Diagnostics GmbH) in the dark till bands became visible. The reaction was stopped by transferring the blot into water. The blot was dried dark.

2.7 Agarose Gel Electrophoresis

To test the size of PCR products and other DNA components agarose gel electrophoresis was performed. A 1 % gel was used, containing 60 μ g/ μ L ethidiumbromide. The DNA samples were prepared by mixing with loading dye (Fermentas). Then 5-10 μ L per sample and 5 μ L 1Kb DNA Ladder (Invitrogen) were filled into the slots. The gels were run in 1 x TAE running buffer for 20 minutes at 135 V. For gel imaging the GeneGenius Bio Imaging System (Syngene, www.syngene.com) was utilized.

The bands on the gel were cut with a scalpel and purified using the QIAquick Gel Extraction Kit (Quiagen GmbH).

2.8 Rapid Amplification of cDNA Ends Polymerase Chain Reaction (RACE PCR)

For *PtPT6* a 5'-RACE PCR had to be performed, because the start codon was not given in the phytozome database.

For rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR) components of the SMART RACE cDNA Amplification Kit and the Advantage 2 PCR Enzyme System (Clontech, www.clontech.com) were used. The 5'-RACE-ready cDNA library was kindly provided by Sandra Irmisch.

The 5'-RACE PCR reaction consisted of 17.5 μ L deionized water, 2.5 μ L Advantage 2 PCR Buffer, 2.5 μ L UPM A, 0.5 μ L Advantage 2 Polymerase Mix, 0.5 gene specific primer 5'-CCAATACCACGTCTCGTATCTGCATC ATCC-3' and 1 μ L 5'-RACE-ready cDNA. The PCR program started with 5 cycles of 94 °C for 30 s, 72 °C for 1.5 min followed by 5 cycles of 94 °C for 30 s, 70 °C for 30 s, 72 °C for 1.5 min and finally 25 cycles of 94 °C for 30 s, 68 °C for 30 s, 72 °C for 1.5 min. The PCR product was diluted 1:50 and taken as template for a nested PCR with 18 μ L H₂O, 2.5 μ L Advantage 2 PCR Buffer, 0.5 μ L dNTPs, 0.5 μ L Advantage 2 POR Buffer, 0.5 μ L dNTPs, 0.5 μ L Advantage 2 POR Buffer, 0.5 μ L diluted PCR. The PCR was run with 26 cycles of 94 °C for 30 s, 68 °C for 30 s and 72 °C for 1.5 min.

Results of the PCR were visualized with gel electrophoresis and bands were cut and purified to extract DNA fragments for sequencing.

2.9 qualitative Real Time Polymerase Chain Reaction (qRT-PCR)

Qualitative real time polymerase chain reactions (qRT-PCRs) were performed in optical 96-well plates using a Stratagene Mx3000P real-time thermocycler (Stratagene, www.stratagene.com). Brilliant SYBR Green QPCR Master Mix (Agilent Technologies, www.genomics.agilent.com) containing a double strand intercalating dye was used to visualize the amplification progress by reading the fluorescence. ROX was used as an internal standard to normalize the fluorescence.

The farnesyl diphosphate synthase gene *PtPT9* was used as housekeeping gene for the qRT analysis of the other prenyltransferases, since the fluctuations of its Ct values (~0,9 Ct) was minimal between control plants and herbivore-treated plants. As negative controls no-template controls were used, containing water instead of cDNA. Three technical replicates and 5 biological replicates were used.

Each reaction contained cDNA and sequence-specific primers in a volume of 25 µL. Primer were designed to bind specifically to only one of the prenyltransferase genes. For each gene at least two primer pairs were designed to assure the finding of a specific pair of primers. The specific primers for the amplified genes are listed in the appended Table A.3. The primer efficiency was tested using 5 different dilutions (1- to 64-fold) and calculated with the standard curve method (Pfaffl, 2001). The thermocycler protocol was performed with an initial denaturing for 10 minutes at 95 °C followed by 40 cycles of 30 seconds with 95 °C for denaturing the double strand DNA, 60 seconds with 55 °C for primer annealing and 60 seconds at 72 °C as the extension phase for the polymerase activity. For primer efficiency tests, the runs were followed by a melting curve analysis consisting of one cycle with 95 °C for 30 seconds, 55 °C for 30 seconds and 95 °C for 30 seconds. The melting curve was supposed to contain just one distinct peak. For each primer pair the products were cloned into the pCR4-TOPO TA vector for sequencing (Invitrogen) and sequenced as described above. 2 µL of the ligation were

applied for the *E. coli* transformation and the cells were plate out on agar plates with 50 μ g/ μ L kanamycin. Six clones from each culture were picked for plasmid preparation and sequencing as described above.

The amplification plots were analyzed with the MxPro QPCR Software (Agilent Technologies).

3. Results

3.1 Identification and Cloning of Prenyltransferase Genes in *P. trichocarpa*

3.1.1 Identification and Cloning of Prenyltransferase Genes

To identify putative prenyltransferase genes a BLAST analysis of the recently sequenced genome of *Populus trichocarpa (P. trichocarpa)* was performed using known prenyltransferase genes from other plant species as references. Seventeen sequences for putative *P. trichocarpa* prenyltransferases were retrieved. They were aligned and an alignment tree was calculated. The results are shown in Figures 3.1. The alignment of the seventeen putative prenyltransferases revealed the four conserved motifs of prenyltransferases, two DD(xx)_{2/4}D and two CxxxC motifs.

Based on the presence of these motifs in the sequences and their clustering with known prenyltransferases in the alignment tree, they were annotated as GPPSs, GGPPSs, FPPSs, LSUs and SSUs (Fig.3.1). The genes *PtPT8* and *PtPT9* both clustered with the FPPS and were annotated as FPPS although *PtPT8* was lacking the first DD(xx)_{2/4}D motif. Genes PtPT16 and PtPT17 lacked the second DD(xx)_{2/4}D motif, but had two CxxxC motifs and clustered with SSUII and were annotated as catalytically inactive SSUIIs. Gene PtPT15 lacked both DD(xx)_{2/4}D motifs but contained one CxxxC motif and clustered with SSU, and was annotated as SSUI. Genes PtPT13 and PtPT14 both contained two DD(xx)_{2/4}D and one CxxxC motifs and thus were thought to be catalytically active LSUs. Genes PtPT12, PtPT10 and PtPT11 were annotated as GGPPSs, they contained two DD(xx)_{2/4}D and one CxxxC motifs each. Genes PtPT6 and PtPT7 clustered with GPPS and were consequently annotated as GPPSs. Genes PtPT4, PtPT5, PtPT1, PtPT2 and PtPT3 did not cluster with any of the known prenyltransferases used. Except for *PtPT2* and *PtPT3*, these all contained both DD(xx)_{2/4}D motifs.



3.1 Alignment Tree and Details of the Sequence Alignment of all 17 putative **Prenyltransferase Genes.** The putative prenyltransferases from *P. trichocarpa* cluster with the known prenyltransferases from other plants. As reference sequences the squences of the geranyl diphosphate synthase 1 of Arabidopsis thaliana (GPPS) with the NCBI accession number NP_001031483.1, the farnesyl pyrophosphate synthase of Humulus lupulus (FPPS) with the GenBank accession number BAB40665.1, the geranyl geranyl pyrophosphate synthase 6 of Arabidopsis thaliana (GGPPS) with the GenBank accession number BAA23157.1, the geranyl diphosphate synthase large subunit of Humulus lupulus (LSU) with the GenBank accession number ACQ90682.1, the geranyl diphosphate synthase small subunit of Humulus lupulus (SSUI) with the GenBank accession number ACQ90681.1 and the class II small subunit of Oryza sativa (SSUII) with the GenBank accession number EAY87007.1 were applied. The four key motifs are shown as details from the complete alignment shown in the appended table A.1. The two CxxxC motifs (where x can be Ala, Leu, Ile, Val, Gly, or Ser) crucial for subunit interactions are marked in yellow. The two DD(xx)_{2/4}D motifs (where x can be any amino acid) involved in substrate and cofactor binding are marked in green. On the basis of the clustering in the alignment tree and the presence of conserved motifs, the genes were annotated as geranyl diphosphate synthases (GPPSs), farnesyl diphosphate synthases (FPPSs), gernylgeranyl diphosphate synthases (GGPPSs), large subunits (LSUs), small subunits class I (SSUIs) and small subunits class II (SSUIIs). The annotations are shown in the middle of the picture.

To confirm the sequences given by the database it was tried to amplify and sequence the open reading frames (ORFs) of the genes. Twelve out of the seventeen predicted prenyltransferases were amplified from cDNA obtained from herbivore induced *P. trichocarpa* leaves. The amplified sequences differed from the database sequences in a few basepairs which could be due to genetic differences in deployed genotypes. The cloned sequences (presented in the appended Figure A.2) were used for further studies.

3.1.2 Signal Peptide Prediction

GPPSs and GGPPSs are localized in the plastids, the location of GPP and GGPP as well as mono- and diterpene synthesis (Vranova et al., 2012). The proteins are targeted to plastids by N-terminal signal peptides (also called targeting sequences) (Orlova et al., 2009). Because sesquiterpene synthesis is localized in the cytosol (Gershenzon and Kreis, 1999), the FPPS does not contain a signal peptide. Since it is known that signal peptides often interfere with the expression of eukaryotic genes in E. coli, plastid-targeted proteins first had to be truncated by removal of their signal peptides. To identify potential signal peptides three different internet prediction programs were used. The results are shown in Table 3.1. For the proteins PtPT12, PtPT11, PtPT5, PtPT2, PtPT9 and PtPT8 none of the programs predicted a signal peptide. For PtPT17, PtPT13, PtPT14 and PtPT6 all three programs predicted targeting sequences. For PtPT15, PtPT16PtPT4, PtPT10, PtPT7 and PtPT3and PtPT1 the predictions were inconclusive about probability, length and destination of the targeting peptide, the likelihood and the target compartment.

