Pyrethrum Secondary Metabolism: Biosynthesis, Localization and Ecology of Defence Compounds

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“Consistency is the last refuge of the unimaginative”

Oscar Wilde

Contents

Chapter 1

General Introduction

1- History and cultivation

Pyrethrum, is a perennial flower with a daisy-like appearance that belongs to the genus *Tanacetum* in the family Asteraceae. The genus *Tanacetum* contains more than one hundred species of which a number are being cultivated as ornamentals and only a few, such as *Tanacetum cinerariifolium* and *Tanacetum coccineum,* recognized as being toxic to insects. However, of these only *T. cinerariifolium*, also known as Dalmatian pyrethrum, is commercially important [1]. Although the use of pyrethrum as insecticide apparently originated in Persia and the Caucasus and was introduced to Europe early in the nineteenth century, this Persian species (*T. coccineum*, Persian daisy) was rapidly replaced by a new species discovered in Dalmatia in 1840, *T. cinerariifolium*. Even though accounts of the discovery of the toxicity of pyrethrum to insects differ, it seems that the discovery of the insecticidal effects of pyrethrum was done by a German woman in Dubrovnik who picked a bouquet of flowers for their beauty. When the flowers withered she threw them into a corner, where a couple of weeks later she found them surrounded by dead insects. Apparently, she associated the death of the insects with the insecticidal properties of the plant [1] .

Dalmatia remained the main source of pyrethrum until World War I, which cut off Dalmatia as source of supply and allowed Japan to seize the market and become the principal producer. In the late 1920's pyrethrum was introduced in the highlands of Eastern Africa, including Kenya, Tanzania, Rwanda and Zaire. Due to the higher flower yield and pyrethrin contents, the production in Africa increased rapidly, and made Kenya twenty years later the main producer in the world. Although over the years there have been many attempts to introduce or expand the growing of pyrethrum to other parts of the world, most did not prove to be economically worthwhile and were discontinued. Only in the last decade the Australian pyrethrum industry grew considerably, and became the world's largest pyrethrum producer, when production in Kenya collapsed due to mismanagement. Consequently, the distribution of pyrethrum production around the world has recently significantly changed. Among the main current pyrethrum producing countries (Australia, Kenya, Tanzania, Uganda, Rwanda, Tasmania, China, Ecuador and Papua New Guinea) [2,3,4], Australia is now the main source of supply. The effort is managed by the Tasmania based company Botanical Resources Australia Pty. Ltd. which is currently expanding the cultivation from Tasmania to the Australian mainland (http://www.botanicalra.com.au). In East Africa local companies are currently being taken over by US and Australian companies to secure a more stable supply of pyrethrins.

2- Constituents of pyrethrum extracts

A typical pyrethrum extract is prepared in two phases: first a crude extract is produced that serves as starting material for refinement later on. Crude extract production involves coarse grinding of the dried flower heads and extraction by percolation with low boiling iso-alkanes. The filtered solution is evaporated to leave a viscous greenish-brown liquid called “oleoresin”. The crude extract is usually partitioned between light petroleum and nitromethane, which leaves most of the inactive materials in the less polar phase. The partitioning solvent is distilled and the resulting residue is dissolved in the final solvent, such as for example the odourless isoparaffins. The concentration of pyrethrins is adjusted to a standard level, and the addition of the food preservative butylated hydroxytoluene (BHT), to prevent loss of pyrethrins by oxidation, completes the production of the final refined pale extract.

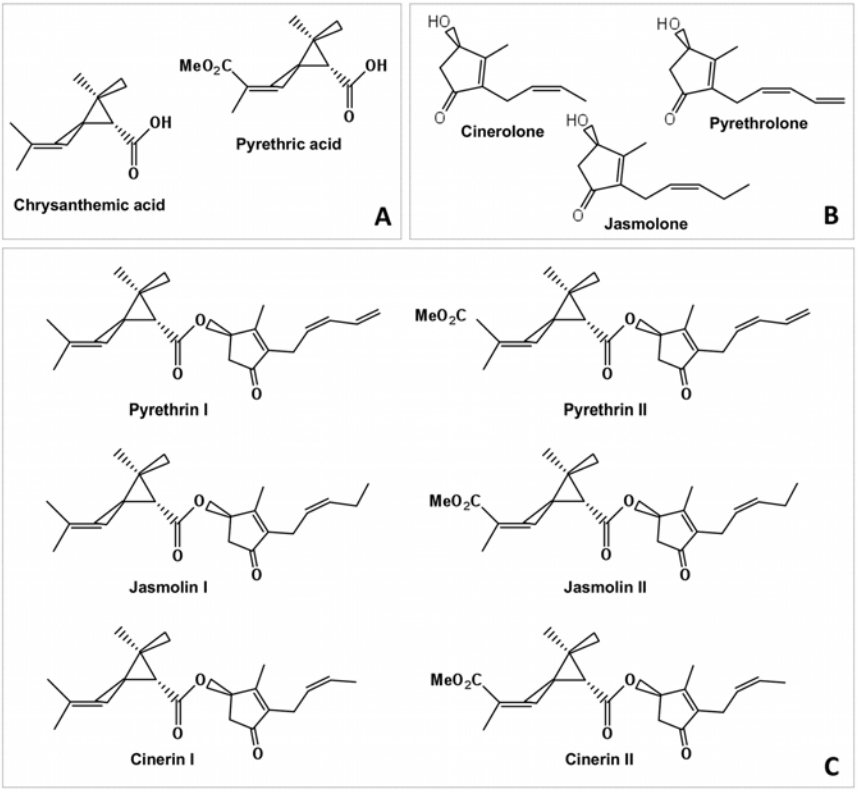

Figure 1: Structures of the six natural pyrethrins (C), the two acid components, chrysanthemic acid and pyrethric acid (A), and the three alcohol components, cinerolone, pyrethrolone and jasmolone (B) [5].

These refined pale extracts are typically composed of 70% pyrethrins, 14% hydrocarbons, 8.5% terpenoids and 7% fatty acids. The fraction collectively known as hydrocarbons results from the deliberate addition of alkanes and isoalkanes during and after processing. The terpene fraction is mainly composed of several sesquiterpenoids such as (*E*)-β-farnesene, pyrethrosin, γ-cadinene, δ-cadinene, γ-muurolene and the pentacyclic

triterpenols taraxasterol, and β-amyrin and α-amyrin. Fatty acids are present in pyrethrum extracts in the form of glycerides. The major fatty acids separated from glycerides are palmitic, linoleic, linolenic and oleic acid. Other compounds that have been reported in pyrethrum extracts are trace metals, carotenoids, flavonoids, polymers of pyrethrins, sesamin and chrysanthemic acid [6]. The active constituents of the pyrethrum extract, the pyrethrins, were identify as esters early in the history of pyrethrum chemistry [7,8,9]. They consist of a combination of either of two acids, chrysanthemic acid and pyrethric acid (Figure 1A), and any of three alcohols, pyrethrolone, cinerolone and jasmolone (Figure 1B). The esters of chrysanthemic acid with these three so-called rethrolones constitute type I pyrethrins, whereas the esters of pyrethric acid are collectively known as type II pyrethrins (Figure 1C) [5].

The outcrossing and self-incompatible nature of pyrethrum makes this plant species highly heterozygous and variable in different traits. Certain features like the ratio of pyrethrins I to pyrethrins II, commonly known as the PI/PII ratio, differ between different origins, likely due to genetic variation. Late maturity flowers appear to have higher levels of PII. An explanation for this decrease in PI/PII seems to be the biochemical conversion of pyrethrins I to pyrethrins II as the flower reaches maturity [6].

3- *Pyrethrum flower: morphology and development*.

The pyrethrum flower head is composed of florets set on a slightly convex receptacle covered on the outside by scales (Figure 2B). The disk florets, representing the yellow flower heart, densely populate the centre of the receptacle and are surrounded by an outer ring of white ray florets. Both disk and ray florets have ribbed achenes, which are seated on the receptacle. Each disk floret has a yellow tubular corolla with a small calyx at its base. The five stamen filaments, rising from the base of the inner surface of the corolla, terminate in elongated anthers that join together to form a cylinder, at the centre of which rises the style from the ovary that opens in the form of two lobes ready for pollination (Figure 2A). The outer disc florets open first and the development proceeds across the disc towards the centre. The ray florets differ in basic structure from the disk florets in that the corolla is enlarged, forming the white petals, and the stamens are absent [10].

The development of the flower head consists of eight stages: 1, well developed closed buds; 2, ray florets are in vertical position; 3, ray florets are in horizontal position and the first row of disc florets are open; 4, three rows of disc florets are open; 5, all disc florets are open; 6, also called early overblown condition; the colour of the disc florets diminishes but the ray florets are still intact; 7, known as late overblown condition; little colour remains in the disc florets and the ray florets are desiccated; and 8, ray florets were shed and flowers are suitable for seed collection (Figure 3A) [10].

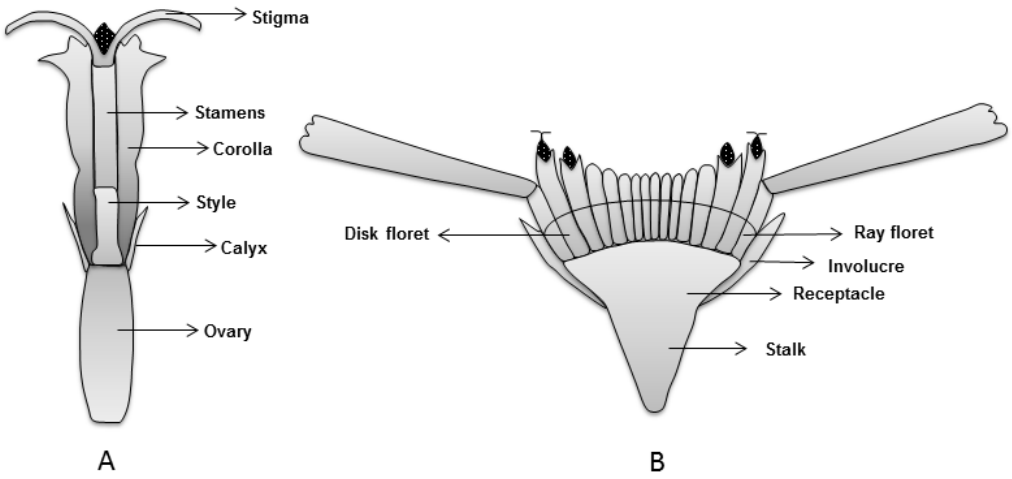

Figure 2: Structure of a pyrethrum floret (A) and a flower head (B) [11]

Early studies on the distribution of pyrethrins in the pyrethrum plant demonstrated the presence of pyrethrins throughout the whole plant, with the highest concentration in the flower heads. According to Figure 3B the accumulation of pyrethrins during flower development occurs in two phases, a phase in which the concentration of pyrethrins increases (stage 1 to stage 4) and a stationary phase in which the concentration has reached its maximum and remains constant (stages 5 to stage 7) [12]. Although the content of pyrethrins per flower head increases with maturity, the pyrethrin content per unit of dry weight is at an optimum in stage 4. The efficiency of extraction is higher with higher relative contents and therefore in East Africa and China flower heads are usually hand-harvested in stages 3 to 5 [13].

