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Identification and characterization of the most efficient feverfew LTP(s) for transport of parthenolide and/or costunolide

Abstract

Parthenolide is the main active compound of feverfew plants which have been used for centuries against multiples afflictions but mostly to reduce fevers and migraines. Parthenolide and its precursor costunolide are both sesquiterpene lactones with medicinal properties. These compounds are hydrophobic and therefore their transport within and outside the cell should involve some kind of mechanism. The precise mechanism is yet unknown but may strongly involve lipid transfer proteins (LTPs). Since their discovery *in vitro*, however, these proteins' function and mechanism remain unsolved as they seems to be secreted and therefore can not fulfil the hypothesized function of intracellular lipid transfers. Understanding how the medicinal compounds are transported outside the cell is of importance in order to be able to increase their production without a limitation factor. Our hypothesis is that a single LTP facilitates the transport of parthenolide and its precursors to the apoplast. In order to test this hypothesis eight LTP candidates have been identified and investigated. Their individual effect on costunolide yields have been tested, LTPs 1 and 2 have shown the best increase in yield whereas LTPs 3, 4 and 8 have showed yields even lower than the control. The combined effect of multiple LTPs on the yields of parthenolide and costunolide, have shown a specificity of the LTP transport, the yields of free costunolide and its conjugates were improved with some LTPs but only the free parthenolide yield was increased with the tested LTPs. The secretion assay has shown that 23% of the free costunolide was present in the apoplast when expressed with LTPs compared to 7.8% in the control. From our eight LTP candidates two seem to have a role in the transport of costunolide and its conjugates to the apoplast.

Introduction

Parthenolide, costunolide and precursors

Parthenolide is the main active compound of feverfew, *Tanacetum parthenium,* a well-known medicinal plant. Feverfew is a bushy perennial that originates from the Balkan peninsula but can now be found in Japan, China, Australia, America, North Africa and Europe in gardens and on the side of the roads (Pareek, Suthar, Rathore, & Bansal, 2011). It has been used since ancient times for multiple afflictions from arthritis to insect bites but mainly, as its common name indicates, against fever and different aches. Feverfew is also sometimes called the 'mediaeval aspirin' (Knight, 1995) . It is now still being used against migraines and its symptoms (Palevitch, Earon, & Carasso, 1997). Feverfew has been shown to also have a therapeutic working on cancer (Guzman et al., 2005), by inducing apoptotic cell death specifically in cancer cells (Mathema, Koh, Thakuri, & Sillanpää, 2012) probably through conjugation with glutathione (Liu et al., 2014). Feverfew synthesises many mono and sesquiterpenes but the most promising compounds for medicinal purposes are parthenolide and its precursor costunolide. Parthenolide constitute up to 85% of the total sesquiterpene lactone content of feverfew plants (Pareek et al., 2011). As the traditional use of its aerial parts indicates, these compounds are mostly found in the leaves and inflorescences more particularly in trichomes on the lower part of the flowers (Majdi et al., 2011).

Sesquiterpene lactones are abundant in Asteraceae with 4000 different sesquiterpenes lactones reported from this important plant family (Liu et al., 2011). Costunolide is thought to be the common precursor for multiple similar compounds with germacranolide, eudesmanolides and guaianolides backbones (Majdi et al., 2011). It counts within its precursors mevalonic acid and farnesyl diphosphate (FPP) originating from the mevalonate pathway (**see Figure 1**). Costunolide has been detected in many other medicinal plants such as *Saussurea lappa* (Robinson et al., 2008) and *Magnolia grandiflora* (Koo et al., 2001). Costunolide itself has also been proven to have a therapeutic effect on cancer as well as having many other medicinal functions for example against viruses and fungi (Liu et al., 2011). Two derivatives 3β-hydroxycostunolide and 3β-hydroxyparthenolide have been found that are more soluble than both costunolide and parthenolide, which may render the formulation of a drug easier. They originate from the addition of a hydroxyl group by a cytochrome P450 enzyme called Tp8878 which appears not to impair the biological activity of the compounds (Liu et al., 2014).

Previous experiments in which the parthenolide pathway was expressed in *Nicotiana benthamiana* plants, yields of parthenolide up to 1.4µg/g fresh leaves were obtained. Even though it seems low it is higher than the amount of parthenolide found in feverfew leaves but lower than in feverfew flowers. However when expressing the parthenolide pathway in *Nicotiana benthamiana*, the bulk of the parthenolide is conjugated. There are two conjugates to be found: the glutathione and cysteine conjugates. Most of the produced parthenolide is conjugated into the cysteine form, it represents almost 94% of the total parthenolide, and 6% is in the glutathione form. Only a mere 0.1% of the parthenolide is in its free form in transformed *Nicotiana benthamiana*, whereas in feverfew flower trichomes 95% of the parthenolide is in its free form (Liu et al., 2014). Conjugation is a process that allows the cell to protect itself from xenobiotics, which are compounds normally not produce in this particular cell or produced in higher concentrations than normal which may be toxic.

Transport and localisation of parthenolide and its precursors

Since parthenolide and costunolide are both hydrophobic, their transport within and outside the cell is complicated and it was not documented yet how and when this transport occurs. In feverfew, parthenolide is found mainly in trichomes on the flowers, it is most likely synthetized in situ since the enzyme TpGas, catalysing the reaction from farnesyl diphosphate (FPP) to germacrene A, was also

found in high concentrations in the trichomes (Majdi et al., 2011). But to optimise and understand fully the synthesis of parthenolide, its transport must be analysed. An unknown element of the transport could be the key to a limitation of parthenolide availability in the apoplast.

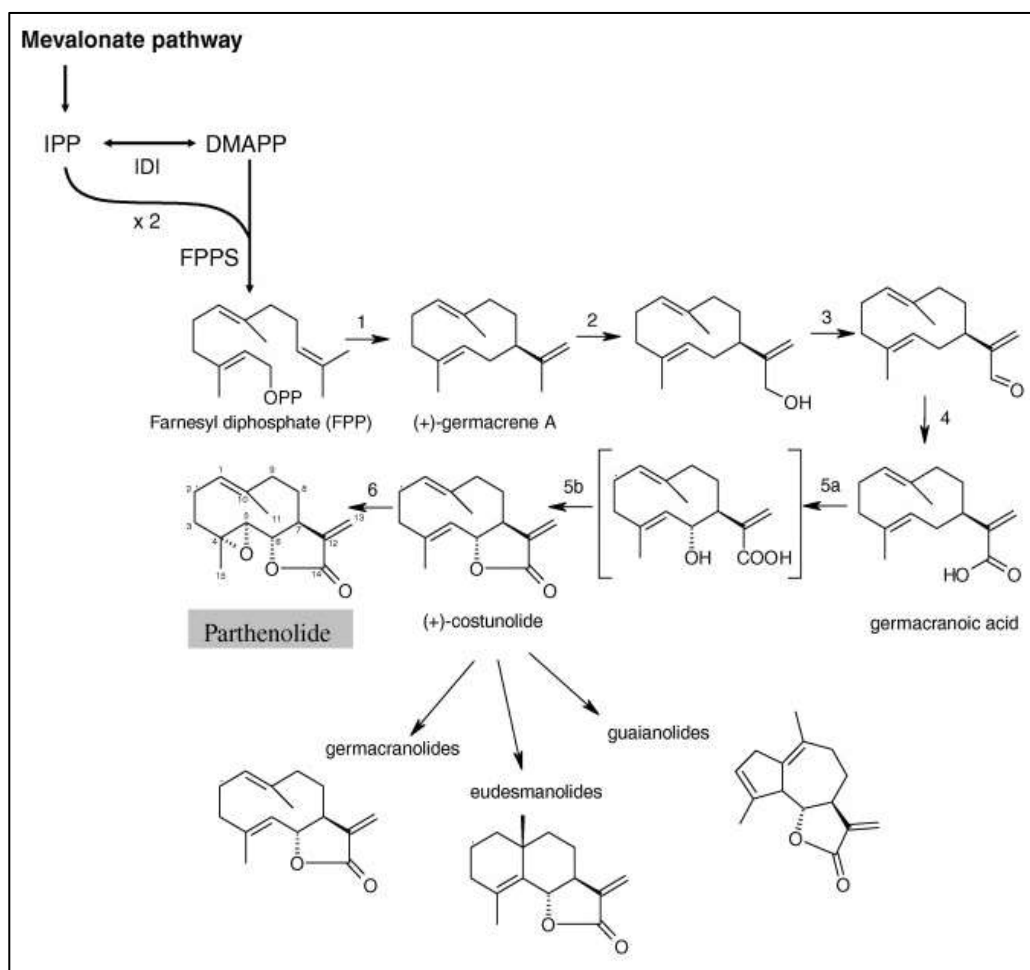

Figure 1 The parthenolide biosynthetic pathway following the mevalonate pathway. 1, germacrene A synthase (TpGAS); 2, 3 and 4, germacrene A oxidase (TpGAO); 5a and 5b, costunolide synthase (TpCOS); 6, costunolide epoxidase/parthenolide synthase (TpPTS). (Majdi et al., 2011)

Transport of hydrophobic compounds especially lipids within and outside the cell has been and still remains an unsolved puzzle. Multiple hypothesis were suggested such as the use of vesicles, spontaneous movements of the lipids, or the involvement of carrier proteins (Kader, 1997). Spontaneous lipid desorption is unlikely since the process is slow and only permits limited amount of lipids to be moved. In both remaining hypothesis, the involvement of carrier proteins, in particular lipid transfer proteins (LTPs), is almost certain but still some doubts remains since little is known about the functions and mode of action of LTPs (Lev, 2010)Error! Reference source not found.. It is not clear yet whether the LTPs function with vesicles or not and whether membrane bound transporters are involved (see **Figure 3**).