Table 3.1 Signal Peptide Prediction for all 17 Prenyltransferase Genes. ChloroP and Predotar state a Y(es) for a predicted presence and a N(o) for the absence of a signal peptide. Predotar additionally states "?" for the possible presence of a signal peptide. TargetP and ChloroP both give the length of the predicted signal peptide, while both TargetP and Predotar predict the location for the protein with C meaning chloroplasts, M mitochondria, S secretory and P plastids. All three programs calculated a score for the probability of their prediction.

	TargetP			ChloroP			Predotar		
	location	SCORE	length	Y/N	length	SCORE	Y/N/?	location	SCORE
PtPT12		0.765		N	9	0.429	Ν		0.99
PtPT11		0.591		Ν	29	0.434	N		0.97
PtPT5		0.922		Ν	16	0.434	Ν		0.99
PtPT2		0.665		Ν	8	0.435	Ν		0.98
PtPT9		0.795		N	12	0.431	N		0.98
PtPT8		0.785		Ν	16	0.427	Ν		0.99
PtPT17	С	0.745	36	Y	36	0.522	Y	Р	0.71
PtPT13	С	0.955	55	Y	55	0.559	Y	Р	0.78
PtPT14	С	0.883	54	Y	54	0.537	Y	Р	0.64
PtPT15	С	0.715	37	Y	37	0.549	?	Р	0.31
PtPT6	М	0.881	44	Y	44	0.535	Y	М	0.56
PtPT16	S	0.315	16	Y	44	0.521	?	Р	0.42
PtPT1	М	0.235	34	Y	62	0.524	?	Р	0.36
PtPT4		0.420		Y	65	0.509	?	Р	0.22
PtPT10		0.534		Y	71	0.512	?	P	0.48
PtPT7		0.656		N	31	0.457	?	Р	0.27
PtPT3	S	0.559	33	N	31	0.434	N		0.94

3.2 Expression and Characterization

3.2.1 Truncation of Prenyltransferase Genes

Because signal peptide prediction (Table 3.1) often did not show a clear pattern, the best truncation site for genes to be expressed in *E. coli* had to be determined by experiment. These truncation experiments were made for the genes of *PtPT15*, *PtPT10* and *PtPT14*. For each gene three primer pairs were designed, amplifying sequences of different length. Figure 3.2 shows the different starting points in an alignment of the three equivalent amino acid sequences. For the shortest truncations soluble His-tagged proteins were obtained for all three proteins (Fig.3.2). For the medium truncations soluble His-tagged proteins were obtained for *PtPT15*. The amounts of protein from *PtPT14* and *PtPT10* were decreased compared to the shortest truncation. For the full length genes no soluble His-tagged proteins were obtained suggesting that the potential full-length signal peptides interfered with the expression. The SDS-PAGE of the purified His-tagged protein is shown in Figure 3.2.

As a consequence, all prenyltransferase genes were expressed as short truncations. The truncation sites are shown in Figure A.1.

a) PtPT10	1 <mark>M</mark> A FSTTISFYDNSLLLKKPINGLINNQPRTPLGHLKF <mark>A</mark> PLKIQATY 46
PtPT14	1 <mark>M</mark> TSVNLGSWFQTSIINKDTGPRSIPISNFLSPGSKSLQIP 40
PtPT15	1 MTSLAATIPAINGNPILPYLPR <mark>S</mark> NTNRPLL30
PtPT10	47 VASPSFKQSFQSQ EVGNKSQKVPLP <mark>A</mark> FEFKEYMINKANQVN87
PtPT14	41 <mark>S</mark> VHQKQRKPMSSICAVLAKEETLQEDQRK <mark>P</mark> TFDFKSYMLQKANSVN86
PtPT15	31 SHRPMTVAVT <mark>A</mark> TSNGTTYN KSYWTSVNDEID61
PtPT10	88 KALDEAVPLQHPQKINEAMRYSLLA - GGKRVRPILCIASCELVGG 131
PtPT14	87 KALDAAVSLKEPAKIHESMRYSLLA - GGKRVRPVLCLAACDLVGG 130
PtPT15	62 AHLKQAIPIRPPLSVFEPMHHLTFA - APRTTAPALCIAACELVGG 105



Figure 3.3 Truncation of PtPT10, PtPT14 and PtPT15. a) Truncation Sites. All three PtPTs were truncated two times. The beginning of the sequences is shown, the complete alignment is shown in Figure A.1. The truncation sites and the start site of the full length gene are marked in red, conserved sites are marked in grey. It was tried to express the full length gene and the two truncated versions in *E. coli.* **b) SDS-PAGE of purified soluble Protein.** The PtPT constructs were inserted into a pASK-IBA 33+ vector containing a HIS-tag and brought into *E. coli.* The expressed soluble proteins were purified with Ni-NTA spin columns binding the HIS-tag, added to the protein by the vector and afterwards separated by SDS-PAGE. For the shortest truncated versions soluble HIS-tagged proteins were obtained for all three PtPTs. For the medium truncated versions soluble HIS-tagged versions were obtained only for PtPT10 and PtPT14. The expression of the full-length genes did not lead to a soluble HIS-tagged protein.

3.2.2 Single Enzyme Assays

For *PtPT9*, *PtPT4*, *PtPT10*, *PtPT16*, *PtPT13*, *PtPT12*, *PtPT14*, *PtPT17* and *PtPT15* soluble His-tagged proteins were obtained, the confirming westernblot is shown in Figure 3.3. The expressed prenyltransferases were assayed as single proteins and in combinations of two proteins to ensure detection of possible subunit interactions. All assays were performed three times with different substrates, once with DMAPP and IPP, once with GPP and IPP and once with FPP and IPP. The diagrams of all single enzyme assays as well as the empty vector control are shown in the Appendix in Figure A.3. Figure 3.4 shows the results of the single enzyme assays for the prenyltransferases with distinct activities.

PtPT9, annotated as FPPS, displayed a distinct FPPS activity with IPP and DMAPP but with IPP and GPP it showed both FPPS and GGPPS activity. With the substrates IPP and FPP PtPT9 showed solely GGPPS activity (Fig.3.4a). Both *PtPT14* and *PtPT13*, annotated as LSUs, showed GPPS activity when assayed with IPP and DMAPP with PtPT14 being slightly more active. With the substrates IPP and GPP or IPP and FPP, PtPT14 and PtPT13 both showed slight GGPPS activity (Fig.3.4d+e). PtPT10, annotated as GGPPS showed GGPPS activity with the substrates IPP and GPP and IPP and FPP (Fig.3.4b). PtPT12 which was annotated as GGPPS as well, demonstrated GGPPS activity only with the substrates IPP and FPP (Fig.3.4c). PtPT4, PtPT16, PtPT17 and PtPT15 showed no activity when assayed alone.



Figure 3.4 Western Blot of the Purified Proteins Used for Enzyme Assays. Crude protein extracts were separated by SDS-PAGE. Proteins were blotted to a membrane and detected with mouse anti-HIS and anti-mouse antibodies. The anti-mouse antibody was coupled to an alcalic phosphatase. Unfortunately the marker was not visible on the blot, but the corresponding SDS-PAGE confirmed the right size of the bands. The smearing of the bands is due to an overload from the membrane.



Figure 3.4 Single Enzyme Assays with distinct Activities. All single enzymes were assayed with three different substrate combinations: IPP and DMAPP, IPP and GPP and IPP and FPP. The assayed enzymes are written in every diagram, the applied substrates are indicated below the enzyme names in braces. The blue peaks in the diagrams represent GPP, the red peaks represent FPP and the green peaks represent GGPP. The Y-axis shows the intensity of the mass spectrometer as counts per second (cps) multiplied by 100,000 and the X-axis shows the retention time in minutes. a) Single enzyme assays of PtPT9 with different substrates. b) Single enzyme assays of PtPT12 with different substrates. d) Single enzyme assays of PtPT13 with different substrates. e) Single enzyme assays of PtPT14 with different substrates.




3.2.3 Combination Assays

Figure 3.5 shows the combination assays with intriguing changes in the activities. PtPT14 and PtPT13 both produced GPP from IPP and DMAPP. This activity increased drastically when these enzymes were assayed together with the SSUI PtPT15. When assayed with the SSUII PtPT16, the product spectrum of PtPT14 and PtPT13 broadens to an additional production of small amounts of GGPP (Fig.3.5c-f). The combinations of PtPT14 and PtPT13 with PtPT15 increased the GGPPS activity when assayed with the substrates IPP and FPP (data not shown).

PtPT9, producing FPP and GPP when assayed with the substrates IPP and GPP changed its activity to an almost solely production of FPP when assayed in combination with PtPT4 (Fig.3.5a). The FPPS activity of the GGPPS PtPT10 was increased by PtPT4 as well when assayed with IPP and GPP as substrates (Fig.3.5b).

In order to quantify the increased GPP production of the combinations from PtPT14 and PtPT13 with PtPT15 assays with three technical replicates were performed. The results are shown in Figure 3.6 as the average peak area from the GPP production with and without SSUI. The GPP production of PtPT14 increased almost 4-fold when assayed in combination with PtPT15, while the activity of PtPT13 increased more than 3-fold. Given that the activity of PtPT14 alone was significantly higher than the activity of PtPT13 the combination of PtPT14 and PtPT15 produced the greatest amount of GPP.