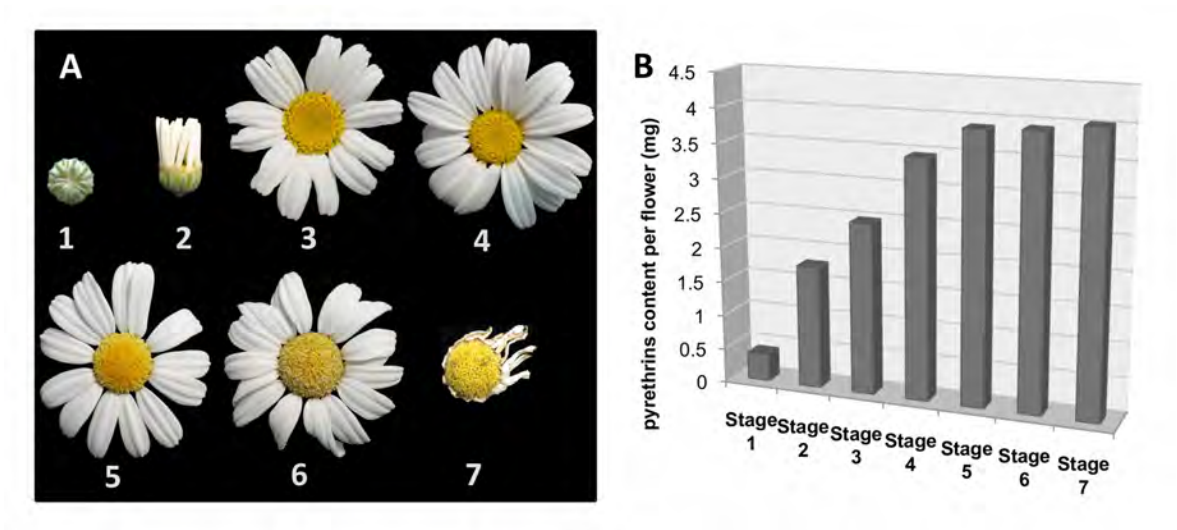

Figure 3: Pyrethrum flower heads at different developmental stages (A) and their corresponding pyrethrin concentrations as determined by Head (1966b) [11].

4- Use in pest control

Even though probably considerably earlier in Persia, the use of pyrethrum in Europe to control insect pests started in the 19th century, where the ground flowers of pyrethrum also known as “Persian insect powder” were used to control pests of medical and veterinary importance, such as bedbugs, fleas, body lice, flies and mosquitoes [1]. It was not until about 1919 that mineral oil-pyrethrum sprays, which are less harmful than powders, were commercially offered for household use [14]. In 1924 the work of Staudinger and Ruzisca, on the identification of the active principles of pyrethrum, allowed improvements of the existing products and boosted pyrethrum’s popularity [1].

The use of pyrethrum in agriculture started in the early 1930’s. In 1934 pyrethrum preparations were already recommended for the control of tulip bulb mite, iris root aphid, black citrus aphid, pea aphid, potato aphid, grape leafhopper, cotton leafhopper, grape trips, bean trips, cabbage worms, tomato fruit worm, parsley caterpillar, red-humped caterpillar and many other insects [15] During the 1930’s and until the advent of DDT (Figure 4), pyrethrum was used as a dust for insects on alfalfa, artichokes, beans, beets, cabbage, corn, pepper, potatoes, spinach and sugar beets among others [15].

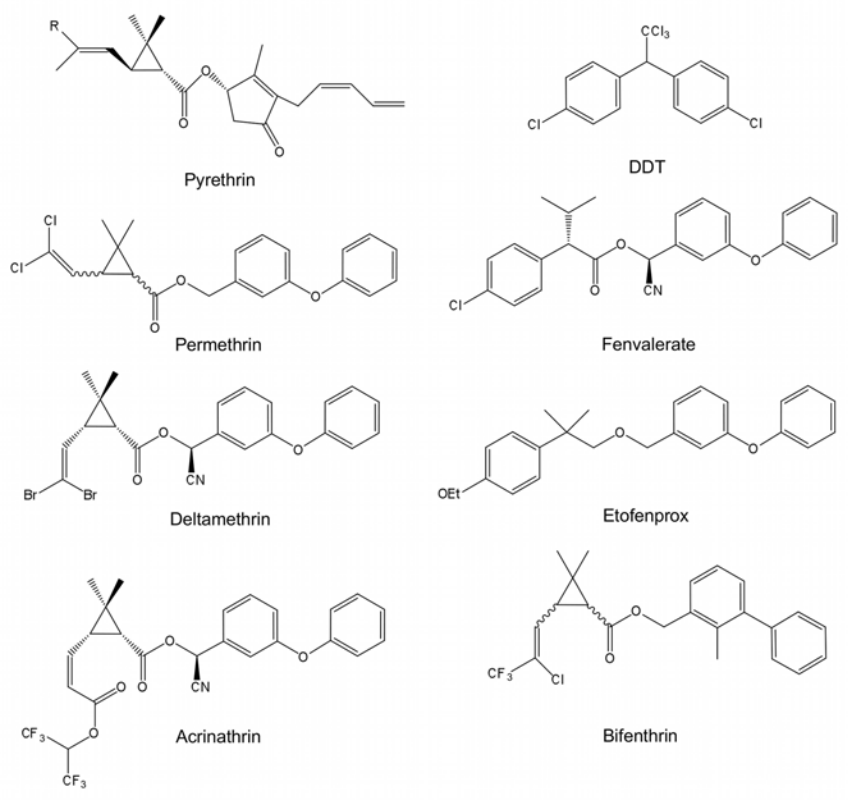

Figure 4: Chemical structures of pyrethrins, DDT and some pyrethroid insecticides [16].

The importance of pyrethrum as an agricultural and horticultural insecticide became replaced by DDT, which was extensively used in World War II to control malaria and

typhus among civilians and troops. However, in 1962, when “Silent Spring” was published by American biologist Rachel Carson this stimulated the development of alternatives to DDT, like pyrethroids, which constituted a group of compounds with less negative effects on the environment and with a wide variety of chemical structures (Figure 4). As a consequence, the use of pyrethrum was strongly reduced [17]. More economical pyrethroids were more stable to sunlight (UV) and air (oxidation) exposure, persisting longer on crops due to physical properties that made them resistant to wash-off by rain. However, the recent general shift of consumer preferences towards more selective, less toxic, less persistent and less environmentally hazardous products, has renewed the interest in the use of pyrethrum, generating a greater demand and encouraging pyrethrum growers to invest resources into expanding production, processing capacity and pyrethrum breeding and agronomy.

5- Mode of action and insect resistance.

The rapid lethal effects of pyrethrins and pyrethroids depend on the ability of these compounds to disrupt the normal functioning of the insect nervous system. Evidence from physiological, biochemical and genetic studies suggests the neuronal voltage-sensitive sodium channel as the principal target. Pyrethrins and pyrethroids share a common mode of action that appears to affect sodium channel function by binding to a unique site on the channel, inducing prolonged macroscopic sodium currents during and after membrane depolarization and a delayed and prolonged opening of single sodium channels [18,19]. There are many possible adaptations that permit insects to survive lethal doses of an insecticide. The majority of cases involve changes in the sensitivity of the target site due to point mutations, or the sequestration/metabolism of the insecticide before it reaches the target due to quantitative or qualitative changes in detoxification enzymes, such as esterases or P450s [20,21]. Unlike resistance due to enhanced metabolic detoxification, which can be compensated by the addition of blocker agents, such as piperonyl botoxide (PBO), knockdown resistance, like in the case of pyrethrins/pyrethroids/DDT, is unaffected by these blocker agents [22]. Molecular genetic analysis of resistance to pyrethroids (and DDT) in insects, provides compelling evidence for the primary importance of sodium channels as target for their insecticidal action. The knockdown resistance trait (designated as kdr) in house fly which confers reduced sensitivity to pyrethrins, pyrethroids and DDT analogs, was shown to be linked to a single mutation in the sodium channel encoding gene [22] (Figure 5). Although resistance to pyrethrins in the field is not common and, so far, only two cases have been reported (cockroaches in the USA and houseflies in Sweden) [23], the fact they share a common mode of action with pyrethroids and DDT has important implications for the use of pyrethrins in insect control, as selection for

resistance by one group of compounds is likely to select for cross-resistance to all other compounds with the same target. Moreover, prior selection for resistance by one of these compounds, like DDT, may lead to a rapid reselection of the same trait by intensive treatment with another of these compounds, like pyrethrins or synthetic pyrethroids. Indeed, the use of massive DDT house spraying applications in West Africa during the malaria eradication campaign in the 1950s greatly increased selection pressure in malaria vectors, leading to the emergence of resistance to pyrethroids [24]. Thus, to avoid compromising a long-term effective use of pyrethins, management of resistance to pyrethrins in target pest populations must take into account the treatment history of these populations with DDT or synthetic pyrethroids [25,26].

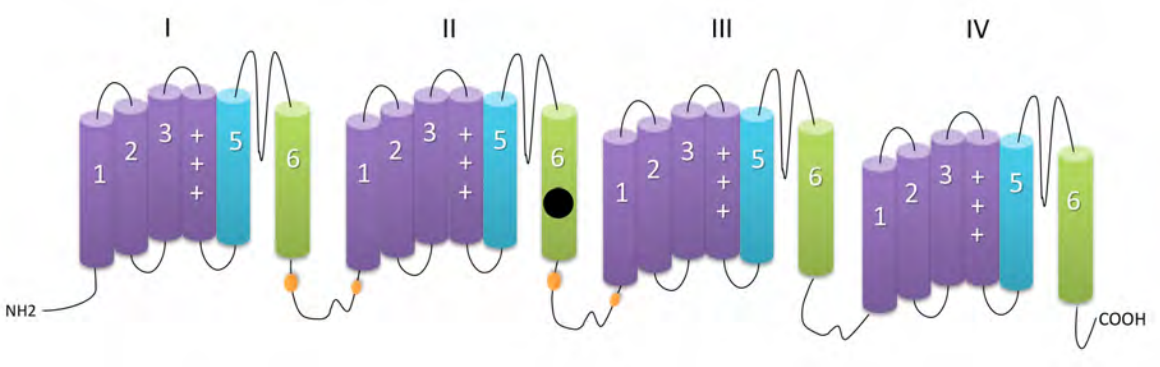

Figure 5: Schematic representation of a sodium channel showing the four internally homologous domains (I to IV), each containing six transmembrane helices (1 to 6) and the location of the mutated residue that confers the *kdr* knockdown resistance (black dot)[22].