Lipid Transfer Protein

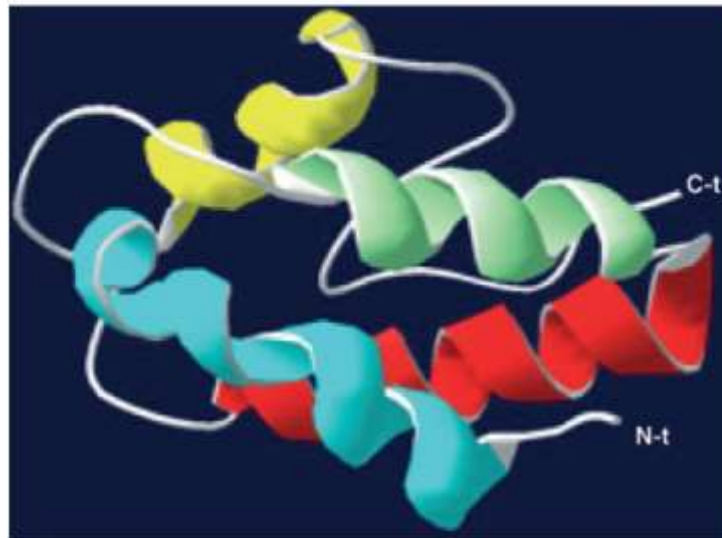

Figure 2 3D model of the LTP Pru p 3, a major peach allergen (Salcedo, Sanchez-Monge, Diaz-Perales, Garcia-Casado, & Barber, 2004).

LTPs were so called when mammal soluble proteins were found that could transfer lipids between membranes within the cell *in vitro*. Proteins with similar *in vitro* activity but evolutionary distinct from mammal proteins were found in higher plants, these proteins are also called LTPs (Yeats & Rose, 2008). Plant LTPs are between 70 and 95 amino acid residues long (an extra piece of 20 to 35 amino acids form the N-terminus signal peptide which is cleaved before the mature protein enters the secretory pathway) (de Oliveira Carvalho & Gomes, 2007) and have eight cysteine residues conserved on specific locations. These cysteine residues form four disulphide bridges which secure the peculiar structure of LTPs with several α-helices which form a hydrophobic tunnel (Kader, 1997) (see Error! Reference source not found.Error! Reference source not found.). The form of this tunnel dictates the specificity and range of lipids compatible with the LTP. The hydrophobic part of the lipid is buried inside the tunnel while the carboxylate portion stays on the outside of the LTP, each part of the lipid interacting with the LTP to form a stable combination**.** LTP transport of lipids does not require energy as it is controlled by the concentration gradient of each lipid individually (de Oliveira Carvalho & Gomes, 2007).

Two plant LTP families have been characterized, LTP1 and LTP2. Members of the LTP2 family have a more flexible conformation allowing for a broader range of lipids to be carried where as members of the LTP1 family are hypothesized to be able to transport two lipids at a time in a tail-to-tail conformation within the hydrophobic tunnel (Yeats & Rose, 2008)Error! Reference source not found.. A distinction between these two families based on the number of amino acids separating the conserved cysteine was used in this project according to the article by Pons et. al.. In this article the LTPs are segregated between LTP family 1 and 2 according to the number of amino acids that separates the eight conserved cysteines. LTP family 1 structure is described as follow "C 9 C 13 CC 19 C 1 C 17 C 13 C" and LTP family 2 structure as "C 7 C 13 CC 8 C 1 C 23 C 6 C" the numbers representing the amount of amino acids between each conserved cysteine (Pons, de Lamotte, Gautier, & Delsuc, 2003).

All known LTPs are synthesized as pre-proteins and possess a signal peptide at their N-terminus (Auclair, Bhanu, & Kendall, 2012), but not an endoplasmic reticulum retention signal (KDEL), thus implying that the protein is bound for the secretory pathway and not to stay within the ER. The role of

LTPs as intra-cellular lipid transfer proteins thus seems unlikely. Moreover, upon observation LTPs were localised near the cell wall and secreted. The experimental evidence now points strongly towards a function in the defence mechanism of plants. LTPs were found to be in high concentration near the epidermis layer of new organs and to have unexpected anti-fungal and -bacterial properties. Furthermore LTP proteins seems to be induced by abscisic acid (ABA), which is a stress response hormone (Kader, 1997).

If the terpene diterpene sclareol is behaving like most, if not all, terpenoids it would indicate the involvement of ATP-binding cassette (ABC) transporters or even more specifically a pleiotropic drug resistance transporter (PDR) also called ABCG transporters. As for LTP, PDR function is not fully understood yet but it is known that they are most likely localised on the cell membrane and are composed of two transmembrane domains (TMD) and two nucleotide binding domain (NBD). Two clusters of PDR have been identified and characterised. Data on cluster I suggests a role in the defence mechanism of the plant whereas cluster II might be more involved in physiological function and more localised in the root system (Crouzet, Trombik, Fraysse, & Boutry, 2006) (Yazaki, 2006). A collaboration between LTPs and PDRs is a likely hypothesis for the transport of sesquiterpene lactones such as parthenolide and costunolide outside of the cell. PDR transporters might transport the complex of LTP with the terpene outside of the cell or the PDR transporter could transport he sesquiterpene lactone outside of the cell where a LTP would transport it further (**see Figure 3**). The secretion of the lipids could also involve vesicles since immature LTPs have a signal peptide. This does not exclude the possibility of a collaboration with a PDR transporter. It is known that the sesquiterpene lactones accumulate outside the trichomes as they can be extracted in chloroform without damaging the trichomes cell wall (Majdi et al., 2011).

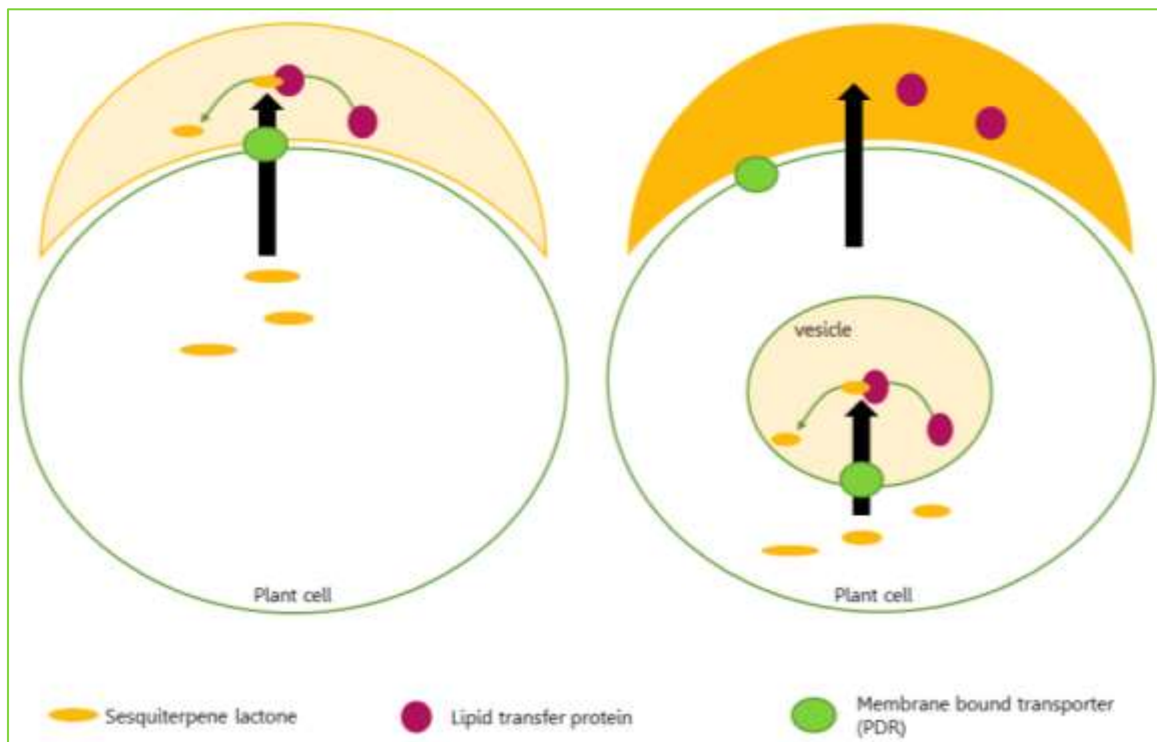

Figure 3 Scheme of the hypothesized transport mechanisms of the sesquiterpene lactones parthenolide and costunolide outside the cell.

The properties observed for LTPs and sesquiterpene lactone individually are very similar, both are claimed to be allergens, anti-fungal, anti-bacterial, and involved in defence mechanisms (Salcedo et al., 2004) (Rodriguez, Towers, & Mitchell, 1976). Moreover, it is suspected that the structure of LTPs without a lipid ligand is not very stable due to its hydrophobic tunnel (Holthuis & Levine, 2005), and

no release mechanism of the lipid by the LTP outside the cell has been found yet. All this leads to the question whether the LTPs and the lipids do part once they are outside the cell. Furthermore, LTPs are present in abundance (Arondel, Vergnolle, Cantrel, & Kader, 2000) which could confirm the hypothesis of a 'one use only' carrier.

In order to be able to increase the production of the medicinal compounds costunolide and parthenolide, the transport system of these lipids and precursors will be investigated. Our start hypothesis is that a single LTP facilitates the transport of parthenolide and its precursors to the apoplast, with the help of a PDR transporter. The eight individual LTPs from glandular trichomes of feverfew flowers were cloned into Agl-0 *Agrobacterium tumefaciens* strain. The individual influence of the eight LTP candidates on the concentration of costunolide and for some parthenolide has been researched. Furthermore combinations of LTPs was tested as well. The extraction of the proteins and metabolites in the apoplast was tested to confirm the secretion of LTPs and metabolites. The main goal was to understand the transport mechanism of parthenolide and costunolide better so as to be able to produce more of these compounds *in planta* for medicinal purposes. To achieve this objective feverfew LTP candidates were identified, the influence of individual and sets of LTPs on the production of costunolide and parthenolide was observed, as was the distribution of costunolide and its conjugates over the apoplast and the cell.