Figure 3.5 Combination Enzyme Assays with Distinct Changes in the Activities. Two enzymes per assay were combined and assayed with three different substrate combinations: IPP and DMAPP, IPP and GPP and IPP and FPP. Only the diagrams are shown where a distinct change in the enzyme activity occurs compared to the single enzyme assays. The assayed enzymes are written in every diagram, the applied substrates are indicated below the enzyme names in braces. The blue peaks in the diagrams represent GPP, the red peaks represent **FPP** and the green peaks represent GGPP. The Y-axis shows the intensity of the mass spectrometer as counts per second (cps) multiplied by 100,000 and the X-axis shows the retention time in minutes. a) Assays of PtPT9 and PtPT4 with the substrates IPP and GPP. b) Assays of PtPT10 and PtPT4 with the substrates IPP and GPP. c) Assays of PtPT13 with PtPT15 and PtPT16 with the substrates IPP and DMAPP. d) Assays of PtPT13 and PtPT15 with the substrates IPP and FPP. e) Assays of PtPT14 with PtPT15 and PtPT16 with the substrates IPP and DMAPP. f) Assays of PtPT14 and PtPT15 with the substrates IPP and FPP.







Figure 3.6 Quantitative Assays of the LSUs PtPT13 and PtPT14 in Combination with the SSUI PtPT15. The purified LSUs PtPT13 and PtPT14 were assayed with the SSUI PtPT15 and with the substrates IPP and DMAPP. Three technical replicates were performed. The average peak area multiplied with 1,000,000 is shown.

3.2.4 The Influence of the Cofactors Magnesium and Manganese on Prenyltransferase Activity

The literature shows divergences in activity and substrate specificity of prenyltransferases when using manganese (Mn^{2+}) or magnesium (Mg^{2+}) as a cofactor and with different concentrations (Fujii et al., 1980; Ohnuma et al., 1993). To determine whether the Mg^{2+} and Mn^{2+} concentrations influence the prenyltransferase activity, assays were performed for PtPT9 which was annotated as a FPPS, PtPT10 annotated as a GGPPS and PtPT14 annotated as a LSU.

The results are shown in Figure 3.7. PtPT9 showed the highest FPPS activity with 0.1 mM Mn^{2+} or 1 mM Mg^{2+} . For PtPT10 and PtPT14 the highest activities were reached with 10 mM Mn^{2+} and 10 mM Mg^{2+} , but the activity with Mg^{2+} was lower than with Mn^{2+} as cofactor. Interestingly, the PtPT14 showed minor GGPPS side activity with Mn^{2+} as cofactor.



Figure 3.7 The Influence of Different Cofactors on Prenyltransferase Activity. Assays with different Mg^{2+} and Mn^{2+} concentrations were performed with the FPPS PtPT9, the GGPPS PtPT10 and the LSU PtPT14. Three technical replicates were performed. The average peak area multiplied with 1,000,000 is shown.

3.3 Gene Transcript Abundance Analysis (qRT-PCR)

To study the transcript accumulation of the prenyltransferase genes in herbivore-damaged well as undamaged as control leaves. а comprehensive gRT-PCR analysis was performed. The relative cT values for each gene are given in figure 3.8a, and the fold changes for transcript accumulation after herbivory are given in figure 3.8b. The genes PtPT4, PtPT6, PtPT7, PtPT16, PtPT13, PtPT17 and PtPT9 were constitutively expressed in undamaged leaves and herbivore damaged leaves while PtPT11 and PtPT12 showed only trace transcript abundance. Compared to undamaged leaves, PtPT16, PtPT14 and PtPT15 showed significantly upregulated transcript accumulation in herbivore-damaged leaves. The strongest upregulation was 34-fold for PtPT15. Gene PtPT10 was upregulated 17-fold, but its basal transcript abundance was very low. Gene PtPT14 exhibited a low basal transcript abundance in undamaged leaves and was 9-fold upregulated after herbivory. PtPT8, 101s60, PtPT1, PtPT2 and PtPT3 could not be amplified from the cDNA suggesting that these genes were not expressed in the tissues analyzed.



Figure 3.8 Relative Expression of Prenyltransferase Genes in Herbivore-Damaged (herbivory) and Undamaged Control (control) Leaves from qRT-PCR. *PtPT9*, *PtPT1*, *PtPT2*, *PtPT3* and *PtPT5* could not be amplified out of the cDNA and the data are not shown. **a)** Relative cT values, shown as mean values of $2^{(-\Delta cT)} \pm$ standard error. **b)** Fold changes comparing control plants and herbivore induced plants. Controls were set one. The mean values are shown \pm standard error, n=5.

4. Discussion

4.1 Prenyltransferases Form a Large Gene Family in Poplar

Seventeen putative prenyltransferase genes were found in the genome of *Populus trichocarpa (P. trichocarpa)*. This is a relatively large number of prenyltransferase genes compared to *Arabidopsis*, which contains two GPPS genes, two FPPS genes and five GGPPS genes. Five of the *P. trichocarpa* prenyltransferase genes did not cluster with known prenyltransferase genes from other plants and were therefore not annotated (Fig.3.1). From these five genes, only PtPT4 was expressed in *P. trichocarpa* leaves (Fig.3.8). Although it lacked the CxxxC motif normally required for prenyltransferase subunit interaction, it modified the activity of PtPT9 and PtPT10 (Fig. 3.5a+b). This implies a new kind of interaction mechanism, maybe similar to the inter-subunit interactions in the homodimers of FPPS and GGPPS. Figure 4.1 gives an overview of all significant prenyltransferase activities in *P. trichocarpa*.

			Substrates	
Name	Combination	IPP+DMAPP	IPP+GPP	IPP+FPP
PtPT9 (FPPS)		FPP	FPP + GGPP	GGPP
	PtPT4	~	FPP	~
PtPT10 (GGPPS)		х	GGPP	GGPP
	PtPT4	х	FPP + GGPP	~
PtPT13 (LSU)		GPP	GGPP	GGPP
	PtPT15 (SSUI)	↑GPP	~	↑GGPP
	PtPT16 (SSUII)	GPP + GGPP	~	~
PtPT12 (GGPPS)		х	x	GGPP
PtPT14 (LSU)		GPP	GGPP	GGPP
	PtPT15 (SSUI)	↑GPP	~	↑GGPP
	PtPT16 (SSUII)	GPP + GGPP	~	~

Figure D)1: Overview of Significant Prenyltransferase Activities from Single Enzyme and Combination Assays and the Annotations for the Prenyltransferases. The products formed with each substrate combination are given. " \uparrow " displays an enhanced production, "~" displays no change in product formation and "x" displays no product formation.

In poplar only one gene coding for a functional FPPS was found, the gene PtPT9. In other plants more than one FPPS are described. Arabidopsis contains one FPPS with housekeeping function and one with expression restricted to specific organs at particular developmental stages (Closa et al., 2010), indicating that the second FPPS is inducible for example by herbivory. Since there is no second FPPS expressed in the cDNA of poplar, it was concluded that poplar contains only one. In wintersweet, which contains only one FPPS as well, it was shown that the transcript abundance correlates with the sesquiterpene emission (Xiang et al., 2010). In poplar the transcript abundance was not elevated by herbivory (Figure 3.8), so it is likely that PtPT9 is not involved in the increased sesquiterpene production after herbivory. PtPT9 displays a distinct GGPPS activity when assayed with IPP and GPP or FPP as substrates (Fig.3.4a). Under natural conditions it is more likely that it produces solely FPP, because only IPP and DMAPP are produced in the cytosol where the FPPS is located. GPP as substrate would have to be transported from the plastids to the cytosol and the FPP produced by the FPPS is required from the terpene synthases which probably have a significantly higher affinity for FPP binding.

Two out of three GGPPS were expressed in *E. coli* and characterized. All three were transcribed in the leaves, although only in very small amounts (Fig.3.8a). The genes *PtPT12* and *PtPT11* were expressed in very low levels in undamaged control leaves as well as in herbivore damaged leaves, whereas *PtPT10* displayed a 17-fold upregulation after herbivory (Fig.3.8b). Since no increased production of GGPP is required after herbivore attack, the upregulation of *PtPT10* is most likely due to its interaction with PtPT4 as described below. An upregulation of the other GGPPS genes under altered conditions with a higher requirement of GGPP is very likely. Such requirements could be present for example during growth periods or in the terpene rich buds of poplar trees.

The predicted prenyltransferases *PtPT8*, *PtPT5*, *PtPT1*, *PtPT2* and *PtPT3* could not be amplified from the cDNA even using several different primer pairs. This led to the assumption that they were either not expressed in

the leaves of undamaged control or herbivore infested trees or not expressed at all. It is possible that these prenyltransferase genes are expressed in other plant organs or under different circumstances. However, the gene structures of *PtPT8*, *PtPT2* and *PtPT3* lack binding motifs important for prenyltransferase activity (Fig.3.1) so it is most likely that they are pseudogenes resulting from incomplete gene duplication and successive degeneration or from deletions in the genome.

The genes *PtPT11*, *PtPT6* and *PtPT7* were amplified and inserted into the pASK-IBA 33+ vector, but could not be expressed in *E. coli* TOP10 cells as soluble proteins. This may result from erroneous folding of the protein, the different codon usage between poplar and *E. coli*, or an accumulation of the expressed protein in inclusion bodies in the *E. coli* cell, which would not be cracked during the protein purification procedure. To determine whether these proteins are formed in inclusion bodies, a western blot from the insoluble fraction could be performed. A co-expression with potential subunit interaction partners could also be performed, because it was shown by Burke and coworkers that inactivity of a prenyltransferase gene product could be caused by erroneous folding and that co-expression could increase the possibility of correct folding (Burke et al., 1999).