6- Pyrethrum benefits and limitations

An ideal insecticide must combat pests at a low dose and in a form and manner that does not harm beneficial organisms. It must degrade sufficiently fast so that residues do not accumulate in the food chain or environment. The insecticide must be readily available, competitive in price and easily formulated for use. Most importantly, it must have low toxicity to humans exposed directly to the compound or to its residues.

In this respect, pyrethrum is rather close to being an ideal insecticide. The single most important feature of pyrethrum is the rapid knockdown against a wide variety of insect pests. Other important characteristics are its low mammalian toxicity and its low persistence, which makes it widely acceptable as safe and environmentally friendly. The low persistence, while considered a benefit from an environmental point of view, also constitutes a limitation, especially in cases where residual activity, in addition to knockdown is equally important and/or required. Another important critical factor affecting the global use of pyrethrum is its cost and availability. Because pyrethrum plants are grown as a labour-intensive agricultural crop, the supply, and to some extent the quality

depend on a variety of biotic and abiotic factors that will ultimately have a great impact on its yield and cost. As a consequence, without major improvements in the long-term stability and supply of pyrethrum, the trend towards using the more cost/benefit enticing synthetic counterparts is unlikely to be reversed in the near future [27,28].

7- *Biochemistry of pyrethrins*

Although considerable knowledge has been gained on different aspects of the chemistry of pyrethrins, there is only limited information available about the biosynthesis of the acid and rethrolone portions of the pyrethrins.

7.1-Biosynthesis of the acid moiety of pyrethrins

The acid moieties are irregular monoterpenes sharing a cyclopropane ring generated by the condensation of two dimethylallyl diphosphate (DMADP) molecules (**3**), in a reaction catalysed by chrysanthemyl diphosphate synthase (CDS) to give chrysanthemyl diphosphate (**4**) [29]. DMADP originates from isomerization of isopentenyl diphosphate (IDP) (**2**). Even though it was shown already in 1961 that [^{14}C] mevalonic acid was incorporated into the acid moiety of pyrethrins when using flowers [30,31], a non-mevalonate route, the 2-C-methyl-D-erythritol 4-phosphate (MEP) (**1**) pathway, was only recently, in 2005, also shown to operate in leaves and seedlings [32]. The MEP pathway is present in the plastids, whereas the mevalonate pathway operates in the cytosol. The plastid targeting peptide at the N-terminus of the CDS enzyme was shown to direct the enzyme to chloroplasts [29,33]. This is the best evidence, therefore, that MEP and not mevalonate-derived substrates are used in the formation of chrysanthemyl diphosphates. The subsequent formation of chrysanthemic acid (**7**) proceeds through dephosphorylation and subsequent sequential oxidations of the (*E*)-methyl group in C1 of chrysanthemyl diphosphate (**4**). Enzymes catalysing these steps have yet to be isolated (Figure 6).

7.2-Biosynthesis of the alcohol moiety of pyrethrins

Although the origin of the acid moiety of pyrethrins is quite clear, there is less certainty about the origin of the alcohol moieties of the pyrethrins, the rethrolones. Rethrolones show chemical resemblance to the plant hormones, jasmonic acid (JA) (**16**) and *cis*-jasmone (**22**). Although it has not been established whether they derive from JA or *cis*-jasmone, they were shown to originate from a common precursor, linolenic acid (**10**) [32]. Indeed, the highest concentration of pyrethrins is found in the achenes of the flowers, a particularly lipid-rich environment. It has been reported that the fatty acids represent 20% of a commercial pyrethrum extract containing 30% pyrethrins, with linoleic acid ($C_{18:2}$) comprising 6.6%, and linolenic acid ($C_{18:3}$) 3% of the total fatty acid fraction [34].

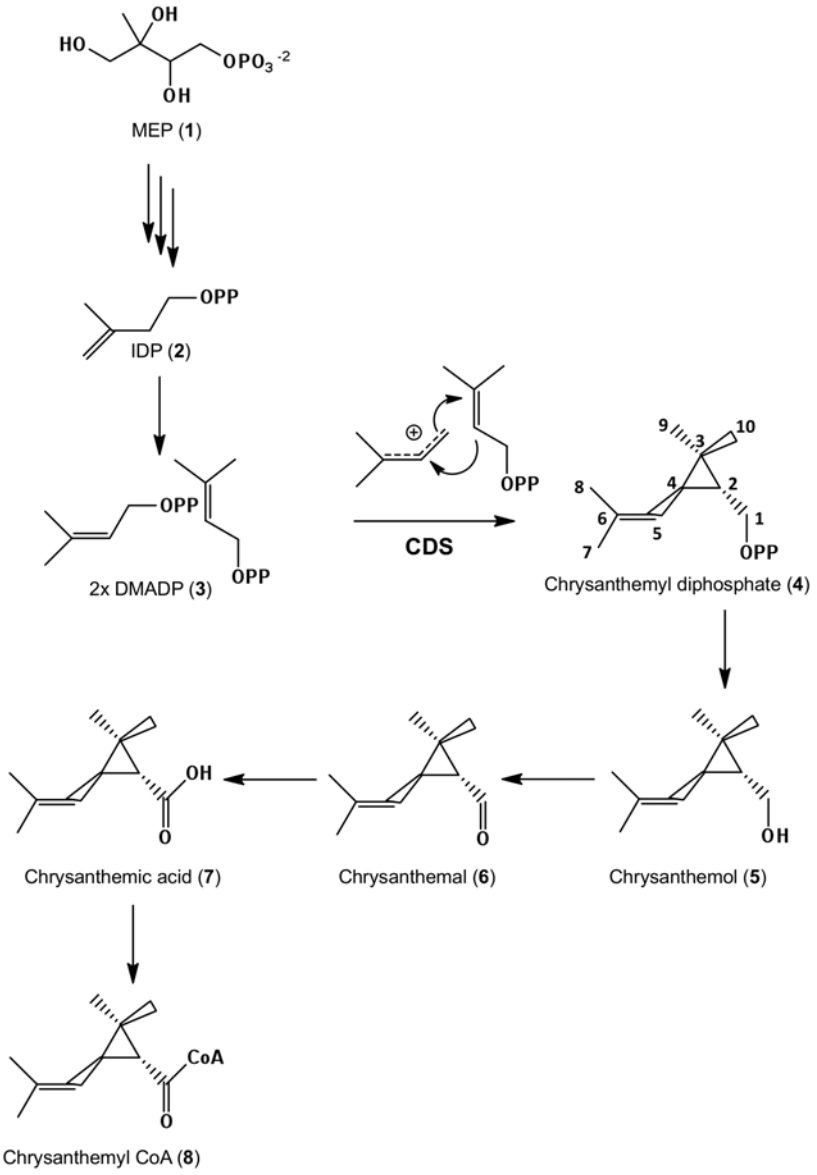

Figure 6: Biosynthetic pathway to Chrysanthemyl CoA, showing the CDS enzyme involved in the first committed step of the biosynthesis of the acid moiety of pyrethrins from 2-C-methyl-D-erythritol-4-phosphate (MEP).

The plant hormone JA derives from the universal oxylipin pathway in plants, which leads from linolenic acid to jasmonates. Jasmonates affect a variety of processes [35], including fruit ripening, production of viable pollen, root growth, and plant responses to abiotic and biotic stresses (insects, pathogens and mechanical damage). Considerable knowledge has been gained especially on the biosynthesis of JA in response to wounding. Although wounding resulted in enhanced content of pyrethrins I and II in pyrethrum seedlings [36], in the flowers, pyrethrin biosynthesis appears to be developmentally and tissue-specifically regulated.

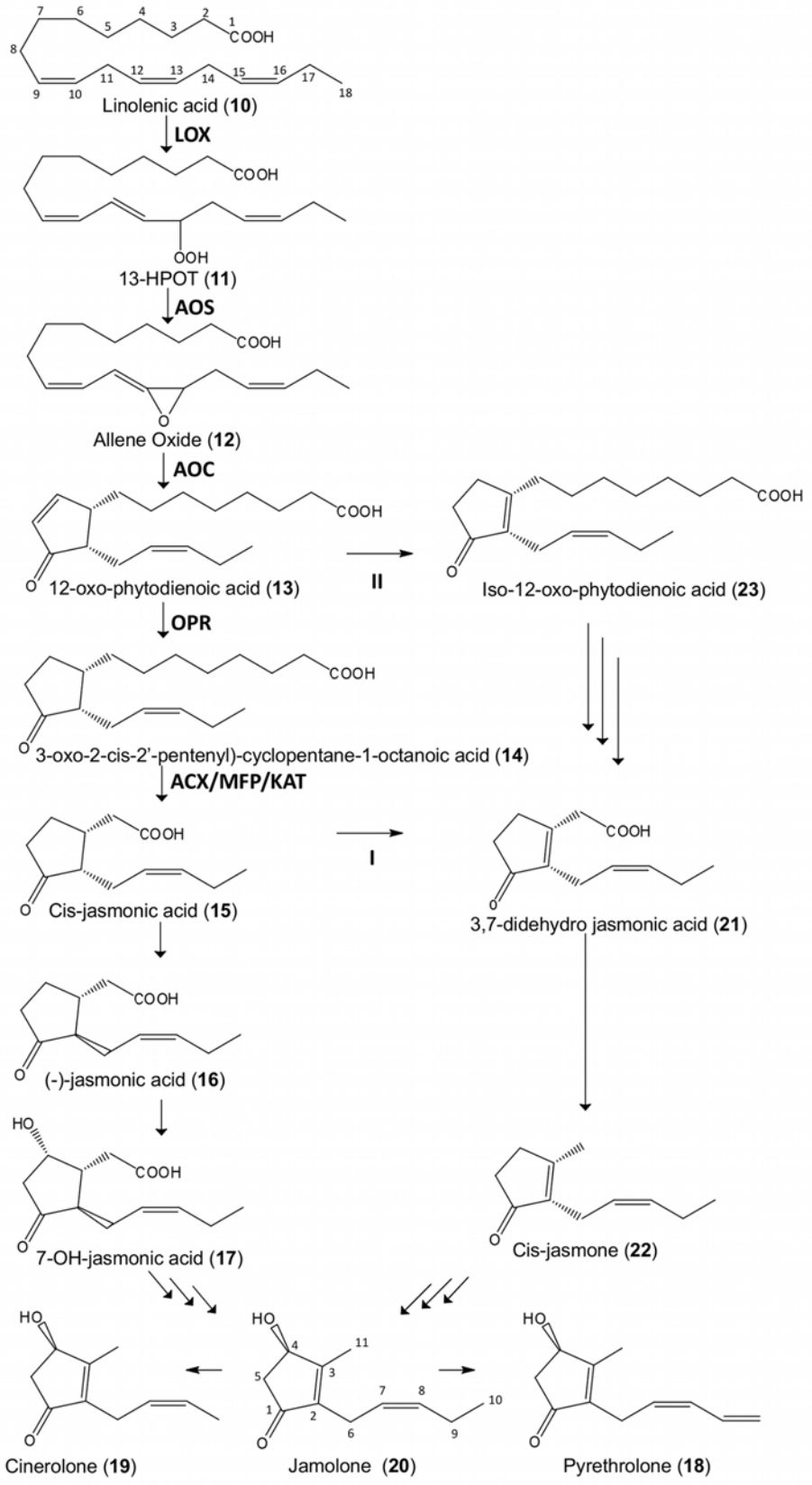

Figure 7: Two possible biosynthetic routes to pyrethrolone as hypothesized by Matsuda et al. (2005) [30]. Both possible precursors 7-OH-jasmonic acid and *cis*-jasmone derived from linolenic acid by the oxylipin or octadecanoid pathway. *Cis*-jasmone can either be derived from *cis*-jasmonic acid (I) or from *cis*-OPDA (II). The biosynthetic enzyme abbreviations are LOX,

lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR, OPDA reductase; ACX, acyl-CoA oxidase; MFP, multifunctional protein; and KAT, L-3-ketoacyl CoA thiolase. Linolenic acid (10) numbering was only added with the purpose of identifying the corresponding carbons in jasmolone (20).