Material and methods

RACE-ready cDNA

First strand cDNA from our feverfew RNA is synthesised for 5′ and 3′ RACE reactions as well as a 5′ cDNA from the control mouse heart RNA. The 5′ feverfew cDNA reaction is prepared with 10µl RNA, and 1.0µl 5′-CDS Primer A in a PCR tube. The tube's content is mixed and spun down. The tube is incubates 3min at 72°C then cooled to 42°C for 2min in a PCR machine. The tube is then spun down to collect the content at the bottom. To just this reaction compared to the 3′end 1µl of SMARTer II A Oligonucleotide is added. 4.0µl of 5X First-Strand Buffer, 0.5µl of DTT (100mM), dNTPs (20mM), 0.5µl RNase Inhibitor (40U/µl) and 2µl of SMARTScribe Reverse Transcriptase (100U) is added to the previous mix. The content is mixed and spun down before incubating it at 42°C for 90min then heated at 70°C for 10min. The first-strand DNA is then diluted with 90µl Tricine-EDTA buffer and then stored at -20°C. The same reaction is made for the 3′ feverfew cDNA but with the start mix as follow: 11µl RNA, and 1.0µl 3′-CDS Primer A. The 1µl of SMARTer II A oligonucleotide is not required for this reaction but the following step are like the 5′ reaction. For the control the only difference with the 5′ feverfew cDNA reaction is that only 1µl of RNA is mixed with the 5′CDS-Primer A so 9µl of sterile H_2O is added.

Primer design for RACE PCR

Gene-Specific Primers for the RACE reaction are designed for the 5′and 3′of every LTP candidates. The primers are between 23 and 28 nucleotides long, have a GC ration between 50 and 70%, a Tm value above or equal to 70°C, are not complementary to the Universal Primer Mix and a 15bp overlap with the pRACE vector is added to their 5′ extremity (unless mentioned otherwise).

Rapid Amplification of cDNA Ends (RACE)

In a PCR tube, 2.5µl of 5′-or 3′-RACE Ready cDNA is mixed with 5µl of 10X UPM, 1µl of 5′or 3′ Gene Specific Primer (10µM), 15.5µl PCR-Grade H2O, 25.0µl 2X SeqAmp Buffer, and 1.0µl SeqAmp DNA Polymerase, to a total of 50µl. This reaction is made for both 5′and 3′ of every LTP candidate as well as the 5′ control mouse heart. Since every primers used have a Tm above 70°C the touchdown PCR program is used. It consists of 5 cycles at 94°C for 30sec followed by 72°C for 3 min, than 5 cycles at

94°C for 30sec, 70°C for 30sec ,and 72°C for 3min, and finally 25 cycles at 94°C for 30sec, 68°C for 30sec and 72°C for 3min.

Characterization of PCR product

10µl GelRed 6X Loading Buffer is added to the samples and the 60µl are loaded on a 1% agarose gel which is run for one hour at 120V. The position of the bands is located under UV light, a picture is taken and then the bands are cut out of the gel and put in separate 2ml tubes. 200µl NTI buffer is added for every 100mg agarose of the samples. The samples are incubated for 10min at 50°C and vortex every 2-3min until the gel is completely dissolved. 700µl of the sample is loaded into the provided spin column and centrifuged 30sec at 11,000g. The flow-through is discarded and the remaining of the sample is loaded and centrifuged. The column is washed twice with 700µl NT3 buffer and centrifuged once more for 1min at 11,000g. In order to get rid of the remaining ethanol the column is incubated 2-5min at 70°C with open lid. The column is then transferred to a new 1.5ml tube, 30µL of NE buffer is loaded directly onto the membrane and left to incubate 1min at room temperature. The PCR product is collected by centrifugation for 1min at 8,000g and the concentration measured with Nanodrop.

In-Fusion cloning of RACE product

7µl of the previously gel purified RACE product is added to 1µl linearized pRACE vector and 2µl In-Fusion HD master mix, on ice. For the control, 1µl pUC19 vector is added to 2µl control insert, 2µl In-Fusion HD mix, and 5µl water. The reactions are incubated at 50°C for 15min. Then, 2.5µl of this reaction is added carefully to a tube competent cells that is thawed on ice. This reaction is incubated 30min on ice, then heat-shock for 45sec at 42°C and again 2min on ice. 250µl of SOC medium is added to the cells which are then placed in the 37°C shaker for at least one hour. In the meantime LB plates are poured. 400ml LB agarose is liquefied in the microwave and then cooled down under water. 200µl ampicillin and 640 µl X-Gal is mixed into the LB medium which is then poured into 16 plates in the flow-cabinet. 20µl, 50µl, and 100µl of the transformed cell are plated per treatment and left to incubate at 37°C overnight. The following day individual colonies are picked, about 14 per treatment, and regrown in 10ml tubes with 5ml liquid LB medium with 5µl ampicillin. The Plasmid DNA is then isolated, with the help of a miniprep kit. 10 grown cultures are selected from the 14 that were picked. The cultures are spin down for 5min at 3,500rcf. The supernatant is discarded and the pellet resuspended in 250µl of buffer P1, containing RNase and lyse blue, and transferred to a 1.5ml tube. 250µl of P2 buffer is added and mixed gently by inverting the tube 4-6 times. Then, within 5 minutes, 350µl of N3 buffer is added and again mixed gently by inverting until the reaction solution loses its blue colour. The tube is centrifuged 10min at 13,000rpm. The supernatant is collected applied to a QIAprep spin column. The spin column is centrifuged 1min at 13,000rpm and the flow-through is discarded. The column is washed with 750µl PE buffer and centrifuged once more. The spin column is then placed in a new 1.5ml tube, 50µl demi water is pipetted directly onto the membrane and incubated for 1min at room temperature to elute the DNA. The plasmid is then collected by centrifuging 1min at 8,000rpm and the concentration is measured with Nanodrop.

Control the presence and length of the insert

An enzyme digestion is realised on the isolated plasmid. 1000ng of plasmid DNA is treated with 1µl EcoRI and 1µl HindIII with 3µl of the appropriate 10xbuffer, here CutSmart buffer (for transformants in Topo vector EcoRI can be used alone). The volume of the reaction is then brought up to 30µl with MQ water. The reaction is left to incubate at 37°C for 1-2 hours. The samples are then loaded onto a 1.5% agarose gel which is run for 1hour at 120V. Pictures of the gel are taken under UV light. Colonies which show the right band are then sent for sequencing with the appropriate M13 primer, or specific primer. Another way used to check the presence and length of the insert is to perform a 25µl

Q5 PCR with M13 primers with 57°C as anneal temperature and isolated plasmid DNA (see Q5 High-Fidelity PCR protocol).

cDNA synthesis

In a PCR tube, 4µl of 5x iScript reaction mix is added to 15µl RNA (feverfew 04-1), and 1µl iScript reverse transcriptase. The reaction is incubated in a PCR machine for 5min at 25°C, 30min at 42°C and finally 5min at 85°C.

Q5 High-Fidelity PCR

In a PCR tube, 10µl of 5xQ5 reaction buffer is mixed with 1µl dNTPs (10mM), 2.5µl M13 forward primer (10µM), 2.5µl reverse primer (10µM), 5µl cDNA, 0. 5µl Q5 High-Fidelity DNA polymerase, and 28.5µl nuclease free water. The program used is dependent on the calculated anneal temperature (NEB Tm calculator) and the expected length of the PCR product. The program consists of a first hold at 98°C for 30sec, followed by 35 cycles. The cycles are as follow: 98°C for 10sec, 30sec at the calculated anneal temperature, and the amount of time needed for the length of the fragment, 20-30sec/kb at 72°C. These cycles are followed by a hold at 72°C for 2min and is then kept at 10°C until the samples are taken out of the PCR machine.

pCR-8-Topo cloning

Before cloning the PCR product an A overhang must be added. For this reaction 5µl of the cloning fragment is mixed with 1µl dATP (10mM), 1µl supertaq buffer, 0.3µl $MgCl_2$ (50mM), 2.6µl nuclease free water, and 0.1µl supertaq. This reaction is incubated 30min at 72°C. Then 4µl of this reaction is added to 1µl salt solution and 0.5µl Topo vector, and left 5min at room temperature. Meanwhile, DH5ɑ competent cells are thawed on ice to which 2µl of the previous reaction is added carefully. The cells are left on ice for 30min, the heat-shock is at 42°C for 30sec, then the cells are placed back on ice for 2min before adding 250µl SOC medium. These cells are then placed in a 37°C shaker for at least 1hour. Meanwhile LB plates with spectinomycin 50 are poured and left to solidify in the flow cabinet. 20µl, 50µl, and 100µl of the cell suspension is then plated and left to incubate overnight at 37°C. The following day cultures are picked are regrown following the same procedure as for pRACE cloning, but with spectinomycin 50 as antibiotic. The plasmid DNA is also isolated like the In-Fusion for the only difference is the back-up of 0.5ml taken before spinning down the liquid culture to be able to make glycerol stocks of the positive colonies. The presence and length of the insert provides insight to which cultures may be positive before sequencing.

Linearizing the plasmid

In order to prepare the samples for the LR reaction the E.coli plasmid has to be linearized. This is achieved by digesting the plasmid with HincII restriction enzyme. This digestion is then run on a gel for more than 1hour and post stained with GelRed for 1hour. A picture of the gel is taken under UV light to localize the cut fragment which should run slower than the uncut plasmid. The cut plasmid is then extracted from the gel.

LR reaction

In a PCR tube, up to 3.5µl (75ng) of the entry clone is mixed with 0.5µl (75ng) of destination vector B7WG2 and the volume of the solution is brought up to 4µl with TE buffer pH 8.0. LR clonase II enzyme mix is thawed 2 min on ice and vortex twice briefly before adding 1µl of it to the previous reaction. The reaction is incubated in a PCR machine for 1hour at 25°C. 0.5µl of Proteinase K is added to stop the reaction and the tube is placed at 37°C for 10min. This reaction is then used to transform E. coli cells like the Topo transformation. The bacteria is plated on plates of LB medium with spectinomycin 50 and streptomycin 50 and kept overnight at 37°C. The next day colonies are picked and regrown in 10ml tubes with 5ml LB medium with spectinomycin 50 and streptomycin 50, they are

kept overnight in a 37°C shaker. The next day the plasmid DNA of the colonies is isolated and samples are send for sequencing with attB primer.