4.2 Formation of GPP, FPP and GGPP by Different Prenyltransferases

4.2.1 The LSUs Produce GPP and their Activity is Increased by the SSUI

The gene products of *PtPT14* and *PtPT13*, both annotated as LSUs, produced significant amounts of GPP. Their activity increased drastically when combined with the SSUI PtPT15 (Fig.3.5c+e). This is consistent with the findings of Wang and Dixon (2009) for GPPS heterodimers in hop. The LSU from hop displayed both GGPPS and GPPS activity, and interaction with the SSU altered the product specificity to increased GPP formation

with enhanced catalytic activity. This parallels the finding that the GPPS activity of PtPT14 and PtPT13 was enhanced although both solely produced GPP from IPP and DMAPP, and GGPPS activity only occurs with IPP and either GPP or FPP as substrates. Other GPPS LSUs described so far are either inactive, as described for mint (Burke and Croteau, 2002), or act as GGPPSs as described for snapdragon (Tholl et al., 2004). In general, SSUs can mediate the chain length specificity of active GGPPS (Burke and Croteau, 2002), activate inactive GGPPS-like proteins (Orlova et al., 2009) or enhance catalytic activity (Wang and Dixon, 2009). The mechanism for this change in specificity and activity is geometric changes at the active site. Chang et al. (2010) showed that the SSU can modulate the shape and size of the active cavity which was the favored hypothesis by Burke et al. (2004) as well. Hsieh (2010) hypothesized that the SSU can stabilize the LSU, thus leading to the full functionality by creating the proper architecture of a functional catalytic site.

4.2.2 PtPT9 Forms the Main Amount of FPP but PtPT10 and PtPT4 may be Involved as Well

PtPT9 showed high FPPS activity when assayed with the substrates IPP and DMAPP (Fig.3.4a). This was expected, because of its annotation as an FPPS. With the substrates IPP and GPP or FPP it showed GGPPS activity as well. In the literature, FPPSs with binary activities are described, for example the FPPS from wintersweet produces GPP next to FPP (Xiang et al., 2010). Because of its constitutively high transcript abundance (Fig.3.8) PtPT9 is thought to produce the major amount of FPP needed for sesqui- and triterpene production in undamaged control leaves as well as for the primary metabolism.

An interesting and unexpected activity was shown by the product of gene *PtPT4*. This gene contains the two $DD(xx)_{2/4}D$ motifs that are necessary for catalytic activity but no CxxxC motif, necessary for subunit interaction (Wang and Dixon, 2009). PtPT4 did not cluster with any of the applied

known prenyltransferases from other plants (Fig.3.1). Unexpectedly, PtPT4 seems to interact with the FPPS PtPT9 and the GGPPS PtPT10. *PtPT10* contains a CxxxC motif but neither the *PtPT9* nor the *PtPT4* contain such, therefore an interaction was thought to be improbable. But the assays displayed a change in product formation, since PtPT4 alone showed no activity, but when assayed with PtPT10 or PtPT9 and with IPP and GPP as substrates, PtPT4 decreased the GGPPS activity and increased the FPPS activity (Fig.3.5a+b). These results indicate an interaction between PtPT4 and either PtPT9 and PtPT10. Since no motif for the interaction in homodimeric prenyltransferases is described to date, it is likely that such or another yet undescribed binding motif is responsible for the interactions with PtPT4.

Altogether it seems possible, that PtPT4 is a new kind of regulatory subunit which increases FPP production, but only accepts IPP and GPP as substrates. Since PtPT9 is probably located in the cytosol and PtPT4 is probably located in the plastids, an interaction of both proteins is unlikely.

4.2.3 GGPP is Produced by a Number of Different Prenyltransferases

The greatest amount of GGPP is most likely produced by the LSUs. The LSU PtPT13 produced GGPP when assayed with IPP and either GPP or FPP as substrates (Fig.3.4d). The combination of PtPT13 with PtPT16 lead to the production of GGPP as a site product when assayed with the substrates IPP and DMAPP. In addition the combination of PtPT13 and PtPT15 caused an increase in GGPP production when assayed with IPP and FPP as substrates (Fig.3.5c+d). The other LSU PtPT14 demonstrated the same pattern (Fig.3.4e and 3.5e+f). The FPPS PtPT9 produced relatively high amounts of GGPP when assayed with the substrates IPP and either GPP or FPP (Fig.3.4a). The GGPPS PtPT10 produced GGPP with all offered substrates in different amounts (Fig.3.4b). The gene product of

PtPT12 which was annotated as a GGPPS, produced significant amounts of GGPP when assayed with the substrates IPP and FPP (Fig.3.4c).

All GGPPS transcripts accumulated at very low levels in undamaged control leaves as well as in herbivore damaged leaves (Fig.3.8). Only PtPT10 was upregulated, but even then the abundance was very low. These results correlate with the low level of diterpenes found in *P. trichocarpa* (personal correspondence with Tobias Köllner, 2010). It is very likely, that GGPPS genes play major roles under different circumstances, where greater levels of GGPP are required. Therefore qRT experiments should be performed, using tissue from different developmental stages and parts of the poplar trees.

4.3 Herbivory Influences the Transcript Abundance of PtPT10, PtPT14 and PtPT15

After herbivore attack, poplar produces a complex blend of volatiles which is dominated by mono- and sesquiterpenes (Danner et al., 2011). This elevated production of terpenes is due to increased terpene synthase gene transcript abundance. However, increased transcript abundance of prenyltransferases might also affect this outcome. To analyze, whether prenyltransferases are involved in herbivore induced upregulation of terpene biosynthesis, their transcript abundance was measured using qRT-PCR. Three genes showed distinct upregulation after herbivore feeding, PtPT10, PtPT14 and PtPT15 (Fig.3.8b).

For biosynthesis of an increased amount of monoterpenes, large amounts of GPP are required. The main sources for this GPP are probably the LSUs PtPT14 and PtPT13 as single enzymes and their combination with PtPT15 as a SSUI. The genes *PtPT15* and *PtPT14* are highly upregulated after herbivory (Fig.3.8b) and therefore thought to be responsible for the increased GPP production. In addition, the interaction of the highly upregulated *PtPT15* with the relatively highly expressed *PtPT13* also leads to elevated GPP production. So in conclusion, the upregulation of *PtPT15* and its interaction with *PtPT13* and the upregulated *PtPT14* are thought to be the main sources of increased GPP levels after herbivory. This high level of GPP on the other hand might be indirectly responsible for increased FPP formation necessary for sesquiterpene production after herbivory, since the FPPS is not inducible as discussed above, but the combination of PtPT4 and PtPT10 with GPP as substrate leads to FPP production as assumed in Chapter 4.2.2. If the FPP is produced by the combination of PtPT10 and PtPT4, the problem occurs that these two enzymes are localized in the plastids whereas the terpene synthases utilizing FPP as a substrate are localized in the cytosol (Wang and Dixon, 2009). In the literature it is shown that exceptions from this rule can occur in particular species and tissues (Nagegowda, 2010). Further, FPP can be exported from the plastids into the cytosol for sesquiterpene formation (Rodriguez-Concepcion, 2006). It has been shown that such translocation through the plastid membrane into the cytosol can occur for IPP, DMAPP, GPP and FPP (Vranova et al., 2012) and it is most likely mediated by specific metabolite transporters (Orlova et al., 2009). Altogether, this suggests that in herbivore-infested poplar trees the LSUs in combination with the SSU produce high amounts of GPP, required for monoterpene formation and as substrate for PtPT4 and PtPT10. Those produce FPP in the plastids, which then is exported into the cytosol. This hypothesized mechanism is illustrated in Figure 4.2. To test this hypothesis, the volatile emission of herbivore-infested plants treated with either fosmidomycin or mevinolin should be measured. Fosmidomycin blocks the MEP pathway by acting as a specific inhibitor of 1-deoxy-Dxylulose-5-phosphate reductoisomerase (Kuzuyama et al., 1998). Since this pathway is responsible for the formation of IPP in the plastids, blocking it would result in a shutdown of the plastid prenyl diphosphate and terpene production. If the hypothesis is correct and the FPP for herbivore-induced sesquiterpene production is formed in the plastids, the plant would not be able to produce the herbivore-induced mono- and sesquiterpenes. The compound Mevinolin has the opposite effect: it blocks the cytosolic MVA pathway by acting as a highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme-A reductase and thus stopping the supply of IPP and DMAPP in the cytosol (Alberts et al., 1980). This would result in a stop of cytosolic prenyl diphosphate and terpene production. If the plant would be able to produce herbivore-induced sesquiterpenes after mevinolin treatment, this would support the hypothesis that the precursor FPP is transported from the plastids to the cytosol. Both treatments, with fosmidomycin and with mevinolin will support or refute the hypothesis.



Figure 4.2: Production of Mono- and Sesquiterpene Precursors Required after Herbivore Attack. PtPT13 and PtPT14 both in combination with PtPT15 produce the major amount of GPP required for monoterpene formation after herbivory. But the GPP is required as substrate for the enzymes PtPT4 and PtPT10 as well. The combination of those two enzymes is responsible for the production of FPP required for sesquiterpene formation after herbivore attack. The FPP which is produced in the plastids is afterwards transported to the cytosol to act as substrate for the cytosolic sesquiterpene synthases.

4.4 Influence of Different Cofactors on the Activity

It was shown, that different cofactors and different concentrations of the cofactor magnesium can influence the product specificity of prenyltransferases (Fujii et al., 1980; Ohnuma et al., 1993). Hence, three of the expressed prenyltransferases were chosen to study such effects, the FPPS *PtPT9*, the LSU *PtPT14* and the GGPPS *PtPT10*.