The biosynthesis of JA starts with the hydroxylation of linolenic acid (Figure 6 (**10**)) catalysed by a chloroplast-localized lipoxygenase (LOX) to give 13-hydroperoxylinolenic acid (**11**). 13-Hydroperoxylinolenic acid is substrate for allene oxide synthase (AOS), which catalyses the formation of an unstable epoxy group resulting in allene oxide (**12**). The subsequent enzyme of the pathway, allene oxide cyclase (AOC), is also plastid-localized and catalyses the stereospecific cyclization of allene oxide to *cis*-(+)-12-oxophytodienoic acid (*cis*-OPDA) (**13**). This establishes the stereochemistry by the formation of the cyclopentenone ring and this intermediate is exported to the peroxisomes where the cyclopentenone ring is reduced to form 3-oxo-2(2'-pentenyl)-cyclopentane-1-octanoic acid (OPC-8:0) (**14**) in a reaction catalysed by a peroxisomal OPDA reductase (OPR). Three rounds of β-oxidative side chain reduction are the last essential step in jasmonic acid biosynthesis, involving the enzymes acyl-CoA oxidase (ACX1), multifunctional protein (MFP) and L-3-ketoacyl CoA thiolase (KAT) [37].Matsuda *et al*. (2005) [32] hypothesized that the alcohol portion of pyrethrins could either arise from 7-hydroxyjasmonic acid (**17**) by desaturation and decarboxylation, or from *cis*-jasmone (**22**) by hydroxylation of the cyclopentenone ring. *Cis*-jasmone might derive from two different pathways [38]. In the first (Figure 7 (step I)), cis-jasmonic acid is transformed (**15**) to the planar unsaturated 3,7-didehydrojasmonic acid (**21**), followed by decarboxylation to cis-jasmone. In the second (Figure 7 (step II)), an isomerisation of the double bond of *cis*-OPDA (**13**) gives rise to the planar iso-OPDA (**23**), which is processed analogously to the jasmonic acid pathway to yield 3,7-didehydrojasmonic acid (**21**). The final step of decarboxylation to get cis-jasmone is proposed to occur spontaneously [38].

The next step in pyrethrin biosynthesis is the ester linkage formation between the acid and the alcohol moieties. A member of the GDSL lipase family of proteins, GLIP, was recently purified from pyrethrum flowers and demonstrated to be capable of transferring the chrysanthemoyl group of the CoA thioester to pyrethrolone to give pyrethrin I (Figure 8) [39].

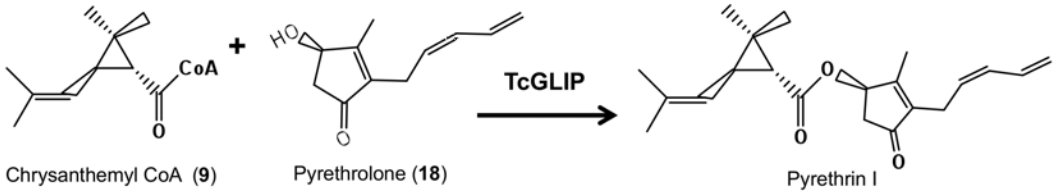

Figure 8: Final step in the biosynthesis of pyrethrins as demonstrated by Kikuta *et al* (2012). Esterification of the acyl-CoA moiety to the pyrethrolone by GLIP to yield Pyrethrin I.

Whether pyrethrolone derives form JA or cis-jasmone, whether the rethrolone ratios are formed before or after esterification and whether pyrethrins type II originate from pyrethrins type I remains to be elucidated. In spite of the considerable variation, in a typical extract from pyrethrum flowers, the proportions within the pyrethrins type I (pyrethrin I (38.0%), cinerin I (7.3%), jasmolin I (4.0%)) are similar to the proportion within pyrethrins type II (pyrethrin II (35.0%), cinerin II (11.7%) and jasmolin II (4.0%)) [10], whereas the ratio PI/PII increases upon flower development [6]. Both of these observations in addition to the fact that free pyrethric acid has never been observed, neither in previous reports [34] nor in our studies, suggest that type I pyrethrins are probably formed first and give rise to type II pyrethrins.

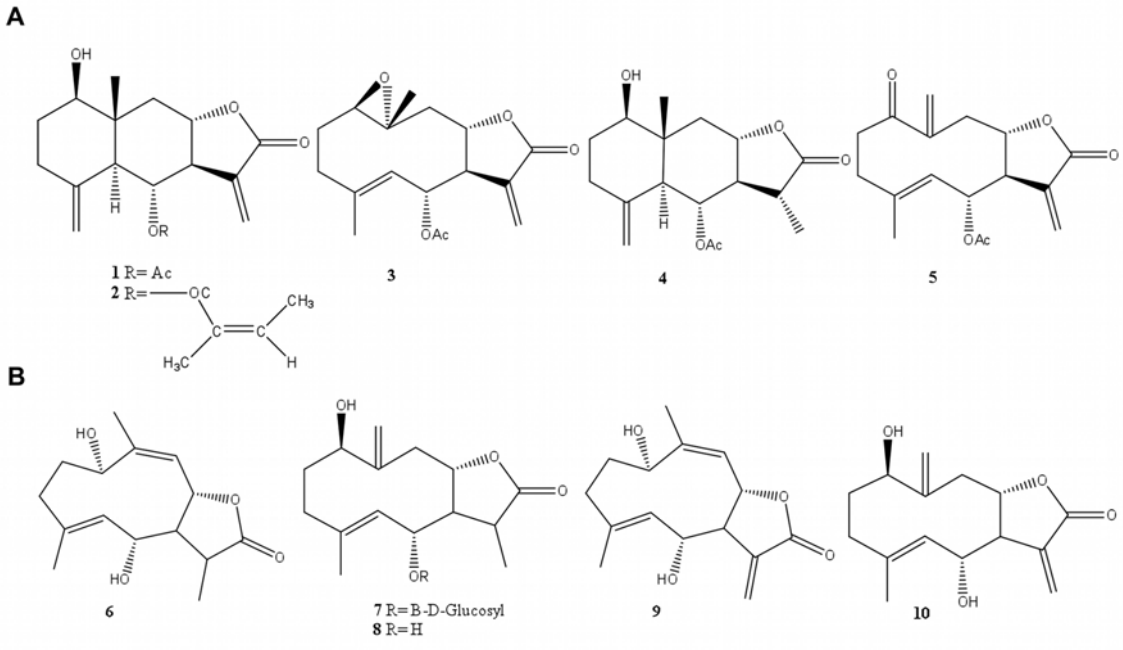

Figure 9: Major sesquiterpene lactones reported for pyrethrum. A, β-cyclopyrethrosin (1), chrysanin (2), pyrethrosin (3), dehydro-β-cyclopyrethrosin (4), chrysanolide (5) [40] . B, (11R)-11,13-Dehydro-Tatridin-A (6), (11-R)-6-O-β-D-glucosyl-11-13 dehydro-tatridin-B (7), (11R)-11,13-Dehydro-Tatridin-B (8) Tatridin-A (9), and Tatridin-B (10) [41]

Sesquiterpene lactone chemistry and biological activity

Several sesquiterpene lactones (STLs) have been isolated from pyrethrum flowers. Pyrethrosin (or chrysanthin) was first isolated by Rose and Haller in 1937 and reported to have low toxicity against mosquito and moth larvae [42]. Later on it was subject of detailed structural investigations by Barton and co-workers [43]. In addition to pyrethrosin, in 1971 Doskotch *et al*. [40] reported the isolation and NMR structural determination of four additional sesquiterpene lactones from pyrethrum flower heads (Figure 9A). Lastly, in 1982 a new report showed the isolation and identification of five more sesquiterpene lactones from pyrethrum (Figure 9B) [41], with significant plant growth inhibitory activity. All of the work done so far on pyrethrum sesquiterpene lactones was focused on structural identification. Enzymes involved in the biosynthesis of these compounds have not been isolated or even hypothesised to exist. Moreover, although the sesquiterpene lactones have been isolated from flower heads and have been reported to have biological activity [44,45,46,47], their exact localization and biological role in pyrethrum remain unclear.

Thesis outline

Although there has been considerable investigation on a variety of aspects of pyrethrum as one of the most important botanical insecticides, the molecular/biochemical bases of the biosynthesis of two of its active ingredients, pyrethrins and sesquiterpene lactones are largely unknown. Hence, this thesis aims to develop knowledge of the biosynthetic pathways leading to pyrethrins and sesquiterpene lactones in *Tanacetum cinerariifolium*. Enzymes involved in different steps of the biosynthesis of these defence compounds are characterized at the molecular and biochemical levels. The cellular organization of the biosynthesis and tissue transport of the products are demonstrated and placed in an ecological context.

Chapter 2 investigates how and where pyrethrin and STL are produced, transported and stored, and gives insights on the biological roles of these two types of compounds in the maternal and embryonic tissues of pyrethrum.

In **chapter 3** cytochrome P450 genes are isolated from a pyrethrum trichome EST library, and the encoded enzymes are assessed on their ability to catalyse the two consecutive oxidative steps leading from chrysanthemol to the acid moiety of pyrethrins, chrysanthemic acid. The transcriptional regulation of the genes during flowering and their tissue specificity in correlation with pyrethrin biosynthesis are also addressed.

Chapter 4 describes the isolation of genes encoding lipoxygenase enzymes, based on the similarity of their transcriptional profile with other pyrethrin biosynthesis related genes. The selected genes are expressed in *E. coli* and the purified enzyme characterized *in vitro* for their ability to catalyse the first step in the biosynthesis of the alcohol moiety of pyrethrins. Like for CDS and the cytochrome P450 enzyme, the tissue specific expression of the selected genes is also investigated.

Chapter 5 addresses the retrieval from the pyrethrum trichome EST library of genes implicated in the costunolide biosynthesis in close relatives of pyrethrum producing medically important STLs. The encoded enzymes are characterized in yeast and *in planta* and evaluated for their involvement in the biosynthesis of putatively identified C6-C7-costunolide-derived and the C7-C8-type STLs reported for pyrethrum.