Agrobacterium transformation

1µl of isolated plasmid of *E.coli* with the LR reaction product is pipetted inside a 1.5 ml tube of 50µl thawed electro-competent *Agrobacterium tumefaciens* cells. The reaction is incubated 30min on ice, then the cells are electroporated. The electroporation machine is set on 200 Ohms and 1.8 Volts. The cells are transferred to a clean, cool, and dry electroporation cuvette. The cuvette is placed in the machine and both buttons are pushed until the sound signal. 500µl SOC medium is added in the cuvette and pipetted up and down to mix. The entirety of the liquid is then extracted from the cuvette and transferred back to the 1.5ml tube. The cells are incubated for one hour at 28°C. 25µl of the culture is plated on LB plates with rifampicin and spectinomycin. The rest of the culture is spun down for 1min at 4000rpm. 350µl of the medium is discarded and the pellet is resuspended in the rest of the medium Therefrom 20 and 50µl is plated. The plates are kept at 28°C, after 2 days 3 colonies are picked and regrown in 5ml liquid LB with the same antibiotics as the plates for 2 days in a 28°C shaker. Glycerol stocks are made and kept at -80°C.

Agroinfiltration

Two days before the infiltration, the plants required for the infiltration are moved to the GMO compartment and signs are put up not to water the plants so that the infiltration will be easier. Furthermore, the desired transformed Agrobacterium tumefaciens colonies are regrown in 50ml tubes with 5ml LB medium with the appropriate antibiotics by breaking a piece of frozen culture. Our colonies with the LTPs construct have rifampicin and spectinomycin resistance while the colonies with the feverfew pathway genes (HMGR, GAS, GAO, COS, PTS), empty vector, and p19 have rifampicin and kanamycin resistance. The cultures are kept overnight in an incubator at 28°C. One day before the infiltration the growth of the colonies is checked and 5ml LB medium with the appropriate antibiotics is added to the cultures. If needed new liquid cultures are made, with a bigger piece of original culture. The cultures are once again kept at 28°C overnight.

On the day of the infiltration the cultures are spun down for 15min at 3,500rcf. In the meantime, agroinfiltration buffer is prepared by adding 20ml $MgCl2$ (500mM) with 20ml MES-KOH (500mM) and 1ml acetosyringone (100mM), the volume is then increase to 1l with demi water. The supernatant LB medium is discarded in GMO waste and the pellet is resuspended in agroinfiltration buffer with plastic 1ml pipets. The volume of each culture is then brought up to 25ml with buffer. The OD600 of the cultures is measured and adjusted to 0.5 by adding buffer. Then the treatment mixture of multiple agrobacterium colonies is made following the agroinfiltration scheme prepared in advance, there should be enough for at least four repetitions. Once the treatment mixes are made in 50ml tubes, the tubes are placed on a roller bench for 2-4hours.

Plants are infiltrated in the greenhouse, the fourth youngest fully developed leaf is infiltrated via a 1ml syringe without needle. The syringe full of treatment mix is applied to the underside of the leaf while applying pressure with a finger on the other side, the plunger is slowly pushed until the liquid does not enter the leaf anymore. The whole leaf is infiltrated with as few infiltration points as possible. The plants are watered after the infiltration without splashing the leaves and the signs to not water them are put down.

On the harvest day pieces of aluminium foil with the treatment and repetition numbers are prepared. A tank a liquid nitrogen is brought to the greenhouse to freeze the leaves immediately. The leaves are harvested individually folded in four and then placed in the appropriate aluminium foil and placed in the liquid nitrogen. Back in the laboratory, the leaves are individually grinded with cooled mortar and pillar to fine powder which is then placed into storage vials which are then stored at -80°C.

Measuring on the triple-quad

50mg of each sample is measured and placed in a clean 1.5ml tube. 300µl of methanol and 50µl of water are added in the fume hood. The samples are vortexed and put in a sonification bath for 15min. The samples are then centrifuged at maximum speed for 15 min. The 200µl of the supernatant is pipetted into a syringe which is fixed to a filter. The liquid is push through the filter into a labelled collection tube. The collection tubes are closed and kept at -20°C until the measurement are made with the triple-quad LC-MS.

Apoplast wash

Leaves from which the apoplast content will be extract are harvested and kept in a petri dish with humid paper towels. The leaves are cut in half along the mid-nerve which is then discarded. The half leaves are cut along their edges as well to facilitate the extraction. The half leaves are washed with demi water to remove any soil or plant sap remaining on the leaves. The half leaves are weighted individually. The half leaves are then placed in beakers according to their treatment and a mortar is placed on top of them so that they will not float when the liquid is added. To measure the metabolite content demi water is used to wash the apoplast, but to run a protein gel a sodium phosphate buffer (0.1M) is used. In both cases the liquid is poured in the beakers until the leaves are completely covered. The beakers are placed in a vacuum chamber and vacuum is applied for 20 min and then slowly released. The half leaves are dried and weighted again. The half leaves are placed in the apoplast wash tube which is then placed in a 50ml tube to collect the apoplast liquid and centrifuged for 15min at 400xg. The half leaves are weighted as well as the liquid collected. The apoplast wash samples are filtered and collected in a 1.5ml tube and 100µl is pipetted into a collection tube to be measured with the triple –quad LC-MS. The half leaves are frozen in liquid nitrogen, grinded and extracted as mentioned previously.

Data processing

The data obtained from for the metabolites is a quantification of the area under the peak from the LC-MS, it is not a direct volume or concentration of the metabolites. The ratio between the three channel results from the Triple-Quad LC-MS, was calculated to check for the reliability of the results. The channel chosen for further analysis was the one with the most consistent ratios and the highest values. The same channels were chosen for the different experiments that were compared. The significance between treatments was calculated with a student t-test based on a two tails distribution and equal variance. Normalisation to each experiment's control was calculated in order to compare multiple experiments with each other. For the data from the apoplast wash experiment some corrections had to be made in regard of leaf size and apoplast volume. This data processing can be found in the appendix.

Results

Characterization of the LTPs

The LTPs of interest for the costunolide and parthenolide pathway were identified by analysing the expression patterns of the genes present in trichomes that were extracted and processed in a previous project. The expression pattern of the candidate LTPs was compared to the expression of the parthenolide pathway genes with GeneMath. Candidate LTPs were identified with the help of the gene library available on the terpmed website. These selections resulted in 13 candidate LTPs. From this list the candidates were screened for the characteristic LTP structure with 8 conserved cysteine. From this analysis 9 candidates remained, one candidate was dismissed at a further stage since its contig was missing a start codon and multiple RACE PCR still did not reveal the presence of a start codon.

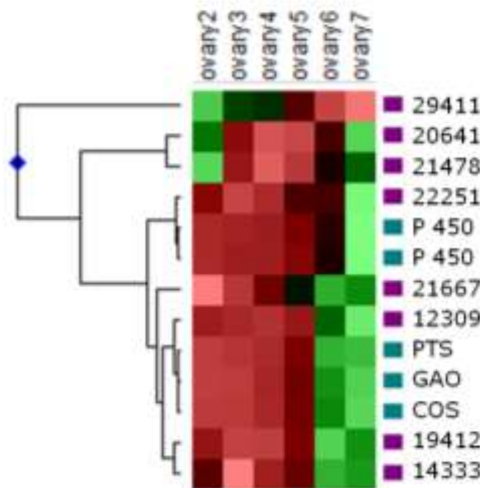

Figure 4 Expression pattern from the feverfew LTP candidates and parthenolide pathway genes from different ovary stages.

From the expression data, LTPs 20641, 21478 and 29411 were found to have a late expression compared to the pathway genes. LTPs 12309, 14333, 19412, 21667, and 22251 are expressed in a similar pattern than the parthenolide pathway genes (**see Figure 4**). In order to get a better sense of the particularities of the selected LTPs multiple analysis were performed. A phylogenetic tree of the selected feverfew LTPs and known plant LTPs from NCBI database was hypothesized with CLC workbench. In this phylogenetic tree, the eight feverfew LTPs were segregated between two main ancestors. 21667, 12309, and 14333 were hypothesized to share one ancestor whereas the other five LTPs would share a different and also relatively closer ancestor (**see Figure 5**). This segregation was further revealed when searching the LTP sequences for a transmembrane domain, the three aforementioned LTPs do have a transmembrane domain whereas the five remaining LTPs do not.

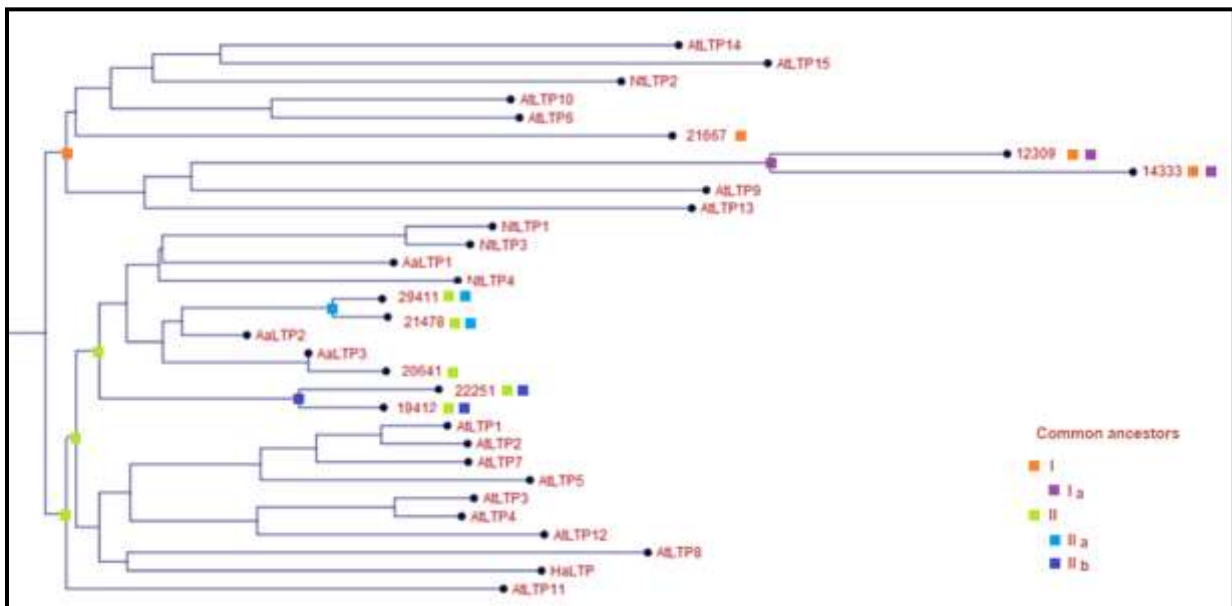

Figure 5 Phylogenic tree of multiple plant LTPs. The common ancestors between the different feverfew LTP candidates are indicated by a colored square. The phylogenic tree was produced with the CLC workbench software.