The FPPS PtPT9, located in the cytosol, seems to have an optimum at Mg^{2+} concentrations around 1 mM and Mn^{2+} concentrations around 0.1 mM (Fig.3.7). Physiological concentrations of Mg^{2+} are 0.4 mM in the cytosol and up to 10 mM in the plastids (Shaul, 2002). The activity is higher with Mn^{2+} as cofactor, which is not consistent with the finding that the pumpkin FPPS requires Mg^{2+} rather than Mn^{2+} (Suga and Endo, 1991) or the fact that in the cytosol only submicromolar concentrations of Mn^{2+} occur (Quiquampoix et al., 1993).

The GGPPS PtPT10 and the LSU PtPT14, both located in the plastids seem to have optimal concentrations of Mg^{2+} and Mn^{2+} at 10 mM or maybe even more which is consistent with the relatively high Mg^{2+} concentrations in the plastids (Fig.3.7). The activity with Mn^{2+} is higher than with Mg^{2+} , which parallels the findings of Suga and Endo (1991), who showed that the rose GPPS required Mn^{2+} rather than Mg^{2+} for GPP formation.

Interestingly, PtPT14 showed GGPPS side activity when assayed with higher Mn^{2+} concentrations. In the assays with Mg^{2+} and Mn^{2+} as cofactors, PtPT14 showed GGPPS activity when having IPP and either GPP or FPP as substrates, but not with IPP and DMAPP (Fig.3.4e). The most likely explanation for this phenomenon is that the high Mn^{2+} concentrations activate the product, in this case GPP, resulting in chain elongation or a conformational change of the enzyme, as hypothesized by Ohnuma et al. (1993).

So in conclusion, the concentrations applied in the assays of 10 mM Mg^{2+} and 0.1 mM Mn^{2+} are mostly near the optimum, although higher Mg^{2+} concentrations could have led to higher activity for the prenyltransferases located in the plastids.

5. Conclusion and Outlook

It was shown that in *Populus trichocarpa* the LSUs have a GPPS activity on their own which is increased by the SSUI and modified to a GGPPS site activity by the SSUII. Some genes coding for functionally active GGPPSs were found. Furthermore a new mechanism was suggested for the FPP production after herbivory. These findings provide the basis for the creation of knock out trees each with one prenyltransferase silenced. These trees will help to elucidate the roles of the single prenyltransferases for the primary and secondary metabolism and especially for the plantinsect interactions.

Further studies should focus on the suggested fosmidomycin and mevinolin assays but should include mutagenesis studies as well to elucidate the binding motifs between PtPT4 and PtPT10, which interact although PtPT4 does not contain the CxxxC motif usually necessary for subunit interaction. And furthermore, the transcript abundance of the prenyltransferase genes under different circumstances like growth and flowering and in different compartments of the tree like buds and roots should be studied.

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Appendix

Table A.1	Official	Names	of the	Prenyltransferases	of Poplar	and	the	Corresponding
Names in	this The	sis.						

Name in Phytozome	Name in this Thesis
POPTR_0011s10190	PtPT1
POPTR_0011s10230	PtPT2
POPTR_0011s10270	PtPT3
POPTR_0001s38950	PtPT4
POPTR_0011s10160	PtPT5
POPTR_0006s13760	PtPT6
POPTR_0010s14860	PtPT7
POPTR_0016s00570	PtPT8
POPTR_0006s00530	PtPT9
POPTR_0004s08970	PtPT10
POPTR_0005s14600	PtPT11
POPTR_0005s14590	PtPT12
POPTR_0005s12860	PtPT13
POPTR_0007s12330	PtPT14
POPTR_0015s04050	PtPT15
POPTR_0004s18610	PtPT16
POPTR_0009s14150	PtPT17

TableA.2ExpressionPrimerforthePopulustrichocarpaPrenyltransferases.The primer are designed for the pASK-IBA33+ vector andthe corresponding restriction enzyme is stated in the last row.

	Forward Primer	Reverse Primer	Restrictio n Enzyme
PtPT4	ATGGTAACCTGCATTAAATGGCAGGACTTCTGAAATTGAAGAAAG	ATGGTAACCTGCATTAGCGCTATGAATCCGTTCGAGATTGTACAG	BspMI
PtPT6	ATGGTAGGTCTCAAATGGCTGAGGTTCCTAAGCTTGCCT	ATGGTAGGTCTCAGCGCTTTTATTTCTTGTGATTACTCTTTGAGT	BsaI
PtPT7	ATGGTAGGTCTCAAATGATGGTAATTGCTGAGGTACCTAAG	ATGGTAGGTCTCAGCGCTTTTATTTCTTGTGATTACTCTTTGAGT	BsaI
PtPT9	ATGGTAGGTCTCAGCGCATGGCAGATCTGAAGTCAACGTTC	ATGGTAGGTCTCATATCATTTCTGCCTCTTGTAAATTTTAGCC	BspMI
PtPT10	ATGGTAGGTCTCAAATGGCATTCGAATTTAAGGAGTACATGA	ATGGTAGGTCTCAGCGCTATTTTGTCGAGTAGCAATATAGTTAG	BsaI
PtPT11	ATGGTAGGTCTCAAATGATGATCCGGAAGGGAAATCAAGTG	ATGGTAGGTCTCAGCGCTATTTTGTCGACTAGCAATATAGCTAG	BsaI
PtPT12	ATGGTAGGTCTCAAATGATGCTCACCAAGTTGAAGCAAGTG	ATGGTAGGTCTCAGCGCTACTCTGTCGCCTAGCAATGTAGT	BsaI
PtPT13	ATGGTAACCTGCATTAAATGCCCACTTTTGATTTCAAGTCTTATAT	ATGGTAACCTGCATTAGCGCTGTTTTGCCTGTAAGCAATGTAATTAG	BspMI
PtPT14	ATGGTAGGTCTCAAATGCCCACTTTTGATTTCAAGTCTTACA	ATGGTAGGTCTCAGCGCTGTTTTGCCTGTAAGCAATGTAATTAG	BsaI
PtPT15	ATGGTAGGTCTCAAATGGCAACTTCCAATGGCACTACTTAC	ATGGTAGGTCTCAGCGCTAACATTGCCGGAAGTGGTCCCT	BsaI
PtPT16	ATGGTAACCTGCATTAAATGGCGCAGTTTGATTTAAAGACTTATTG	ATGGTAACCTGCATTAGCGCTAATTGACTCGCCAAAACTGAAACC	BspMI
PtPT17	ATGGTAGGTCTCAAATGTCAAAAACACCCCAGTTTGATTTAAA	ATGGTAGGTCTCAGCGCTACTTGACTCACCAAAACTGAAACC	BsaI

	Forward Primer	Reverse Primer
PtPT4	GTACAAGGGTAGCGGTACTG	CAAATAGGCTAGATGCCTGC
PtPT6	AACGGAAACTGGCAGTGAAG	CAGCTGTCGCTAGTAATG
PtPT7	CATCAACCCTCATCGTATGG	AGCGGCTGAGGCAAGCTTAG
PtPT9	GGTTGCCCAAGGTTGGTCTTATTGC	TGAGTAGTAGGCGGTCTTGTACTG
PtPT10	AAGTGGGCAACAAGTCACAG	AAGCCGCTGGCATTGCTAAC
PtPT11	GAGCACACTGGAGTACATTC	TCAAATCCTTGCCGGCGGTC
PtPT12	AGCGATGCCATTGGCATGTG	GCGAAACGTTCTTGGTCTTG
PtPT13	GTTGCTGGACAAGTTGTG	TCCAATACTCCTCGCGTATC
PtPT14	TGGCGAAAGCTATTGG	CGGTTCCTCCACCTAATATG
PtPT15	TGCTCCAGCCTTGTGCATAG	ACCATCCCATCTCCTGTTAG
PtPT16	GGATTGCTAGCTGGTGCTAC	CTACACTCTCGCCGTATTTC
PtPT17	GATTGCTAGCCGGTGCCAAG	CCTCCGCTACCTCTATAGCC

Table A.3 Applied qRT Primer for the *Populus trichocarpa* Prenyltransferase Genes.

D. D	
PtPT1	I MTCRN LD FGRPKLHLVACS FSSSDSIHRYSVRNYAKSVSKSCNR 44
PtPT2	
PtPT3	
PtPT4	1 MMSMTCHN LD FGRAKLDLVACGCSSNAS I DRYSVRNYAKSVSKSCNR 47
PtPT5	
<i>GPPS</i>	1MLFTRSVARISSKFLRNRSFYGSSQ 25
PtPT6	1MSLSWILRITRNGSHW 16
PtPT7	
PtPT8	
FPPS	
PtPT9	
GGPPS	1MLCKIIIMRPC 11
PtPT10	1MAFSTTISFYDNSLLLKKPI 20
PtPT11	
PtPT12	
LSU	1MSSVNLTWVQTCSMFNQAG 19
PtPT13	1MTSVNLGPWVOITSIINKKT 20
PtPT14	1MTSVNLGSWFO-TSIINKDT 19
PtPT15	1 MTSLAATIPA 10
SSUI	
SSUII	1 MALSS 5
PtPT16	1MVVSSLIAISSP 12
PtPT17	1 MALS LAALPS 10

Figure A.1 Complete Sequence Alignment. The truncation sites are marked in red, conserved sites are marked in grey. As reference sequences the sequences of the geranyl diphosphate synthase 1 of *Arabidopsis thaliana* (GPPS) with the NCBI accession number NP_001031483.1, the farnesyl pyrophosphate synthase of *Humulus lupulus* (FPPS) with the GenBank accession number BAB40665.1, the geranyl geranyl pyrophosphate synthase 6 of *Arabidopsis thaliana* (GGPPS) with the GenBank accession number BAA23157.1, the geranyl diphosphate synthase large subunit of *Humulus lupulus* (LSU) with the GenBank accession number ACQ90682.1, the geranyl diphosphate synthase small subunit of *Humulus lupulus* (SSUI) with the GenBank accession number ACQ90681.1 and the class II small subunit of *Oryza sativa* (SSUII) with the GenBank accession number ACQ90681.1.