In **chapter 6** the most important findings of this thesis are summarized and discussed.

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Chapter 2

Bidirectional Secretions from Glandular Trichomes of Pyrethrum Enable Immunization of Seedlings

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Abstract

Glandular trichomes are currently only known to store mono- and sesquiterpene compounds in the subcuticular cavity just above the apical cells of trichomes, or emit them into the headspace. We demonstrate that basipetal secretions can also occur, by addressing the organization of the biosynthesis and storage of pyrethrins in pyrethrum flowers. Pyrethrum *(Tanacetum cinerariifolium)* produces a diverse array of pyrethrins and sesquiterpene lactones for plant defense. Highest concentrations accumulate in the flower achenes, which are densely covered by glandular trichomes. The trichomes of mature achenes contain sesquiterpene lactones and other secondary metabolites, but no pyrethrins. However, during achene maturation the key pyrethrin biosynthetic pathway enzyme chrysanthemyl diphosphate synthase, is expressed only in glandular trichomes. We show evidence that chrysanthemic acid is translocated from trichomes to pericarp, where it is esterified into pyrethrins which accumulate in intercellular spaces. During seed maturation, pyrethrins are then absorbed by the embryo and during seed germination the embryo-stored pyrethrins are recruited by seedling tissues, which, for lack of trichomes, cannot produce pyrethrins themselves. The findings demonstrate that plant glandular trichomes can selectively secrete in basipetal direction monoterpenoids, which can reach distant tissues, participate in chemical conversions, and immunize seedlings against insects and fungi.

Introduction

The perennial herbaceous plant *Tanacetum cinerariifolium* (Asteraceae), also known as pyrethrum, is an economically important crop used for the production of a group of potent insecticidal secondary metabolites collectively called pyrethrins [1]. Despite the economic significance of pyrethrins, many aspects of their biosynthesis and cellular localization are poorly understood. Pyrethrins occur throughout the aerial parts of the plant, but are concentrated in the dry fruits (achenes, fruits containing a single seed) at levels that are 10-20 times higher than in leaves [2]. Both the biseriate, capitate glandular trichomes on the outside of achenes and secretory duct-like structures inside the pericarp of achenes have been suggested to be involved in the production and storage of pyrethrins [3,4]. Evidence supporting a role for trichomes is the reported association of a pyrethrin-void mutant line with the absence of both pyrethrin-containing ducts and trichomes [3], and the correlation between pyrethrin content and the number and development of trichomes on ovaries and leaves [5,6]. However, direct evidence for the role of trichomes in pyrethrin biosynthesis and storage is lacking.

A typical pyrethrum flower hexane extract consists of six pyrethrin esters: pyrethrin I (PI), cinerin I (CI), jasmolin I (JI), and pyrethrin II (PII), cinerin II (CII), and jasmolin II (JII) (Figure 1) [5], and a variety of other compounds including waxes, fatty acids, glycerides, carotenoids and terpenoids such as *E*-β-farnesene, γ-cadinene, and sesquiterpene lactones [7]. The pyrethrin esters derive from the condensation of a terpenoid acid and an oxylipin pathway-derived alcohol moiety [8]. The terpenoid acids are irregular monoterpenoids with c1'-2-3 linkages between two dimethylallyl units, and can either be chrysanthemic acid (CA, Type I) or pyrethric acid (PA, Type II) (Supplemental Figure 1 online) [9]. The alcohol part consists of pyrethrolone, cinerolone, or jasmolone which are linoleic-acid derivatives and share the biosynthetic pathway with jasmonic acid. The formation of chrysanthemyl diphosphate (CPP) from two molecules of dimethylallyl diphosphate (DMAPP) by chrysanthemyl diphosphate synthase represents the first dedicated step in the biosynthesis of pyrethrins, and was demonstrated in pyrethrum and *Artemisia tridentata* [9,10]. *CDS* evolved relatively recently after a gene duplication from an ancestral farnesyl diphosphate synthase (*FDS*) gene. In that process it acquired a plastid targeting signal, which enabled access to DMAPP derived from the methylerythritol-4-phosphate (MEP) pathway [9,11]. Very recently, a second dedicated enzyme, *Tanacetum cinerariifolium* GDSL Lipase-like Protein gene (*TcGLIP*), responsible for the specific esterification of chrysanthemoyl-CoA thioester and pyrethrolone to form pyrethrin I was discovered [12].

Generally, glandular trichomes are involved in the synthesis, storage and emission of a broad array of secondary metabolites [13]. In the *Asteraceae* family, glandular trichomes are particularly rich in sesquiterpenoids [14,15,16,17]. Indeed, apart from pyrethrins, pyrethrum extracts also contain a variety of sesquiterpene lactones (STLs), such as pyrethrosin (chrysanthin) [18,19], chrysanin, dihydro-β-cyclopyrethrosin, chrysanolide, and β-cyclopyrethrosin [20,21]. Sesquiterpene lactones are generally characterized by a five-membered α-methylene-γ-lactone ring. Many also contain additional oxidized positions, and they may be conjugated to glycosides [22,23]. Like pyrethrins, sesquiterpene lactones have been implicated in plant defense. They protect plants against insects, pathogenic bacteria and fungi, or play allelopathic roles [24]. STLs were specifically described to be cytotoxic [25], antibacterial [26], antifungal [27], phytotoxic [28], and germination inhibitors [23].

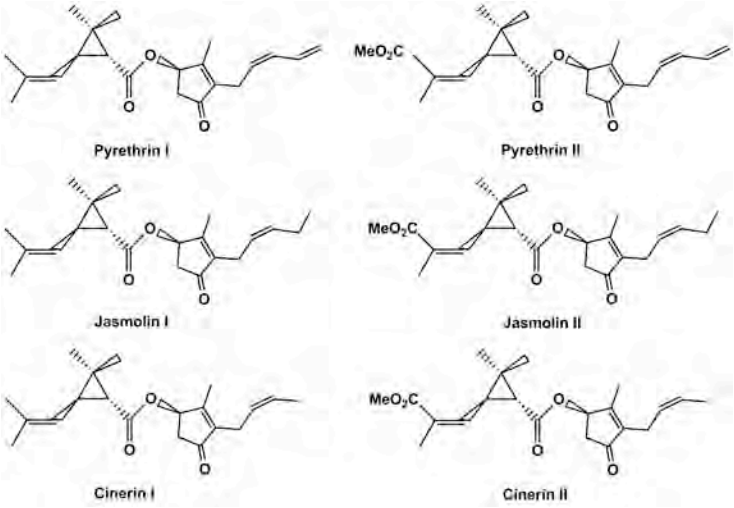

Figure 1: Structures of Natural Pyrethrins.

The terpenoids produced in biseriate capitate glandular trichomes are thus far known to be secreted towards the apical side, resulting in a droplet of secondary metabolites in an extracellular cavity covered by a thick cuticular membrane [29,30,31]. The secretion of metabolites in basipetal direction, across the epidermis, has not been described. In this report we investigate the role of pyrethrum trichomes in the biosynthesis and secretion of precursors of pyrethrins and of sesquiterpene lactones. We identify the sites of biosynthesis, storage and deployment of both compounds by chemical, transcriptional and enzymatic analyses in achene trichomes, pericarp tissues, and seedlings. The potential novel ecological roles of these compounds are investigated by following their fate in germinating seeds and evaluating their effects against herbivorous insects and a seedling pathogen.

Results

Localization of Oil-like Substances in Pyrethrum Achenes by CryoSEM

To obtain structural information on the localization of oil-like substances in pyrethrum achenes, cryoSEM images of surface and cross sections of the achenes of disk florets from different flower developmental stages were prepared. Glandular trichomes occur mainly in 4 out of 5 indentations between 5 ribs in the longitudinal direction of the seed (Figure 2A,B), in a density of 650±15 glands per achene for the genotype we evaluated. On the basis of surface density, mature achenes have a 6-fold higher glandular trichome density (74±6 trichomes/mm^2) than the combined abaxial and adaxial sides of leaves (12±2 trichomes/mm^2). Biseriate capitate glandular trichomes consist of a layer of two juxtaposed basal cells (Figure 2C, bs), a layer of two stalk cells (Figure 2C, stc), and three layers of two head or secretory cells (Figure 2C, sec). Cross sections of the glandular trichomes show that the two cell pairs below the apical cell pair contain chloroplasts, as visible in Figure 2C (arrows), but the apical cells do not. Each trichome is covered with a sac-like cuticular membrane that seems to adhere firmly to young trichomes, but inflates as it fills with oil after opening of the corolla (Figure 2C Os). Transverse sections of the ovaries of open disc florets with fully developed embryos (prior to desiccation) showed similar oil-like substances as in the trichomes to be present in, sometimes air-filled, intercellular spaces of the pericarp surrounding the developing embryo, as well as in the space immediately contacting the epidermis of the cotyledonary tissue of the embryo, but at this immature stage none in the dense cell tissue of the embryo itself (Figure 2D arrows).

Pyrethrum Glandular Trichomes Store Sesquiterpene Lactones, but not Pyrethrins

To identify the content of glandular trichomes, we analyzed, by GC-MS and GC-FID, chloroform extracts of (i) complete achenes, (ii) trichomes from achenes (using chloroform dipping; see Materials and Methods) and (iii) achenes after removal of trichome content using chloroform dipping. The GC-MS profiles obtained from trichomes and achenes after chloroform dipping were completely different (Figure 3). The achene content remaining after chloroform dipping consisted mainly of the six pyrethrin esters CI, JI, PI, CII, JII,PII (Figure 3B, peaks 1 to 6 respectively) and the thermally induced isoform of PI (Figure 3B, peak 3a) [32] at an average total concentration of 46.9±3.1 µg/mg (GC-MS), or 65.4±4.6 µg/mg (GC-FID) determination (one seed ≈ 1 mg). The profile obtained for seed trichomes included compounds (A-J) at an average total concentration of 10.9±1.0 µg/mg (GC-MS) or 6.2±1.3 µg/mg (GC-FID) per seed.