Two families of LTPs are described in the introduction. Four of the selected feverfew LTPs fit perfectly the description of the LTP family 1: 19412, 22251, 20641, and 21478. 21667 structure is closely similar to the description of the LTP family 2 structure but the three remaining feverfew LTP candidates do not relate to either family (**see Figure 6**). The three remaining LTP candidates are

12309, 14333, and 29411. 12309 was found to have a high similarity with hybrid proline-rich proteins and 14333 was found to be xylogen like. These results explain why these two LTPs do not fit in the two families described previously as well as their unusual length. Following the typical LTP structure all the candidates have a signal peptide which cleaves the first 21 to 28 amino acids. However for 29411, the two first conserved cysteine are situated before the cleaving site of the signal peptide which is an irregularity compared to the "classic" LTP structure. Despite their signal peptide for secretion only 14333 and 21667 have N-glycosylation sites (N-$X_{(\text{not P})}$- S or T), 3 and 1 respectively. No O-glycosylation sites (O-S or T) were found.

The findings from the multiple bioinformatics analysis are summarised in **Table 1** with also the new numbering of the LTP candidates based on that information. The new LTP numbering will be used in the remaining of the report.

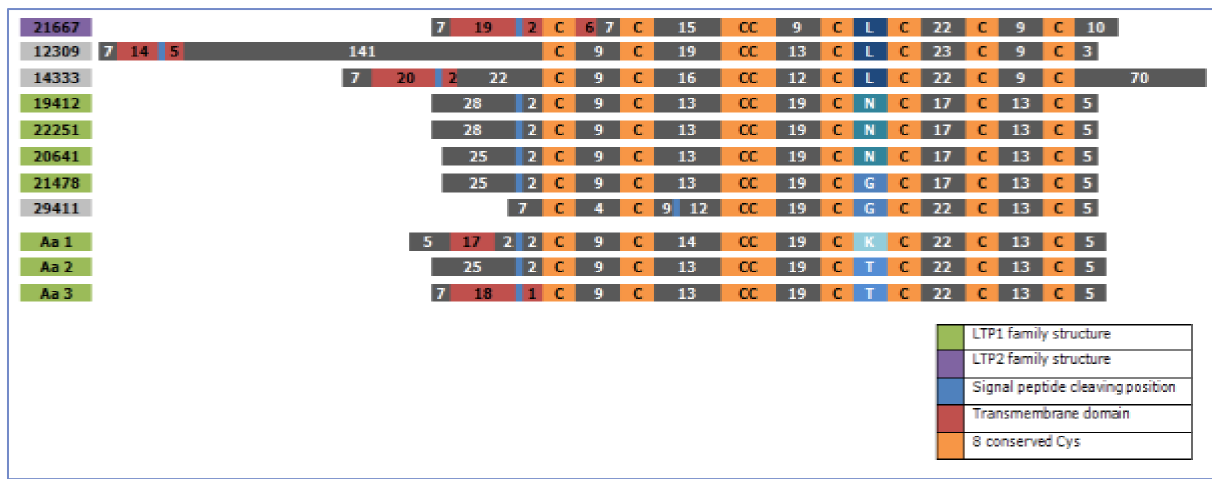

Figure 6 Alignment of the feverfew LTP candidates according to the conserved cysteine positions and three artemisia LTPs. The letter between the two conserved cysteines represents the single amino acid between them, the numbers represents the amount of amino acids between two cysteines.

Table 1 Summary from the bioinformatics analysis of the eight LTP candidates. These data from the aforementioned analysis were summarised in this table .

LTP	contig #	Common ancestor	expression pattern	LTP length (nucleotides)	signal peptide	transmembrane domain	Class or family
1	21667	I	simultaneous	336	yes	yes	LTP 2 family
2	12309	I_a	simultaneous	756	yes	yes	Proline-rich like
3	14333	I_a	simultaneous	594	yes	yes	Xylogen like
4	19412	II_b	simultaneous	360	yes	no	LTP 1 family
5	22251	II_b	simultaneous	360	yes	no	LTP 1 family
6	20641	II	late	351	yes	no	LTP 1 family
7	21748	II_a	late	351	yes	no	LTP 1 family
8	29411	II_a	late	303	yes	no	?

Expression of the parthenolide pathway and three LTP candidates

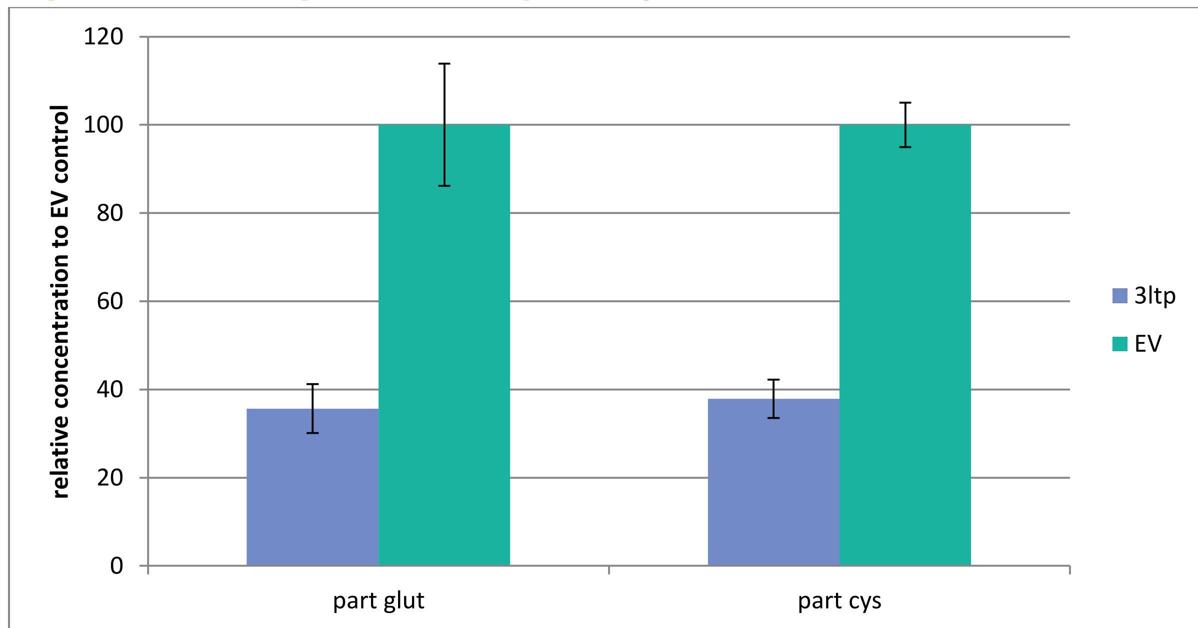

Figure 7 Relative concentration of parthenolide conjugates with the expression of 3 LTPs and the parthenolide pathway compared to the control without LTPs .

An experiment where the parthenolide pathway (PTS PW) was expressed was repeated three times. In these experiments a mix of LTPs 1, 3, and 4 was used, these were the first LTP genes to be successfully transformed in Agl-0. From these experiments we can observe that for both parthenolide conjugates only 35% and 37% was produced compared to the control. The difference in the parthenolide production is significant for both conjugates (**Figure 7**). Unfortunately free parthenolide is present in such low quantities that it can not be reliably measured. However it does seem that there is more free parthenolide when its pathway is expressed simultaneously to the three LTPs.

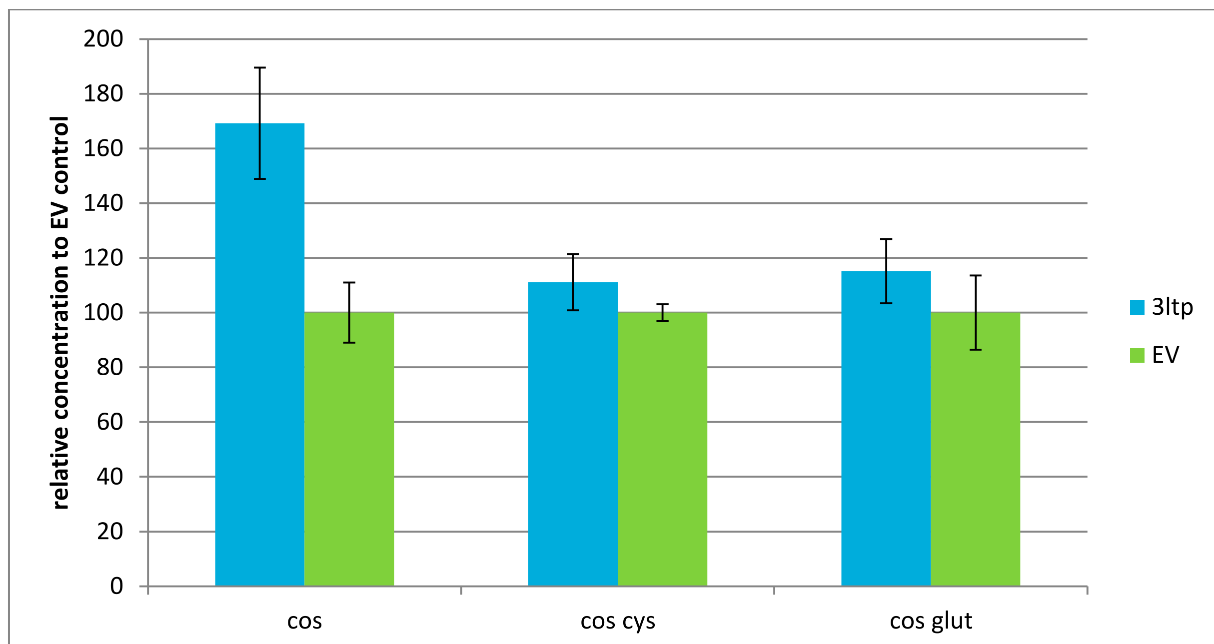

Figure 8 Relative concentration of costunolide and its conjugates with the expression of 3 LTPs and the parthenolide pathway compared to the control without LTPs .