PtPT1 PtPT2	45 DYG FRRMVCCRRD I ARCRVSS TKTPQALLA RVAEGP TGLLNLK 8 1 VAEGP TVLLNLK 1	87 12
PtPT3 PtPT4 PtPT5	48 DYGSRRLVCSRRDIARCRVSPTKTPETLPA GVAEGPAGLLKLK	90
GPPS PtPT6	26 SLASHRFAIIPDQGHSCSDSPHKGYVCRTTYSLKSPVFGGFSHQLYH 17 FVSHRPSSYGLLLSNNSHSSTRKVFNCRETYSWTVPSFHVFKHQIHH	72 63
PtPT7 PtPT8	1 MAAVGFALINPHRMDSCSPTILTLLLQRETYSWTVPSLHVFKHQIHH	47
PtPT9	1 MAGLRSKFMEVYSIL	15 15 21
PtPT10 PtPT11	21 NGLINNQPRTPLGHLKFAPLKIQATYVASP SFKQSFQSQEVGN (63
PtPT12 LSU	20 RSRSSTFNLLHYHPLKKVPFSFQTPKQRRPTSSFSSISAVLTEQEAV (66
PtPT13 PtPT14	21 RSRSRPMSNFLSPGLKTLQIASTHQRQRKP MSSICAVLTEEET (20 GPRSIPISNFLSPGSKSLQIPSVHQKQRKP MSSICAVLAKEET (53 62
SSUI	11 INGNPILPYLPRSNTNRPLLSHRPMTVAVTMSR 3 1MSR 3 6 FSMSIPFAKIPSTSKSTRFIPIRASSAAAA	40 3 35
PtPT16 PtPT17	13 LTTTIYLP - VKSPNLYTKILGKMSKIQCSL	42 40
PtPT1 PtPT2	88 KESREPVSLTNLFEVVADDLLNLKKNLRSIDA AENMVL 13 KESREPLLLTNLEEVVAADLOTLKONLWSIDA AENTVL	125 50
PtPT3 PtPT4	1 KESREPVSLTNLFEVVAADLQTLKQNLWSIVA AENTVL 3 91 KDSRESISITNLFEVVADDLQTLNQNLQSIVG AENPVL 3	38 128
PtPT5 GPPS	73 QSSSLVEEELDPFSLVADELSLLSNK <mark>L</mark> REM <mark>V</mark> LAEVPKL	110
PtPT6 PtPT7 PtPT8	64 QSSSLVEEQLDPFSLVADELSLLANRLRSMVI AEVPKL 1 48 QSSSLVEEQLDPFSLVADELSVLANRLRSMVI AEVPKL 8	101 85
FPPS PtPT9	16 KSELLNDPAFEFTDDSRQWVERMLDYNVPGG KLNRGL 5 16 KKELLEDPAFEWSPDSRDWVDRMLDYNVPGG KLNRGL 5	52 52
GGPPS PtPT10	32 IASSDSEFISYMNNKAKSINKA <mark>L</mark> DNSIPLCNNSVPLWEPVLEV 64 KSQKVPLP <mark>A</mark> FEFKEYMINKANQVNKALDEAVPL QHPQKI	74 102
PtPT11 PtPT12	1 MIRKGNQVNKALDEAVPL QHPRKI 2 1 MLTKLKQVNEALEEAVPL QHPIKI 2 67 TEDEFOKSTENEKSYMVOKANSVNOALDAAVL	24 24 105
PtPT13 PtPT14	64 LQEDQHKPTFDFKSYMUQKANSVNQALDAAVLLKEPQMI 64 LQEDQHKPTFDFKSYMIQKANSVNKALDTAVTLKEPAKI 63 LQEDORKPTFDFKSYMLOKANSVNKALDAAVSLKEPAKI	103 102 101
PtPT15 SSUI	41 ATSNGTTYNKSYWTSVNDEIDAHLKQAIPI RPPLSV 4 THENHHVPTSTSIVVSASITADIEAHLKQSITL KPPLSV	76 42
SSUII		

P+PT1	126 MSAAFO I FCAC - C KEMRPALVELISRATVEVVC 157
$D_{t}D_{T}$	51 MSAAFOIVCAC C KPMPDAIVFIIS PAPP 78
D+DT3	30 MSAAFOIVSAC C KPM 53
$P_{P} D T_{A}$	129 MSAAFOIFGAG. G
PtPT5	1 MSGAROK FGAG - G
GPPS	111 A SA A EVERKOGVOG
DITS DITE	102 A SAAFY FERROVOC KOFOT FLIMA TAINVDILETE 122
$P_{1}P_{1}P_{1}$	86 A SA A FY FERMOVED
$P_{1}P_{1}P_{1}$	SUNSARE FRANCIES KAPATIVE EDMA TAEN (KIEETI 122
	535 VIDS 10 LEKG - 0 KELTER FLT
CCDDS	75 UKAMDYTIISC C KDUDDMICIVAC PLVC 100
DEDT 10	102 NEAMONT LESS - C KRYRE LARC EVAC
PlPII0 DPTI1	105 NEAMNTS LEAG - 0
P(PIII)	25 HEAMRIS LEAG - G KRWRFILL IASC E LVG
	16 HEAMORIS LEGA - G KDUDDULCISAC FLUC 122
	100 ILE AMATS LEAGTON KRYRTY LE LAAC PLUC 130
PUPIIS DPTIA	103 HESMATS LEAGE C KRVRTV CLAAC DIVC 130
PUP114	102 ILE SMRTIS LEAGTON - CONTACT - CONTAC
	12 HEDMANI VESA D DNA DSI CIAAC FLVC 70
	72 HS AND VAVI CONCETARDAD DVI CVAAC
05011 D+DT16	12 IISAMATAV BIGT-AGNEGIAKATIV BOVAAC
$P_{P} P_{T} P_{T$	62 1 EAM T S VEAR - GA - KRAFT WOVAAC FIFC 100
<i>FUE117</i>	SUTEAMATSVEAK - GA KNATFVMCVAAC EEFO 100
PtPT1	158 DLTTEHORLAELLEMIHTASLIHDDL LD 185
PtPT1 PtPT2	158 DLTTEHQRLAEIIEMIHTASLIHDDL LD 185 79 KEHTTEHRHLAEIIEMIHTASLIHDGV LD 107
PtPT1 PtPT2 PtPT3	158 DLTTEHQRLAEIIEMIHTASLIHDDL LD 185 79
PtPT1 PtPT2 PtPT3 PtPT4	158 DLTTEHQRLAEIIEMIHTASLIHDDL LD 185 79
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5	158 DLTTEHQRLAEIIEMIHTASLIHDDL LD 185 79
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS	158
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6	158
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7	158 DLTTEHQRLAEIIIEMIHTASLIHDDL LD 185 79
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8	158 DLTTEHQRLAEIIIEMIHTASLIHDDL LD 185 79
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS	158
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9	158 DLTTEHQRLAEIIEMIHTASLIHDDL LD 185 79
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS	158 DLTTEHQRLAEIIEMIHTASLIHDDL LD 185 79
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10	158 DLTTEHQRLAEIIEMIHTASLIHDDL LD 185 79
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11	158 DLTTEHQRLAEIIEMIHTASLIHDDL LD 185 79
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12	158 DLTTEHQRLAEIIEMIHTASLIHDDL LD 185 79
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU	158
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13	158
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14	$158 - \dots - D L T T E H Q R L A E I I E M I H T A S L I H D D L - L D 185$ $79 - \dots - K E H T T E H R H L A E I I E M I H T A S L I H D Q V - L D 107$ $54 - \dots - E H T T E H R H L V E I I E M I H T A S L I H D D V - L D 190$ $27 - \dots - T T E H R R L A E I I E M I H T A S L I H D D V - L D 52$ $148 I G E S T D I V T S - \dots - E L R V R Q R G I A E I T E M I H V A S L L H D D V - L D 185$ $139 T G S E - \dots - G D A L T T E L R K R Q Q S I A E I T E M I H V A S L L H D D V - L D 176$ $123 T S G V E T T T R G V G D A L T T E L R K R Q Q S V A E I T E M I H V A S L L H D D V - L D 167$ $77 - \dots - A L G W C I E W L Q A Y F L V L D D I - M D 97$ $77 - \dots - A L G W C I E W L Q A Y F L V L D D I - M D 97$ $103 - \dots - G Q E S T A M P A A C A V E M I H A A S L I L D D L P C M D 132$ $131 - \dots - G D E E L A M P T A C A L E M I H T M S L I H D D L P C M D 160$ $53 - \dots - G D E E L A M P A A C A V E M I H T M S L I H D D L P C M D 160$ $134 - \dots - G D E S V A M P A A C A V E M I H T M S L I H D D L P C M D 163$ $131 - \dots - G D E S V A M P A A C A V E M I H T M S L I H D D L P C M D 163$ $131 - \dots - G S E S M A M P A C A V E M I H T M S L I H D L P C M D 163$ $131 - \dots - G S E S M A M P A C A V E M I H T M S L I H D L P C M D 163$ $131 - \dots - G S E S M A M P A C A V E M I H T M S L I H D L P C M D 163$ $131 - \dots - G S E S M A M P A C A V E M I H T M S L I H D L P C M D 163$
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14 PtPT15	158
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14 PtPT15 SSUI	$\begin{array}{cccccccccccccccccccccccccccccccccccc$
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14 PtPT15 SSUI SSUI	$\begin{array}{cccccccccccccccccccccccccccccccccccc$
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14 PtPT15 SSUI SSUI SSUI PtPT16	$\begin{array}{cccccccccccccccccccccccccccccccccccc$