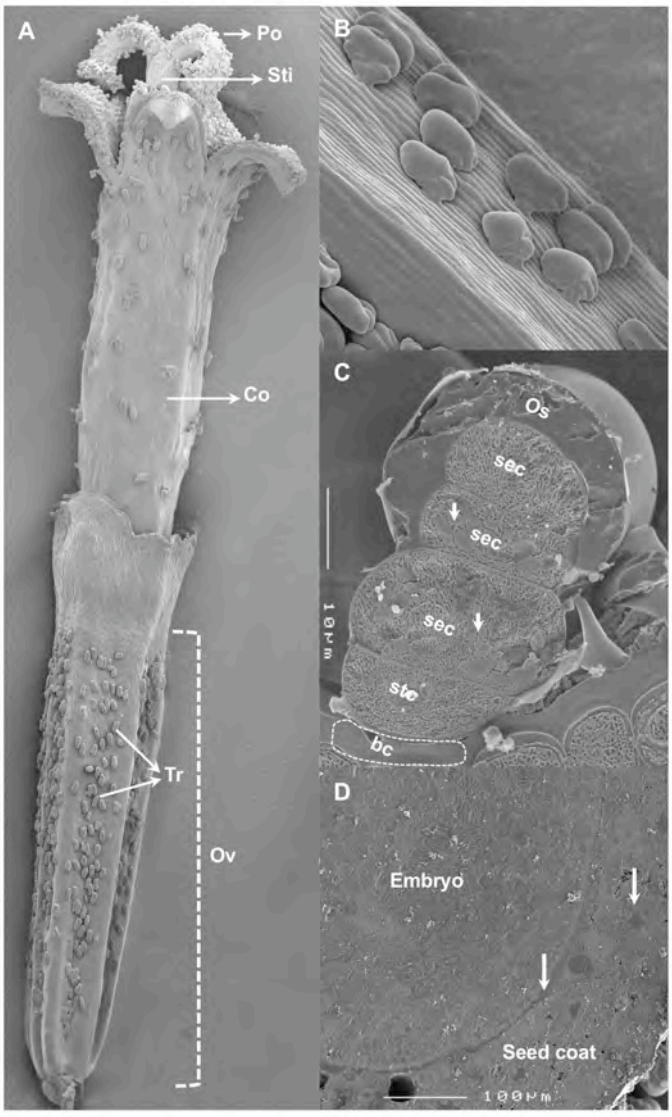

Figure 2: Localization and morphology of pyrethrin stores and pyrethrum glandular trichomes. A, CryoSEM image of a complete disk floret showing the highest density of glandular trichomes in the indentations between the ribs of the ovary. B, closer view of trichomes. C, side view of a fully developed trichome indicating the location of basal and stalk cells and the presumed secretory cells and the oil-sac. D, perpendicular cut of a disk floret showing the oil in the intercellular space between the embryo and the seed coat and in the intercellular space of cells (arrows). Ov, ovary; Co, corolla; Tr, trichome; Sti, stigma; Po, pollen; bc, basal cell; stc, stalk cell; sec, secretory cell; and Os, oil sac.

Comparison of the mass spectrum of each trichome constituent with the NIST library resulted in a list of putative identities, which are summarized in Supplemental Table 1 online. Based on previous characterizations of pyrethrum extracts, dihydro-β-cyclopyrethrosin (Figure 3C peak F, KI: 2454) and β-cyclopyrethrosin (Figure 3C peak M, KI: 2683) could be assigned, while the others require further analysis to determine their exact STL identity. Complete achene extracts yielded a quantitative combination of what was detected in chloroform-dipped achenes and in the chloroform that was used for the dipping (Figure 3A). This demonstrates that chloroform dip extraction was very effective in removing all chloroform soluble contents from the trichomes without extracting the seed interior. Microscopic examination of vegetative tissues showed that glandular trichomes are present on both sides of the surface of leaves, but not on seedling cotyledons. To obtain further information on their chemical composition, the content of leaf trichomes was extracted by chloroform-dip as well, and compared to the whole ground leaf before and

after dip. We confirmed that pyrethrins also in leaves are confined to the leaf interior (Figure 3E), that the leaf trichomes do not contain pyrethrin esters (Figure 3F), and that leaf trichomes contain the two major STLs of the seed trichomes (Figure 3F, peaks C and E), but lack the others (Figures 3F and 3C). Seedling extracts (no true leaves) did not contain any sesquiterpene lactones, but were high in pyrethrin content (Table 1, column 4; Figure 6, peak E).

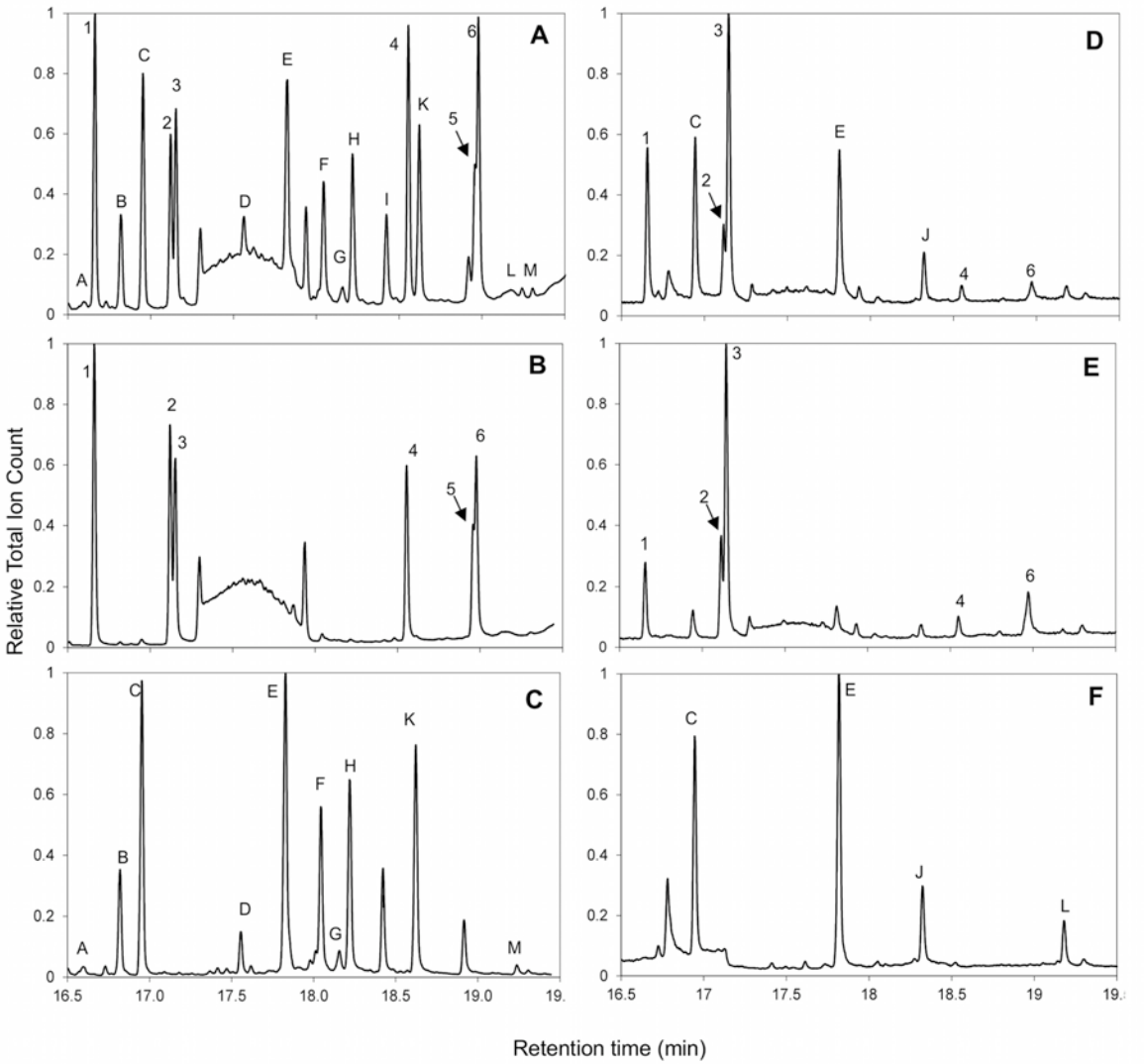

Figure 3: GC-MS analysis of pyrethrum seed and leaf oil constituents. Chloroform extracts of complete seeds including trichomes (A), seed contents after trichome extraction (B), and contents of seed glandular trichomes only (C), complete young leaves including trichomes (D) leaf contents after trichome extraction (E), and contents of young leaf glandular trichomes (F). The major pyrethrin components identified by mass spectrometry are labeled by numbers: 1, cinerin I; 2, jasmolin I; 3, pyrethrin I; 4, cinerin II; 5, jasmolin II; 6, pyrethrin II; and the major sesquiterpene lactones by letters: A to M (See Supplemental Table 1 online for GCMS identification details)

Pyrethrin Biosynthesis Gene *CDS* Is Expressed in Trichomes, and Pyrethrin Esterification Enzyme *TcGLIP* in the Pericarp

Having established that pyrethrins, unlike sesquiterpene lactones, are not stored in the glandular trichomes, we first investigated the expression of pyrethrin and sesquiterpene biosynthetic genes across different flower stages in relation to the accumulation of the product. As described above, CDS catalyzes the formation of the unique monoterpene precursor of pyrethrins, CPP, whereas farnesyl diphosphate synthase 1 (FDS1) catalyzes the biosynthesis of the sesquiterpene precursor farnesyl diphosphate (FPP). TcGLIP, a GDSL lipase-like protein, was recently reported to catalyze esterification of terpene acids and lipid alcohol substrates into pyrethrins [12]. The expression patterns of *CDS, FDS1,* and *TcGLIP* were analyzed by RT-qPCR and normalized to the *GAPDH* reference gene (see also Supplemental Table 2 online). Figure 4 shows that the pyrethrin content of flower stages S1-S7 (bud to overblown) correlated with the gene expression of both *CDS* and *TcGLIP*, Fifty percent of pyrethrins were already produced before the first disc florets opened (stage 2) at a point where embryos were not yet formed, and corresponding to the peak of transcription of both *CDS* and *TcGLIP*.

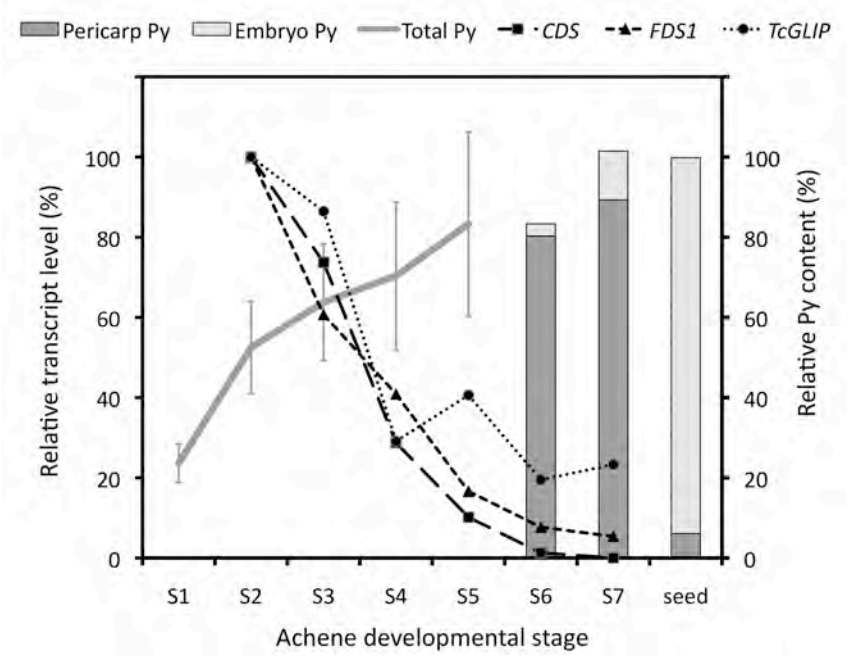

Figure 4: Correlation of pyrethrin (Py) product accumulation and gene expression across all stages of achene development. Py content was measured in whole flowers of stages 1-5 (S1-S5). For the final stages S6-S7 and dry seeds, embryos and pericarps were micro-dissected and separately examined for Py content. Peak areas were determined using specific masses 123 or 133. For the graph S5 and S6 were matched to have the same relative content, but S6 will be somewhat higher. Transcript levels of *CDS, FDS1* and *TcGLIP* were measured in dissected ovaries

stages S2-S7 and normalized against *GAPDH*. All data were determined in triplicate. STE error bars were left out for most data for better visualization.