For the same samples, from leaves in which the parthenolide pathway was expressed as well as a mix of LTPs 1, 3, and 4, the content of costunolide and its conjugates was also measured. This data showed that there was significantly more free costunolide (170%) when the LTPs were co-expressed than in the control. Both conjugates levels were also higher (about 111%) than the control although not significantly (**Figure 8**).

Expression of the costunolide pathway with the eight LTP candidates

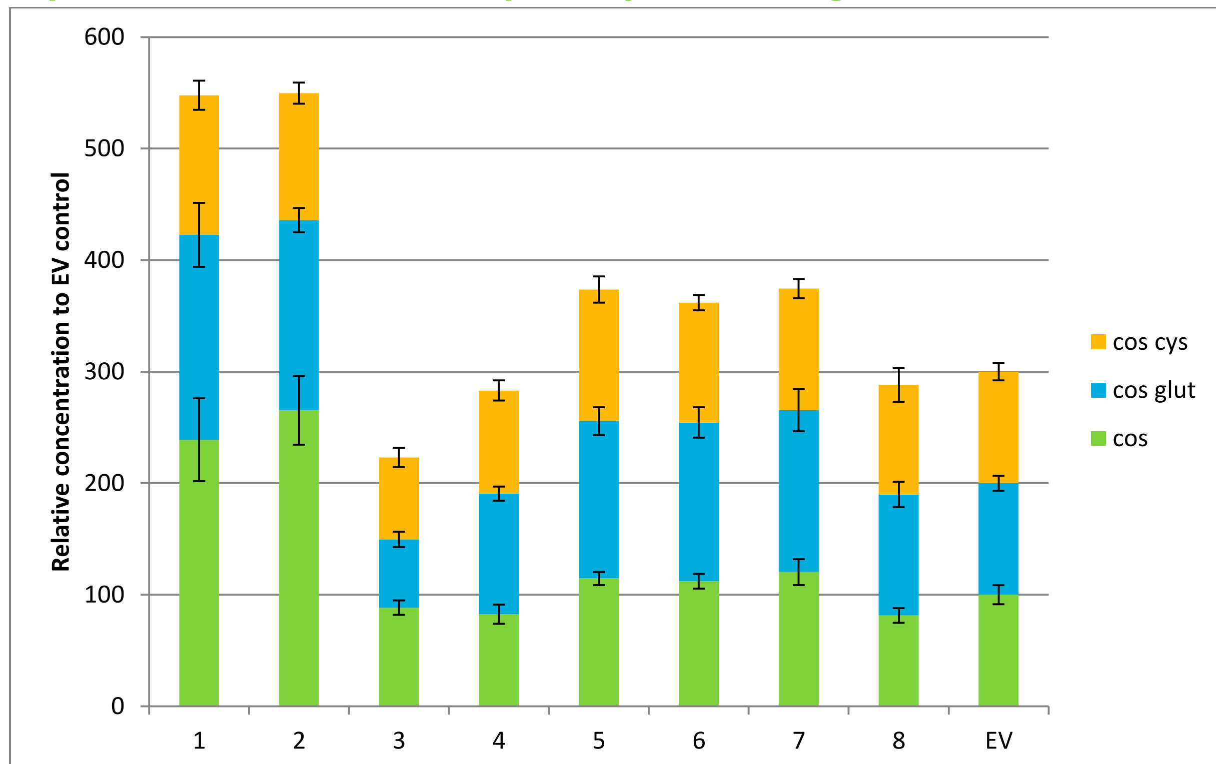

Figure 9 Relative concentration of costunolide and its conjugates with the expression of each individual LTP candidate and the costunolide pathway compared to the control without LTP .

In this experiment the costunolide pathway was co-expressed with each LTP candidate individually and a control with empty vector (EV), this experiment was repeated twice and the data were combined. LTPs 1 and 2 showed the highest increase in production of costunolide and its conjugates compared to the control, the difference for costunolide and glutathione was significant. LTPs 3, 4, 8 showed a decrease in the production of costunolide and its conjugates compared to that of the control. LTPs 5, 6 and 7 showed a slight increase compared to EV but is was not significant for any compound.

LTPs 1 and 2 have a significantly increased content of free costunolide compared to the control, more than 250%. The same can be said about costunolide glutathione but to a lesser extend as the content is there about 180% of the control. LTPs 5, 6 and 7 show have similar yields, their free costunolide content is about 115% and their costunolide glutathione is about 140% of the control. LTP 4 and 8 have a very similar free costunolide (about 81%) and costunolide glutathione (about 108%) content then the control, whereas LTP 3 has a lower free costunolide content of 88 % and a significantly lower costunolide glutathione content of 61% compared to the control. The amount of costunolide cysteine was rather unchanged throughout the different LTP treatments the only significant difference to be found was between different LTPs and LTP 3 (**Figure 9**).

Determination of the apoplast costunolide content with the expression of the costunolide pathway

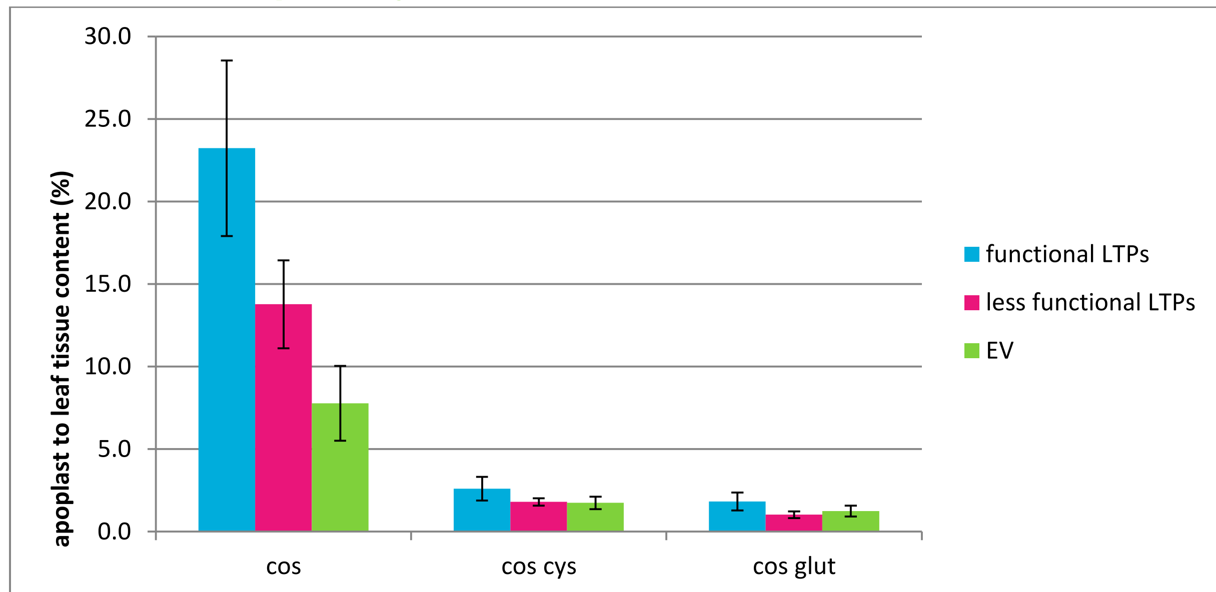

Figure 10 Percentage costunolide and its conjugates present in the apoplast compared to the content remaining in the leaf tissue.

From the experiment where the apoplast content was extracted the following data was obtained after correcting for the leaf weight and apoplast volume, as mentioned in material and method part. In this experiment the two functional LTPs used were 1 and 2, and the two less functional LTPs were 7 and 8. The data shows that more free costunolide was present in the apoplast with the functional LTPs (23.2%) than with the less functional LTPs (13.8%) and the control (7.8%). The percentage of both conjugates in the apoplast was very low no more than 2.6% for costunolide cysteine and no more than 1.8% for costunolide glutathione. However for both conjugates the higher percentage in the apoplast was obtained with the functional LTPs (**Figure 10**). The data from this experiment can not be compared to others since it was performed without p19 and harvested 5 days after the infiltration due to technical hindrances, furthermore this is based on the total amount of costunolide per milligram fresh weight whereas other experiment results are based on an extraction of a sample of the leaves.

Discussion

The goal of this project was to find feverfew LTPs involved in the transport of costunolide and parthenolide and to characterize them. To this end eight LTP candidates were identified and analysed to be able to understand and explain possible deviation in biological experiments. This analysis revealed that three LTPs had a late expression pattern compared to the parthenolide pathway genes. Three LTPs had a transmembrane domain, and two were longer than the classical LTP structure described in literature, revealing the variety of LTPs present in feverfew trichomes. Biological assays in *Nicotiana benthamiana*, in which the parthenolide or costunolide pathway was expressed transiently alongside the LTP candidates were performed. These assays revealed the increase in yield of costunolide and its conjugates with some LTPs and a slight decrease with others compared to the control with EV. This was not found back with parthenolide conjugates which implies a specificity of the LTP candidates.