PtPT1	186 E S G M R A	
D+DTO	108 F SD MP D A 114	
FIF12		
PtPT3	80	
PtPT4	191 ESDMRRGKETVHOLYGTRVAVLAGDFMFAOSSWY - LAN LE 229	
	52 FSDMK LEINTMULACDEMEAOSS CAOSSWYLANIE 87	
PIPIS	33 ESDMK EFTN IMVEAGDFMFAQ33 CAQ33WIEAN EE 87	
GPPS	186 DAD TRRGVGS LNVVMGN KMSVLAGD FLLSRACGA LA 221	
PtPT6	177 DADTRRGIGS LN LVMGN KVAVLAGD FLLSRACVA LA 212	
D+DTT	168 DAD TRRC LOSIN FYMCN KVAVLACDELLSRAC 100	
	100 DAD I KKO I OS LA FYMON KYAY BAOD I BESKACI I I I I I I I I I I I I I I I I I I	
PtPT8	······································	
FPPS	98 N S V T R R G Q P C W F R V P K V G L I A A N D G I L L R N H I P R I L K K H F K G K S Y 142	
PtPT9	98 SSHTRRGOP CWFRLPKVGLIAANDGILLRNHIPRILKNHFRDK - AV 142	
GGPPS	133 DDS LRRGIP INHKVFGEKISI LASNALMSLAVKQI LASISLG VI 176	
PtPT10	161 NDDLRRGKPTSHKIFGEDTAVLAGDALLSLAFEH - VARNTKN VS 203	
PtPT11	83 NDDLRRGMPTSHKVFGEDTAVLAGDALLALAFEH - VARNTKN VS 125	
D+DT10	83 NDDI PRCKPTNHKVECECIALIACDALISIAFEH - LASKTKN VS 125	
<i>FUF112</i>	I CANDE LA REAL THINK TO E CHAT BACKAGE A LA CAPELLA LA VALUE A VA	
LSU	164 NDD LRRGKPTNHKVFGEDVAVLAGDALLAYAFEH - VAVSTVG VP 206	
PtPT13	161 NDD LRRGKS TNHIVFGEDVAVLAGDALLAFAFEH - I AVS TLN VS 203	
PtPT14	160 NDDLRRGKPTNHIVFGEDVAVLAGDALLSFAFEH-IAVSTIHVS 202	
D+DT1E	123 TONDAD ICHSECSNIELLTODOMVDEGLE LLAKSDDITONN 173	
PIPIIS	135 IGN KAK IGHS FOSN IE EE IGD GMVFFGEE EE AKSDDE IQNN 175	
SSUI	101 SPSPS PVIHNSYDPSIQLLMPDAI LPLGFE LLAQSYNPAQNN 142	
SSUII	136 A A P T R R G R P S T H A A Y G T D M A V L A G D A L F P L A Y T H - V I A H T P S P D P V P 181	
D+DT16	141 DDPSPRGOPSNHTLYCVDMALLAGDALEPLGESH-LVSOTPS-DLVP 185	
DUD717		
PtP117	139 DDPSKRGQPSNRKI I GVDMAI DAGDALFPLGFRH - I VSH I PS - DLVP 183	
PtPT1		
PtPT1		
PtPT1 PtPT2		
PtPT1 PtPT2 PtPT3	98 N I E L I K L I S <mark>S</mark> V A <u> </u>	
PtPT1 PtPT2 PtPT3 PtPT4	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 CDDC	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6	98 N I EL I KL I S SVA	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 EDD0	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9	98 N I EL I KL I S SVA	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11	98 N I EL I KL I S SVA	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11	98 N I EL I KL I S SVA	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12	98 N I EL I KL I S SVA	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT12	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14	98 N I E L I K L I S S VA	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14 PtPT15	98 N I EL I KL I S 98 N I EL I KL I S 230 N I EV I KL I S 97 222 A LKN TEVVA L LA TAV - E H LVT GE TME I TSS TEQ	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14 PtPT15 SSUI	98 N I ELIKLISS VA	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14 PtPT15 SSUI SSUI	98 N I E L I K L I S S VA	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14 PtPT15 SSUI SSUI	98 N I E L I K L I S S V A	
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14 PtPT15 SSUI SSUI SSUI PtPT16	98 N I E L I K L I S S VA	

D+DT1	
PIPTO	195 I I K TASLIAAS I KGAAT F SOVD SN VCVLI I EFGKN LOLS FOVV 255
PtP12	115 YYKTASLTAASTKGAATFSGVDSDVCVLTYEYGKNLGLSFQVV 157
PtPT3	110 YYKTASLIAASTKGAAIFSGVDSNVCMLMYEYGKNLGLSFQVV 152
PtPT4	266 LIKSYYK TASLIAASTKGAAIFSGVDSSVSMQMYDYGKNLGLSFQVV 312
PtPT5	98 <u>-</u> TASLIAASTKGAAIFSGVDSNVCMLMYEYGKNHGLSFQVV 137
GPPS	261 MQKTYYKTASLISNSCKAVAVLTGQTAEVAVLAFEYGRNLGLAFQLI 307
PtPT6	252 MQKTYYKTASLISNSCKAIALLAGQTTEVAMLAFEYGKNLGLAYQLI 298
PtPT7	222 MOKTYYKTASLISNSCKAIALLAGOTTEVAMLAFEYGKNLGLAYOLI 268
PtPT8	1 MAGENLDNHVDVKNLLVEMGTVF0V0 26
FPPS	185 R LVOY KTAYYS FYL PVA CALVMAGEN LDNHVDVKNVL LEMG TY FOVO 231
P+PTQ	185 R LVOVKTAVYS FYL PVA CALLIMAGEN I DNHVDVKNI LVFMG TVFOVO 231
CCPPS	
D+DT10	
PIPIIO DIDIII	244 EI INVIKTAK LLEAAVVCGATMGGADATSVERUKKARSTGLLFOVV 290
PTPTTT	100 EY I H V H K I A K L L E A A V V C G A I M G G A D A I S V E R L R KY A R S I G L L F Q V V 212
PtPT12	166 EY I HVHKTAKLLEASAVCGA I I GGADDASI ERLRKYARSI GLLYQVV 212
LSU	248 EFIHLHKTAALLEASVVLGAILGGGTDEQVEELRSFARCIGLLFQAV 294
PtPT13	245 E F I H I H K T A K L L E G A V V L G A I L G G T D E E V E K L R R Y A R S I G L L F Q V V 291
PtPT14	244 EFIHVHKTAKLLEGAVVLGAILG <mark>G</mark> GTDEEVEKLRTYARSIGLLFQVV 290
PtPT15	210 DHVCKKKEGELHS <mark>CAGAV</mark> GAILG <mark>G</mark> GTEEEIEKLRRYGLYV <mark>GLMQG</mark> VL 256
SSUI	187 D C S G R E K E G K M H A C A A A C G A I L G D A N E E E T E K L R T F G L Y V G M I Q 230
SSUII	221 MKVLTKKFGEMAECSAACGAMLGGAGPDEEAALRRYGRTIGVLYQLV 267
PtPT16	221 E F V Q E K K Y G E M G E C S A V C G G L L A G A T D D E I R R L R R Y G R A V G V L Y Q V V 267
PtPT17	219 E FVQEKK FGEMGECSAVCGGL LAGAKDDE I QRLRRYGRAVGV LYQVV 265
PtPT1	236 DD LLD FTOSA PLPGK PAGSDLAKGN LTAPVI FALEOSPKLRELLETE 282
P+PT2	158 DD LIDETOSAELPGKPAGSDIAKGNITAPVIEALEOSPKIELIETE 204
P_{PT3}	153 DD LLD FTOSAFI PCKPA CSDLAKCNI TA UTAL FOSPKI RELIETE 109
$P_{P} P T A$	313 D LI D FTOSA FOI GKPAGSDI AKGNI TADVI FALFOSPKI PELLESE 350
	129 DI I DETOSA EL DEVOS EL ACOLARON LA VILLA D
GPPS	308 DD LDFTGTSASLGKGSLSDTRHGVTTAFTLFAMEEFFQLREVVDQV 334
PtP10	299 DDVLDFTGTSASLGKGSLSDTRHGTVTAPILFAMEEFPQLCSVTDRG 345
PtP17	269 DDVLDFTGTSASLGKGSLSDTRHGTVTAPTLFAMEEFPQLRSVTDWG 315
PtP18	27 DDCLDCFGDPE11GK-1GTD1EDFKCSwLVVKGME1CNEEQKKLLHE 72
FPPS	232 DDY LDCFGHPDVIGK - IGTDIEDFKCSWLVVKALEIATEEQKKMLFE 277
PtPT9	232 DDYLDCFGAPETIGK-IGTDIEDFKCSWLVVKALEICNEEQKKLLHE 277
GGPPS	269 DDVLDETKSSEELGKTAGKDLITGKLTYPKVM 300
PtPT10	291 DD I LDVTKSSEELGKTAGKDLASDKATYPKLM 322
PtPT11	213 DD I LDMTKSSEELGKTAGKDLTSDKATYPKLI244
PtPT12	213 DD I LDATKSSEELGKTAGKDLASNKATYPKLM244
LSU	295 DD I LDVTKSSQELGKTAGKDLVADKATYPRLM 326
PtPT13	292 DDILDVTKSSQELGKTAGKDLVADKVTYPKLM 323
PtPT14	291 DDILDVTKSSQELGKTAGKDLVADKVTYPRLM 322
PtPT15	257 SNWVE
SSUI	
SSUII	
	268 DD I RSA
PtPT16	268 DD I RSA
PtPT16 PtPT17	268 DD I RSA