In a next step, we wanted to know whether pyrethrin biosynthesis occurs in trichomes or the pericarp. To that end, trichomes, ovaries and ovaries from which trichomes had been largely removed were isolated from stage-3 flowers. The efficiency of removing trichomes from the ovaries was 85%, as established by using the STL compound E (exclusively present in trichomes) as an internal standard. Expression of *CDS* in ovaries with trichomes was 5.9% of the expression in trichomes alone, and removing 85% of the trichomes, further reduced *CDS* expression in the ovaries by 76% (1.4% of trichomes). This correlation suggested that virtually all *CDS* expression observed in ovaries is derived from the trichomes (Figure 5A). This was supported by the finding that *CDS* was not expressed at all in seedlings at the cotyledon stage, which do not have any trichomes (Figure 6B; microscopic analysis not shown).

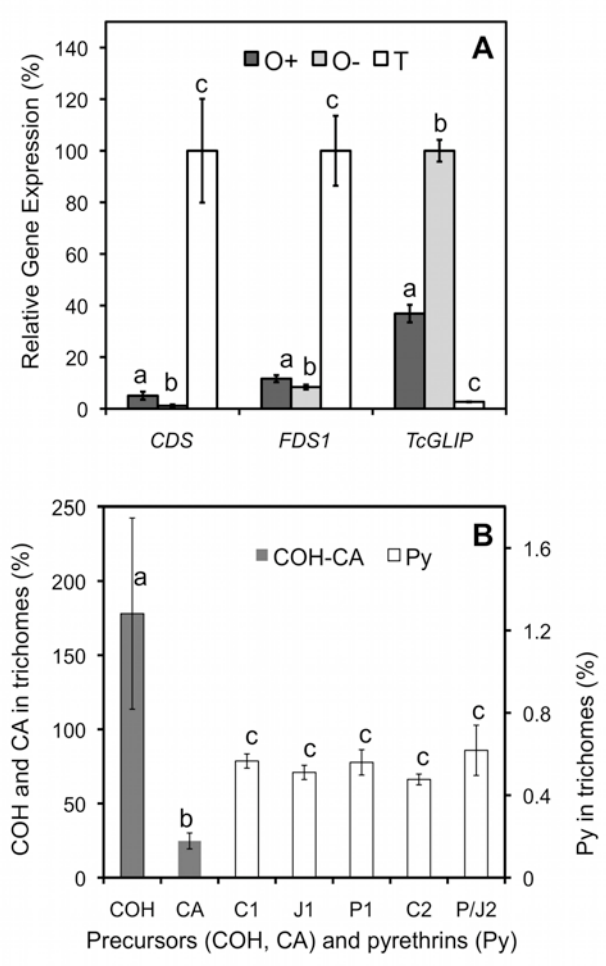

Figure 5: The terpene precursors of pyrethrin biosynthesis are synthesized in trichomes and transported to the pericarp. Panel A: Relative gene expression of *CDS, FDS1,* and *TcGLIP* in ovaries with trichomes (O+), ovaries with 85% less trichomes (O-), and, isolated glandular trichomes (T) after normalization against the *GAPDH* gene. Lower case letters indicate significant differences for that gene relative to the other tissues ($P < 0.05$). Panel B: The relative content of precursors chrysanthemol (COH) and chrysanthemic acid (CA), and pyrethrins (Py – right y-axis) that can be traced to be specifically contained in trichomes and not in pericarp (see also Supplemental Tables 2 and 3 online, peak areas of specific masses were integrated per compound). Lower case letters indicate significant differences. Replication n=3. Error bars represent standard errors.

To verify these observations at the enzyme level, we did CDS enzyme assays on crude protein extracts of seedlings, ovaries with trichomes, ovaries with trichomes partially removed and trichomes alone. Except for the seedlings, all these tissues showed background chrysanthemol even without the addition of DMAPP. However, its concentration increased 5-8 times in the presence of DMAPP and subsequent phosphatase treatment (Table 1, Control vs. DMAPP), showing that most of the chrysanthemol originates from CDS enzyme activity. These *de novo* CDS enzyme activities closely match the estimated trichome numbers in the different tissue preparations as calculated from the sesquiterpene lactone (peak E) content. Comparing the specific CDS activity per unit protein we observed a 30-fold difference between trichomes and ovaries with less trichomes, but the activity in the latter preparation also obviously derived from the remaining trichomes, and the actual difference in specific activity between pericarp and trichomes is, therefore, much higher (Table 1). Furthermore, just as in the transcript analysis, CDS activity was not detected in seedlings, which are devoid of trichomes.

Table 1: Specific chrysanthemol diphosphate synthase (CDS) activity in crude protein extracts of seedlings, ovaries and trichomes

Tissue[a]	Treatment[b]	Chrysanthemol[c] (area/µl x1000)	Trichome lactones[d] (area/µl x1000)	Protein (ng/µl)	Specific CDS Activity (area/ng)
Seedling	Control	0	0		
	DMAPP	0	0		
	De novo	0 (0%)	(0%)	114	0
Ovaries with trichomes	Control	42 ± 1.6	712 ±166		
	DMAPP	211 ± 27	537 ±212		
	De novo	169 (100%)	(100%)	176	193
Ovaries with less trichomes	Control	9.2 ± 4.8	229 ± 76		
	DMAPP	70 ± 0.8	178 ± 67		
	De novo	61 (36%)	(32%)	132	93
Glandular trichomes	Control	9.9 ± 4.8	130 ± 62		
	DMAPP	51 ± 18	110 ± 104		
	De novo	41 (24%)	(20%)	2.80	2973

[a] CDS activity was based on extracts of 250 mg tissue. Glandular trichomes were removed from 250 mg ovaries with ~68% efficiency and recovered with ~20% efficiency, column 4.

[b] In the treatments *De novo* refers to the subtraction of chrysanthemol content of controls from DMAPP incubated extracts

[c] Chrysanthemol peak areas of specific mass 123 are given (n=3). In brackets relative total CDS activity is given

[d] Trichome lactones are peak areas of specific mass 83 (n=3) of sesquiterpene lactone (STL) compound E and representative of trichome numbers in various tissue preparations. In brackets calculated relative trichome numbers are given averaged for Control and DMAPP

The expression of *TcGLIP* was surprisingly different from that of *CDS*. Its expression in the ovaries with trichomes was 14-fold higher than the expression in trichomes alone, and removing 85% of the trichomes further increased *TcGLIP* expression to a 39-fold difference with trichomes (Figure 5A). Also in seedlings *TcGLIP* expresses at levels similar to leaves which is unexpected because in seedlings there is no *CDS* gene expression or CDS enzyme activity at all (Figure 6B).

The expression of *FDS1,* involved in sesquiterpene and other isoprenoid precursor production is intermediate between *CDS* and *TcGLIP*. Expression in ovaries with trichomes was 18% of the expression in trichomes (5-fold lower), and removing 85% of the trichomes reduced *FDS1* expression in the ovaries by 57% compared with the ovaries with trichomes, resulting in 13-fold lower *FDS1* expression compared with trichomes (Figure 5A). *FDS1* expressed equally high in seedlings compared with leaf tissues (Figure 6B). Just like *TcGLIP, FDS1* is also expressed in non-trichome tissues, although at 8- to 13-fold lower levels, which is in line with its broader role in plant metabolism.

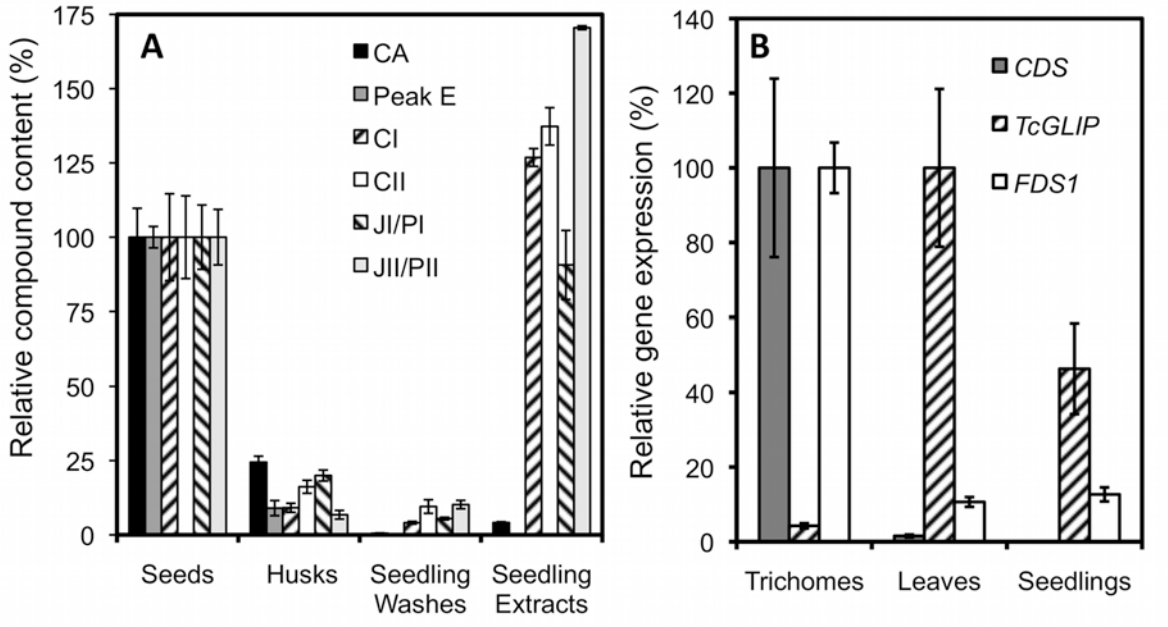

Figure 6: Pyrethrins from embryos protect seedlings. Panel A: Relative peak areas (specific masses) of chrysanthemic acid, STL peak E and the different pyrethrin esters in husk extracts, seedling washes and seedling extracts relative to intact seeds (100%). Panel B: Relative gene expression of CDS, TcGLIP and FDS1 in trichomes, leaves and seedlings after normalization with GAPDH. All data were determined in triplicate. Error bars represent standard errors. See Supplemental Table 2 and 4 online for supporting data.