Parthenolide pathway expression

The parthenolide pathway was infiltrated in *Nicotiana benthamiana* alongside a mix of three LTPs, LTP 1, 3 and 4. Measuring the amount of free parthenolide was very difficult both with triple-quad and orbi-trap LC-MS, the concentration was too low to have trustworthy results. However it seems like the presence of the LTPs boosted the yield of free parthenolide compared to the control (data not shown). This low concentration could be explain by the early harvest date which was necessary since the combination of HMGR and LTP 3 starts causing necrosis three days after infiltration (data not shown). The build-up of free parthenolide may be measurable a few days later if LTP 3 is not present in the experiment. HMGR is essential for the experiment as it was shown to boost the production of free costunolide up to 7-fold and its conjugates up to 20-fold (Liu et al., 2014). However both conjugates were in high enough concentrations to be measured and analysed since more than 90% of parthenolide is conjugated (Liu et al., 2014). Both conjugates' yields were decreased with the LTPs compared to the control, it implies a specificity of the used LTPs towards the free form of parthenolide and not its conjugates. It could be that the three LTP used here (LTP 1, 3, and 4) are not compatible with the parthenolide conjugates. The other LTP candidates may have a better compatibility with parthenolide conjugates, which then would increase the production of parthenolide in its conjugated and free form. Also waiting 5 days after the infiltration to harvest could increase the yield of the total parthenolide.

The relative costunolide yields were increased with the LTP mix compared to the control. The free costunolide level was significantly increased with the LTPs but it was in relatively low amounts compared to its conjugates and probably only represents less than 10% of the total amount of costunolide like for parthenolide. Contrary to the parthenolide conjugates, the costunolide conjugates yields were increased compared to the control. This increase though not significant implies that the costunolide conjugates are compatible with the feverfew LTPs expressed. The increase in relative costunolide yield indicates that a part of costunolide is exported outside the cell which means that this part of the costunolide product is not available for parthenolide synthesis anymore which would explain the lower relative yields in parthenolide conjugates. The increase in free parthenolide observed could be the result of its direct secretion before it had time to be conjugated.

Costunolide pathway expression

The costunolide pathway was expressed in *Nicotiana benthamiana* alongside the eight individual LTP candidates and EV as a control. Two LTPs, LTP 1 and 2, showed an increase in costunolide under its free and conjugated form, they have very similar total costunolide relative yield but LTP 1 has slightly more costunolide conjugates than LTP 2 that has more free costunolide. LTP 4, and 8 showed a decrease in costunolide compared to the control, they did not show any difference in the conjugates to free costunolide ratio. LTP 5, 6, and 7, showed a slight increase in costunolide compared to the control, but again they did not show any difference in the conjugate to free costunolide ratio. LTP 3 resulted in a steeper decrease in costunolide yields than LTP 4 and 5, it is mainly due to a relative low conjugate yields, the case of LTP 3 is discussed further in another paragraph. In every treatment the relative amount of costunolide cysteine conjugate was relatively unchanged, this could be explained by the fact that the conjugation to cysteine is irreversible and in an experiment with parthenolide it represented 94% of the total parthenolide content (Liu et al., 2014) but otherwise little is known about cysteine conjugation. However the relative yields of costunolide glutathione were more diverse and often even significantly different between treatments.

During the secretion assay, it appeared that mostly free costunolide is being transported outside of the cell. The secreted percentage of free costunolide was three fold higher with the mix of functional LTPs than without LTPs. The percentage free costunolide with the less functional LTPs was twice as high as the control. This implies that even with a low compatibility the LTP candidates still transport

some free costunolide outside the cells. The percentages of conjugates transported out of the cell were a little increased but it remain low nonetheless. This confirms that the increase in relative yield of free costunolide is due to its transport outside the cell by the LTPs . It also explains why the relative yields of the conjugates had not any consequential differences between LTP candidate treatments and control. As the total amount of costunolide was calculated in the secretion experiment the results are more practical to analyse and conclusions can be drawn, which is not really the case with the relative yields used in other experiments. A quantification of the peak area from the LC-MS chromatograms is not precise enough to make any concrete conclusions, therefore in future experiment a scale of standard concentration should be measured to make a calibration curve so as to be able to really measure the concentration of costunolide and parthenolide (Majdi et al., 2011).

LTP 3 paradox

Experiments with LTP 3 gave confusing results, in the first repeat with parthenolide pathway expression, LTP 3 seem to have a positive effect on the yield of free costunolide, this confirmed two repeats of this experiment done previously. The increase in free costunolide yield was even higher than with LTP 1. However in the last repeat the yield of free costunolide with LTP 3 was worse than that of the control. Similarly when the first repeat with the costunolide pathway was performed the levels of free costunolide was lower than the control. An explanation for this unexpected result could have been the loss of quality of the stock of Agrobacterium, to remedy this a new transformation into Agrobacterium was performed using the same E.coli plasmid that was used previously. However, when using the new culture the same low free costunolide relative yield was obtained again. The E.coli plasmid was send for sequencing again and came back fully positive, so the fault was not the loss of the gene in the E.coli plasmid with the destination vector. The loss of LTP-like effect can not be explain thus far, but it appears to be more complex than the loss of the gene or a mutation.

Furthermore, leaves treated with LTP 3 and costunolide of parthenolide pathway, always showed early signs of necrosis independent of the costunolide yields. LTP 3 was the only LTP to induce necrosis already three days after the infiltration. This is the reason why all the leaves were harvested three days after the infiltration unless P19 was not used. LTP 3 clearly had an effect on the leaves when co-expressed with HMGR (data not shown) which was used as a booster for the costunolide and parthenolide production. An explanation could reside in the nature of LTP 3, it was found to be very similar to the xylogen type proteins. Xylogen proteins are extracellular arabinogalactan (AGP) proteins first found in *Zinnia elegans,* they appear to be involved in xylem differentiation though little is known about these proteins yet. AGPs are known for their wide variety in functions and structure. Xylogen proteins are a chimeric AGP containing a LTP domain. Xylogen structure is composed of a signal peptide, a non-specific LTP domain, one or more AGP domains and a glycosylphosphatidylinositol (GPI) anchored sequence (Kobayashi, Motose, Iwamoto, & Fukuda, 2011). The AGP domains consist essentially of amino acids alanine, serine, proline, threonine and glycine, which are all abundant in the LTP 3 sequence. The AGP domain also implies a rich glycosylation (Ma, Ma, Zhao, Qi, & Zhao, 2014). All of these features can be found back in LTP 3 except for the GPI anchor, so LTP 3 could also be another kind of proteins related to xylogen type proteins. A LTP involved in transporting a hydrophobic compound outside the cell has been described that has a GPI anchor (DeBono et al., 2009) called LTPG. This LTPG probably belongs to the xylogen family thus being a member of this family does not mean that LTP 3 can not be involved in costunolide transport.

Characterization of LTPs

The most important features of an LTP are the eight conserved cysteine residues which maintain the peculiar hydrophobic tunnel (Kader, 1997). Based on the position of these cysteine residues an alignment of the feverfew LTP candidates was made. This alignment revealed the diversity in structure of the LTP candidates. Two of the candidates were found to be longer than the "usual" LTP length of

100 amino acids. One of these candidates was LTP 3 which as discussed previously may be a xylogen type protein. The other was LTP 2 which was found to be a hybrid proline rich proteins (HyPRP). HyPRPs are a very large family of putative cell wall proteins. Their N-terminal domain even though very variable contains repetitive and proline rich domains and their C-terminal domain has the typical cysteine residue pattern of LTPs (Dvořáková, Srba, Opatrny, & Fischer, 2012). This LTP 2 was actually one of two best performing LTPs in this research.

The other best performing LTP was LTP 1 which was identified as an LTP from the LTP family 2. Only one of our eight candidates corresponded to the structural description of the LTP family 2, four candidates were identified as members of the LTP family 1. The families diverge in the eight cysteine residue motif, different numbers of amino acids separate the conserved cysteine residues which ultimately results in a different conformation of the α-helices that composes the hydrophobic tunnel. LTP family 2 was found to be composed of five α-helices instead of the four described for the LTP family 1 members, however the size of the hydrophobic tunnel was found to be similar in both families they only have another conformation (Pons et al., 2003). However the distinction between LTP from family 1 or 2 may not be the explanation of the improved costunolide and parthenolide transport. In a similar research on artemisinin transport, a similar sesquiterpene, the most effective LTP was identified to be *Artemisia annua* LTP 3 (aaLTP3) (unpublished data). This LTP is a member of the LTP 1 family, but like our feverfew LTP candidate 1, 2 and 3 it has a transmembrane domain at its N-terminal. The presence of the transmembrane may not be significant since it is before the cleaving site thus not present in mature LTPs. However, from the phylogenic tree it appears that aaLTP3 is most similar to feverfew LTP 6. LTP 6 did show a slight increase in costunolide yield but is was not has effective than LTP 1 and 2.

One feverfew LTP candidate did not fit any description found, LTP 8. LTP 8 differs from the description of both LTP families and it was not found to be similar to another defined class of proteins yet. The distance between the cysteine 1 and 2 is too small and the distance between cysteine 2 and 3 is too large, which would indicate that the second cysteine residue shifted from the usual position. Furthermore, LTP 8 lacks a signal peptides cleavage site before the conserved eight cysteine residue motif, one can be found between cysteine 2 and 3 which would break the LTP conformation. One explanation could be that LTP 8 is meant for a cytoplasmic function, like the intracellular transport of lipids. The diversity of LTPs should be further analysed as it may hint to their function.