DIDE		
PtPT1	283 FRVSVSLDEATELVMDCGGTERAQELAKERADLATQNLRSLPRGS	327
PtPT2	205 FRVSGSLDEATELVMDCGGIERAQELAKERADLAIQNLRSLPRGS	249
PtPT3	200 FHESGSLDEATELVMDCGGIERAOELAKERADLAIONLRSLPRGS	244
PtPT4	360 FCESGS LDEALELVKSSGG LERAOFLAKEKADLA LON LRCLPOGS	404
P_{PT5}		172
	107	401
GPPS	335 EKDP KN V D I A LE I LGKS KG I Q KAKE LAMEH AN LAAAA TOS LF E I DN E	401
PtP16	346 FDKPENIDAALEYLGKSRGIQRTRELAAKHANLAAAAHDSLPETDDE	392
PtPT7	316 FDKPEN I DVA LEY LGKS RGIQRTRELAAKHAN LAAAAIDS LPETDDE	362
PtPT8	73 NYGKPDPANEAQGKALYNDLNLQGVFADYESKTYEKLITSIEDHP	117
FPPS	278 HYGKGDEASVKKVKELYKALDLEGVFADYENASYOKLIKSIEAHPKE	324
PIPT9	278 NYGKA DPANVA KYKA LYHELN LOGYFADYE SKSYEK LLASI EAHPSK	324
CCDDS	201 ADEVAKED IN A DEVAKED IN REACENTICE DESD.	306
		340
PIPIIO	323	348
PtPT11	245	270
PtPT12	245	270
LSU	327	352
PtPT13	324 GIEKSREFAEKLLNEARELLAGFDOE	349
PtPT14		348
D_{PT15}		280
		209
SSUI	244	267
SSUII	290 GMDRALGIVEELKAQAKMEADRFGDKYGE	318
PtPT16	297	324
PtPT17	295	322
1		
PtPT1	328 YOPALEEMVLCNLERVH	344
PtPT1 PtPT2	328 YQPALEEMVLCNLERVH	344 266
PtPT1 PtPT2 PtPT3	328 YQPALEEMVLCNLERVH	344 266 261
PtPT1 PtPT2 PtPT3 DtPT4	328 YQPALEEMVLCNLERVH	344 266 261
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5	328 YQPALEEMVLCNLERVH	344 266 261 421
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5	328 YQP A LEEMVLCNLERVH	344 266 261 421 176
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS	328 YQP A L EEMVLCNLERVH	344 266 261 421 176 422
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6	328 YQP A LEEMVLCNLERVH	344 266 261 421 176 422 413
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7	328 YQP A L EEMVLCN LERVH	344 266 261 421 176 422 413 383
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8	328 YQP A L EEMVLCNLERVH	344 266 261 421 176 422 413 383 134
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS	328 YQP A L EEMVLCNLERVH	344 266 261 421 176 422 413 383 134 342
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9	328 YQP A L EEMVLCNLERVH	344 266 261 421 176 422 413 383 134 342 342
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS	328 YQP A L EEMVLCN LERVH	344 266 261 421 176 422 413 383 134 342 342 343
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10	328 YQP A L EEMVLCN LERVH	344 266 261 421 176 422 413 383 134 342 342 343 265
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11	328 YQP A L EEMVLCN LERVH	344 266 261 421 176 422 413 383 134 342 342 342 343 365 297
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11	328	344 266 261 421 176 422 413 383 134 342 342 343 365 287
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12	328 YQP A L EEMVLCNLERVH	344 266 261 421 176 422 413 383 134 342 342 343 365 287 287
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU	328 YQP A L EEMVLCNLERVH	344 266 261 421 176 422 413 383 134 342 343 365 287 287 369
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13	328 YQP A L EEMVLCNLERVH	344 266 261 421 176 422 413 383 134 342 343 365 287 287 369 366
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14	328 YQP A L EEMVLCNLERVH	344 266 261 421 176 422 413 383 134 342 343 365 287 287 369 366 365
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14 PtPT15	328 YQP A L EEMVLCNLERVH	344 266 261 421 176 422 413 383 134 342 343 365 287 287 369 366 365 312
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14 PtPT15 SSUI	328 YQP A L EEMVLCNLERVH	344 266 261 421 176 422 413 383 134 342 343 365 287 287 369 366 365 312 279
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT11 PtPT12 LSU PtPT13 PtPT14 PtPT15 SSUI SSUI	328 YQPALEEMVLCNLERVH	344 266 261 421 176 422 413 383 134 342 343 365 287 287 369 366 365 312 279 344
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT6 PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT12 LSU PtPT13 PtPT15 SSUI SSUI SEVII	328 YQP AL EEMVLCN LER VH	344 266 261 421 176 422 413 383 134 342 342 343 365 287 287 369 366 365 312 279 344
PtPT1 PtPT2 PtPT3 PtPT4 PtPT5 GPPS PtPT7 PtPT8 FPPS PtPT9 GGPPS PtPT10 PtPT12 LSU PtPT13 PtPT14 PtPT15 SSUI SSUI PtPT16	328	344 266 261 421 176 422 413 383 134 342 343 365 287 287 369 366 365 312 279 344 347

SP#PT10
> PtPT4 GC1AGGTCGAAGTTCCCCAACAAAGACCCCTGAGACTTTACCTGCGGGGAGTGCGAAAGGACTCGCAAGGACTTCGAAAATGAAGAAGATCGAAGAAGACTACTTCAATAACAAATTGTTGAAGGGACCAGGCTGAGAAGAAGTCGAAGGACTCAAGTAGTGCGACGAGAGAACTGCGACGAGAGAACTGCGACGAGCAGCACGCCGGCGAAGAAGTCGAAGGAGCGCCCGCGGAAGAAGTCGAAGGAGCGCCCGGCGAAGAAGTCGACGGACG
> PtPT7.1 Arggranded and and argentic ar
> PtPT7.2 ATGGTTAATTGCTGAGGTACCTAGGCTGCCGGGTGCTTTTTTAAATTGGGAGTACAGGGAAGGGGTCCGTCC
>
> PEPT12 ATGCTCacCAAGTGAAGCAAGTGAACGAAGCACTAGAAGAGGCAGTGCCCTTGCAACACCCCATTAAAATCCATGAAGCAATGAGAATTGCTCCTCTTGGTAATGGAAAAGGAATGTGCCGATTTATGCATTGCTTCATGGATTGGTCAGGTCAGGACGACGACGACGACGACGACGACGACGACGACGACG
> PtPT17 TCAMAACCCCCAGTTGATATAGACTTATTGGACAACCTTGATTCAGAAATCAATC
> PPT9 Artogcargarceased concentration of the conc
>
> PPT16 AGCCCAANTCTTTACACTAMATACTTGGCAMANTGCAMANTCAATOTTCTTTATCAGTTCAAGGCCAATCAAGGCAGCCCCGGTTTGATTTAAAGACTTATTGGACAACCTGATGGGGAAATCAATC
PtPT11 ATGATCCGGAAGGGAATCAAGTGAACAAGGACTAGATGAGGGAGTGCCCTGCAACATCCCAGAAAAATCCATGAAGACCATGAGATATTCCTCCTCTGAGGAGGTGACGGTGACGGCCACAGACCATAAAGTTTTTGGTGAGATATAGCAGGTGACGTGACCACGACGACGACGACGACGACGACGACGACGACGACG
> PtPT14 cccctrttrgatticangectacamagecantegeration and cattered the construction of th
> PtPT15
GCAACTTCCAMTGGCACTACTTACAACAGGTCTTACTGGACCTCTGTAMTGATGAGAATGAGAGGCCATTCCCATTGAGCCACCCCATTGAGCCACCATTTGAGCCATGCACCATTGAGCCAGCC

Figure A.2 Sequences that were Actually Amplified and Inserted into the pASK-IBA33+ Vector. The nucleotide sequences are shown in the FASTA format. Nucleotides that differ from the database are marked in red, in the truncated sequences, a start codon was inserted by the vector.



Figure A.3 Single Enzyme Assays. All single enzymes were assayed with three different substrate combinations: IPP and DMAPP, IPP and GPP and IPP and FPP. The assayed enzymes are written in every diagram, the applied substrates are indicated below the enzyme names in braces. The blue peaks in the diagrams represent **GPP**, the red peaks represent **FPP** and the green peaks represent **GGPP**. **a)** Assays of the empty pASK IBA33+ vector with different substrates. **b)** Assays of PtPT4 with different substrates. **c)** Assays of PtPT9 with different substrates. **d)** Assays of PtPT10 with different substrates. **e)** Assays of PtPT12 with different substrates. **f)** Assays of PtPT13 with different substrates. **g)** Assays of PtPT14 with



different substrates. **h)** Assays of PtPT15 with different substrates. **i)** Assays of PtPT16 with different substrates. **j)** Assays of PtPT17 with different substrates.
















Selbstständigkeitserklärung

Hiermit erkläre ich an Eides statt, dass ich die vorliegende Arbeit selbstständig und nur unter der Verwendung der angeführten Quellen und Hilfsmittel angefertigt habe.

Jena, den 23.Juli 2012

Nora Pauline Petersen