Functions of LTPs

We separated the LTP candidates in two categories according to their expression pattern compared to that of the parthenolide pathway genes in either simultaneous or late. This segregation was made based on the hypothesis that LTPs with a different expression pattern would have a different function. From this analysis we can see that the two most effective LTPs ,LTP 1 and 2, share a similar expression pattern than the parthenolide pathway genes. LTP 2 has the most similar expression pattern followed by LTP 3 and 4, which were not effective, and then LTP 1. Although two not effective LTPs share a similar expression pattern as well, it appears that the LTPs in charge of the transport of costunolide and parthenolide are expressed simultaneously to the pathway genes. The LTP candidates that were not found to be effective with costunolide and parthenolide probably transport another lipid out of the trichomes that has a similar expression pattern or have completely different purposes like in plant defence mechanisms like wax formation (García-Olmedo, Molina, Segura, & Moreno, 1995) (Pyee, Yu, & Kolattukudy, 1994).

In this research we proved that LTPs have an influence on the yield of costunolide, parthenolide and their conjugates. We were able to demonstrate that the presence of LTPs increased the amount of costunolide secreted outside the cell though we couldn’t find an answer to how the LTPs transport it outside the cell. Since none of our candidate LTPs are membrane bound when mature, they are free

to disperse once they are secreted which would not be the most efficient use of resources. The transport of LTP therefore probably happens in the vesicles where the LTPs are confined. LTPs are reported to be present in high numbers (Kader, 1997) which could indicate that they are a one use only transporter and then disperse into the apoplast. On the other hand, in this case were the sesquiterpene lactone is secreted to the surface of the glandular trichomes, the LTP once secreted will be on the surface of the trichome cell wall, the LTPs being small enough to pass through the cell wall pores (DeBono et al., 2009). LTPs are known to be a main component of wax layers (Pyee et al., 1994) and present in high quantities on fruit skin (García-Olmedo et al., 1995), so since there would be minimum diffusion, the secreted LTPs could transport more than one sesquiterpene before degrading.

Future prospects

The secretion of costunolide by LTP 1 and 2 has been proven, however the most effective LTPs for parthenolide have not been found yet. Therefore, an assay with the co-expression of individual LTPs and the parthenolide pathway should be performed as well as a secretion assay. In this project it appeared that one of the most effective LTP is not just a LTP but a hybrid proline rich protein as well, this either indicated that the LTP family is broader and more complex than expected or that other LTP-like proteins also have a role in transport of hydrophobic compounds.

The conjugation of both costunolide and parthenolide should be researched to better understand where and when it happens, furthermore little is known about the cysteine conjugates, our hypothesis is that it is a degraded form of glutathione conjugates. The biological significance of the conjugates should also be further researched, though they have been reported to have a decreased activity on cancer cells, since they are more stable and easily produced than the free form parthenolide (Liu et al., 2014).

The way LTPs transport the hydrophobic compounds out of the cell still has not been discovered. The involvement of membrane bound carriers could be tested repeating these experiments and co-expressing feverfew trichome membrane bound carriers, especially PDR. The localization of the LTPs could be analysed by fusing a fluorescent tag to the LTP gene and observing their location over a certain time span, which could indicate whether vesicles are involved or not. The specificity of the LTPs could be tested further by co-expressing them with another sesquiterpene pathway such as the artemisinin pathway. Another approach to the problem could be to analyse the lipid layer on top of the glandular trichomes of feverfew flowers. This layer, if one of our hypothesis is true, should contain the specific parthenolide LTPs in high concentrations which could show on a protein gel. Puzzling out how the LTP transport hydrophobic compounds outside the cell may also give an insight to the broad and diverse protein group that share the peculiar eight cysteine residue motif.

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Appendix

Processing of the data from the apoplast wash experiment:

LTP treatment	replicate	Weight Before Infiltration (mg)	Weight After Infiltration (mg)	Weight After Centrifuge (mg)	Liquid Volume (ul)	apoplast volume	LC MS data apoplast samples		total apoplast content	extracted apoplast	apoplast content still in leaf	LC MS data leaf samples		content in full leaf	not apoplast content	% in apo
fuctional LTPs (1 and 2)	1	453	659	534	66	206	cos	47608.95	980744.3	314219	666525.2	cos	14266.07	5332657	4666132	21.01836
							cos cys	779876.9	16065465	5147188	10918277	cos cys	2507693	9.37E+08	9.26E+08	1.734075
							cos glut	326364.1	6723100	2154003	4569097	cos glut	1203821	4.5E+08	4.45E+08	1.509387
	2	301	435	380	26	134	cos	15.3	205.02	39.78	165.24	cos	4597.266	1222873	1222708	0
							cos cys	562.888	7542.699	1463.509	6079.19	cos cys	1004635	2.67E+08	2.67E+08	0
							cos glut	41.245	552.683	107.237	445.446	cos glut	65998.92	17555713	17555268	0
	3	457	683	521	95	226	cos	69719.94	1575671	662339.4	913331.2	cos	14700.48	5361265	4447934	35.42478
							cos cys	1644456	37164703	15622331	21542372	cos cys	2392390	8.73E+08	8.51E+08	4.367373
							cos glut	425927.4	9625959	4046310	5579649	cos glut	865801.4	3.16E+08	3.1E+08	3.103365
	4	448	653	532	60	205	cos	16295.69	334061.7	97774.16	236287.6	cos	7419.109	2762876	2526589	13.22185
							cos cys	496128.2	10170627	2976769	7193858	cos cys	1606994	5.98E+08	5.91E+08	1.720188
							cos glut	82239.92	1685918	493439.5	1192479	cos glut	519034.8	1.93E+08	1.92E+08	0.877643
less functional LTPs (7 and 8)	1	498	737	620	82	239	cos	10143.18	242422	83174.08	159247.9	cos	12449.36	5403024	5243776	4.623043
							cos cys	262257	6267942	2150507	4117435	cos cys	1399985	6.08E+08	6.03E+08	1.038639
							cos glut	45565.7	1089020	373638.8	715381.5	cos glut	746487.9	3.24E+08	3.23E+08	0.336886
	2	436	633	510	91	197	cos	83673.72	1648372	761430.8	886941.4	cos	31166.55	11126459	10239517	16.09814
							cos cys	642352.6	12654347	5845409	6808938	cos cys	1776224	6.34E+08	6.27E+08	2.017263
							cos glut	472851.5	9315174	4302948	5012226	cos glut	2182824	7.79E+08	7.74E+08	1.203113
	3	830	1263	982	207	433	cos	34978.63	1514574	724057.5	790516.9	cos	13382.2	9198924	8408407	18.01262
							cos cys	428846.3	18569047	8877119	9691927	cos cys	1299951	8.94E+08	8.84E+08	2.100822
							cos glut	222303	9625721	4601673	5024049	cos glut	1123688	7.72E+08	7.67E+08	1.254331
	4	773	1189	900	178	416	cos	33710.14	1402342	600040.5	802301.4	cos	14888.57	9379799	8577498	16.34908
							cos cys	432342.1	17985431	7695689	10289742	cos cys	1410111	8.88E+08	8.78E+08	2.048268
							cos glut	237338.3	9873271	4224621	5648650	cos glut	1219367	7.68E+08	7.63E+08	1.294766
control (EV)	1	399	581	474	84	182	cos	53767.21	978563.2	451644.6	526918.7	cos	26776.23	8884354	8357435	11.70889
							cos cys	791042.2	14396968	6644754	7752213	cos cys	1969662	6.54E+08	6.46E+08	2.229387
							cos glut	799254.8	14546436	6713740	7832697	cos glut	2335826	7.75E+08	7.67E+08	1.896056
	2	626	847	742	188	221	cos	14007.78	309571.8	263346.2	46225.66	cos	21851.07	11349446	11303220	2.738793
							cos cys	282338.7	6239686	5307968	931717.8	cos cys	2309861	1.2E+09	1.2E+09	0.52049
							cos glut	85624.81	1892308	1609746	282561.9	cos glut	1635185	8.49E+08	8.49E+08	0.222878
	3	557	799	638	131	242	cos	11227.69	271710	147082.7	124627.3	cos	16260.44	7261912	7137284	3.806911
							cos cys	340072	8229742	4454943	3774799	cos cys	1075336	4.8E+08	4.76E+08	1.727232
							cos glut	237876.6	5756613	3116183	2640430	cos glut	1150670	5.14E+08	5.11E+08	1.12599
	4	670	956	735	153	286	cos	21444.9	613324.3	328107	285217.2	cos	9849.318	5067474	4782257	12.825
							cos cys	448956.9	12840168	6869041	5971127	cos cys	1016717	5.23E+08	5.17E+08	2.482968
							cos glut	281115.3	8039899	4301065	3738834	cos glut	893814.6	4.6E+08	4.56E+08	1.762638

average % in apoplast	cos	cos cys	cos glut
fct LTPs	23.221662	2.60721229	1.83013165
less fct LTPs	13.770722	1.80124819	1.0222741
control	7.7698986	1.74001921	1.25189065

std error	cos	cos cys	cos glut
fct LTPs	5.3100206	0.71859019	0.54070274
less fct LTPs	2.6662071	0.22065214	0.19851971
control	2.2650467	0.37739808	0.33076896

t-test	cos	cos cys	cos glut
fct LTP / less fct LTP	0.208519	0.357564	0.24728
fct LTP / control	0.056636	0.378999	0.455406
less fct LTP / control	0.187961	0.907419	0.624658

Formulas:

- Apoplast Volume = Weight After Infiltration – Weight Before Infiltration
- Total Apoplast Content = Apoplast Volume / 10 (measured sample) x LC MS data apoplast sample
- Extracted Apoplast Content = Liquid Volume / 10 (measured sample) x LC MS data apoplast sample
- Apoplast Content still in Leaf = Total Apoplast Content – Extracted Apoplast Content
- Content in Full Leaf = Weight After Centrifuge / 50 (extracted sample) x LC MS data leaf sample x 350 (extraction fluid) / 10 (measured sample)
- Not Apoplast Content = Content in Full Leaf – Apoplast Content still in Leaf
- Percentage in Apoplast = Total Apoplast Content / Not Apoplast Content x 100