Chrysanthemic Acid is Exported from Trichomes in Basipetal Direction to the Pericarp

The transcript and enzymatic results strongly suggest that biosynthesis of the terpene precursors of pyrethrins by CDS occurs in the trichomes, but that the esterification step most likely occurs in the pericarp. This implies that there should be transport of CDS products from the trichomes to the pericarp. We hypothesized that biosynthetically active trichome tissues, should contain transient, low concentrations of terpene precursors and possibly also pyrethrins if trichomes would perform that step as well, despite the low transcript levels of *TcGLIP*. To analyze this, the chemical composition of trichomes (knocked off in liquid nitrogen) derived from biosynthetically active ovaries (stage 3, just after opening of the flower), was compared with the chemical composition of intact ovaries (supplemental Table 3) and ovaries from which the trichomes had been removed (Figure 5B). After normalization against trichome-derived peak E, which is specific for trichomes it was established that chrysanthemol (COH) is exclusively present in the trichomes (Figure 5B, COH, 178±64% is not significantly different from the trichome specific peak E distribution, 100±20%, see Supplemental Table 3). COH is the primary product of CDS after dephosphorylation, and this finding directly supports our claim that all enzyme activity is contained in the trichomes. In a next step COH is oxidized into chrysanthemic acid (CA), but trichomes only contained 25±5% CA, with the remaining 75% being in the pericarp. Apparently CA is exported from the trichomes to serve as a substrate for TcGLIP, which is localized in the pericarp to make pyrethrins. Indeed, the relative content of pyrethrins was extremely low in trichomes (0.55%), and there was no significant difference in concentration between these esters relative to the complement in the pericarp from which the trichomes were isolated.

In conclusion, these experiments show that the terpene precursor of pyrethrin biosynthesis, CA, is synthesized via COH in the trichomes, and is then transported to the pericarp, where it is used by the pericarp-specific TcGLIP to synthesize pyrethrins (Figure 7).

Pyrethrins Accumulating in Pericarp Tissues, are Absorbed by the Embryo and End up in Seedlings

Pyrethrins appear to accumulate in the intercellular space of the pericarp surrounding the developing embryo based on the observation of electron-dense areas, suggestive of lipophilic metabolites in Figure 2C and our finding in Figure 3B that pyrethrins represent the major class of compounds extractable by organic solvents from pericarp and embryo tissues. To obtain better evidence on the localization of pyrethrins during seed maturation and dessication we separated and analyzed the pyrethrin content of embryo and pericarp

tissues of flower stages 6 and 7 and mature seeds (Figure 4). The content of pyrethrins decreases in pericarp tissues from stage 7 to mature seeds, while it increases at the same rate in the embryo during that phase. These observations prompted us to investigate what happens after seed germination. GC-MS analysis of the chemical composition of seeds, husks, and seedlings showed that all pyrethrin esters originally present in the achene (Figure 6A, seeds), but only 4% of CA, were present in seedling extracts after germination. Only 7-24% of these esters and CA remained in the husk after the seedling had emerged. Dipping of the seedlings in chloroform, to wash off any compounds present on the surface, resulted in 4-10% of pyrethrins (Figure 6A). The STLs, on the other hand, did not migrate to the seedling as they were not detected in any seedling tissue after germination, and only a small amount remained in the husk.

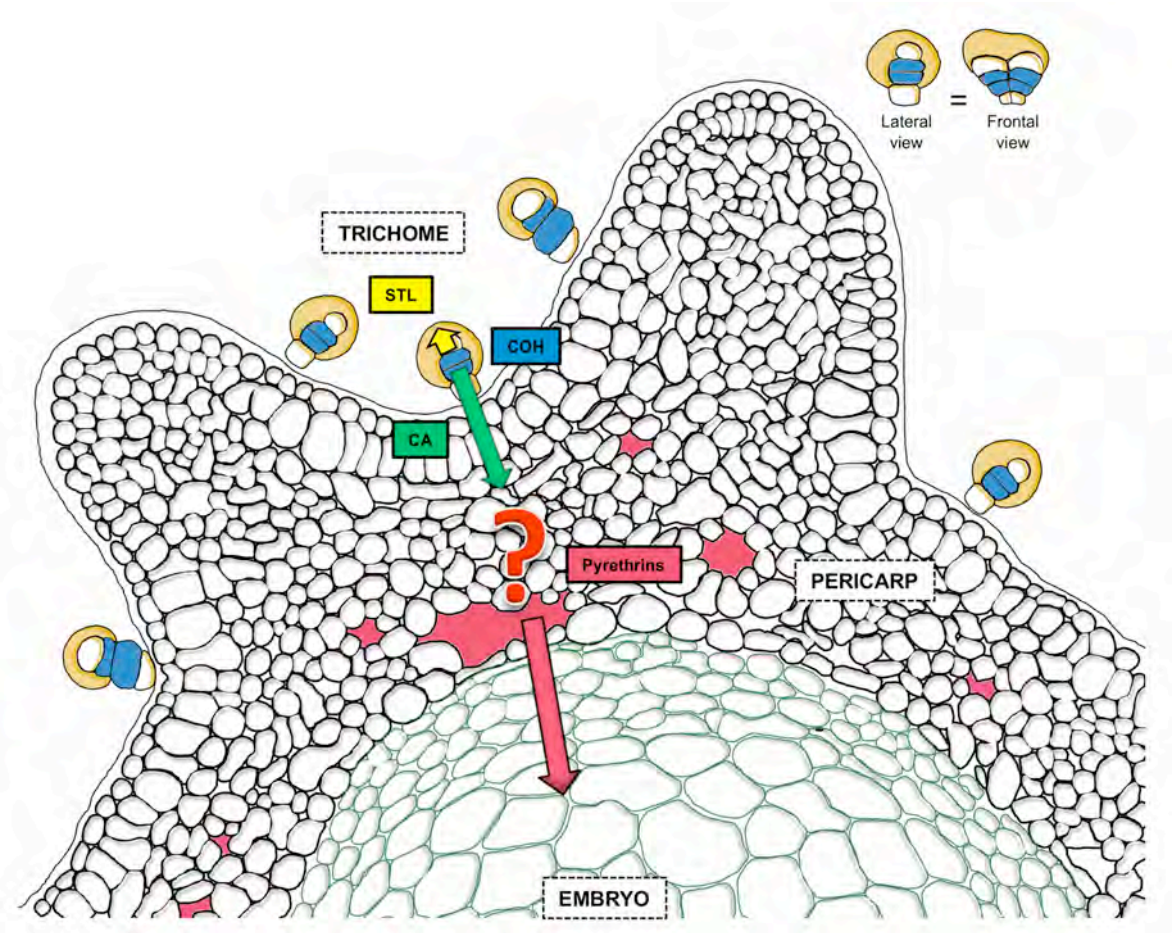

Figure 7: Proposed scheme of bidirectional secretions from glandular trichomes of pyrethrum (*Tanacetum cinerariifolium*) that enable immunization of seedlings. Sesquiterpene lactones (STL) are secreted in apical direction from the white apical cells to the subcuticular space (yellow). In the blue subapical cells the terpene substrate of pyrethrin biosynthesis is synthesized. This leads to the formation of chrysanthemyl diphosphate and chrysanthemol. Chrysanthemol is oxidized into chrysanthemic acid and then exported to the pericarp (green arrow). The enzyme TcGLIP which forms pyrethrin esters by joining chrysanthmoyl-CoA to lipid alcohols resides in the pericarp and is active in the apoplast according to Kikuta et al (2012), but the question mark

indicates that it is unclear where and how the esterification occurs precisely. Pyrethrins are subsequently accumulating in the intercellular spaces of pericarp, surrounding the embryo. During seed maturation they are absorbed into the embryo. Seeds germinate with high pyrethrin concentrations in the seedlings. Trichomes are a biseriate stack of 5 paired cells oriented along the groove of the seed. The inset shows the lateral and frontal views of the trichomes for the purpose of clarity. The figure was based on an original CryoSEM image shown in Supplemental Figure 2 online.

To distinguish whether pyrethrins in the seedlings were synthesized *de novo* or recruited from the embryo, the expression of *CDS, TcGLIP* and *FDS1* was analyzed in seedlings. *FDS1* and *TcGLIP* were expressed in leaves and seedlings, whereas *CDS* was not expressed at all in seedlings (which do not have trichomes) at both the RNA and enzyme level, while it was highly active in the true leaves of older seedlings, which are rich in glandular trichomes (Figure 6B, Table 1). The lower concentrations of CA and STLs may be explained by loss of trichomes during seed germination, metabolization, or release to the medium. In the case of CA it may be that the TcGLIP activity in the cotyledons is using the remaining maternal CA to synthesize new pyrethrins, which could explain the relatively higher levels compared to the original seeds. These combined results demonstrate that pyrethrins are translocated from the pericarp to inner tissues of the maturing embryo, and finally to inner tissues of the germinating seedling (Figure 7).

Pyrethrum Seed Trichome Content is Insect Antifeedant and Pyrethrins have Antifungal Properties.

The association of pyrethrins and trichome STLs with leaves and germinating seedlings suggests that they may have multiple roles in plant defense. The effects of pyrethrins against insect herbivores have been published [1]. To also know the potential effects of the STL trichome content on insect herbivores, we assayed its effects on a specialist (*Pieris brassicae*) and a generalist (*Mamestra brassicae* L.) caterpillar. At the highest tested dose (300 ng/mm^2). the trichome oil showed deterrent activity against both the specialist (Figure 8A) and the generalist (Figure 8B) caterpillars ($P<0.05$). At a lower concentration (60 ng/mm^2) only the specialist caterpillar was affected ($P < 0.05$) (Figure 8A).

The effects of pyrethrins and trichome oil against pathogens have not been tested before. The fungal pathogen *Rhizoctonia solani* primarily attacks belowground plant parts such as the seeds, hypocotyls, and roots, but can also infect aboveground plant parts such as pods, fruits, leaves and stems. It is an important pathogen of pyrethrum causing root rot and wilting of the plants [33]. The most common symptom of *Rhizoctonia* disease is

referred to as "damping-off" characterized by non-germination of severely infected seeds, whereas infected seedlings can be killed either before or after they emerge from the soil. To test the toxicity of trichome oil and pyrethrins against this fungus, we assayed radial growth at two concentrations (70 and 220 µg/ml). At a concentration of 220 µg/ml, pyrethrins slowed radial growth of *R. solani* by 50% whereas trichome oils reduced growth by 30%. After 3-fold dilution the trichome oil was ineffective whereas pyrethrins still inhibited fungal growth significantly by 38% (Figure 8C). The average weight of seedlings was 3.5 mg and one seed contained 45-65 µg pyrethrins. The concentration pyrethrins in the seedlings is, therefore, about 1.5-2% (w/FW) which is approximately 70-100 fold higher than the concentration yielding 50% inhibition of *R.solani* radial growth *in vitro.* Pyrethrins may, therefore, prevent or inhibit germination of spores of pathogens, and hence contribute to seedling survival.

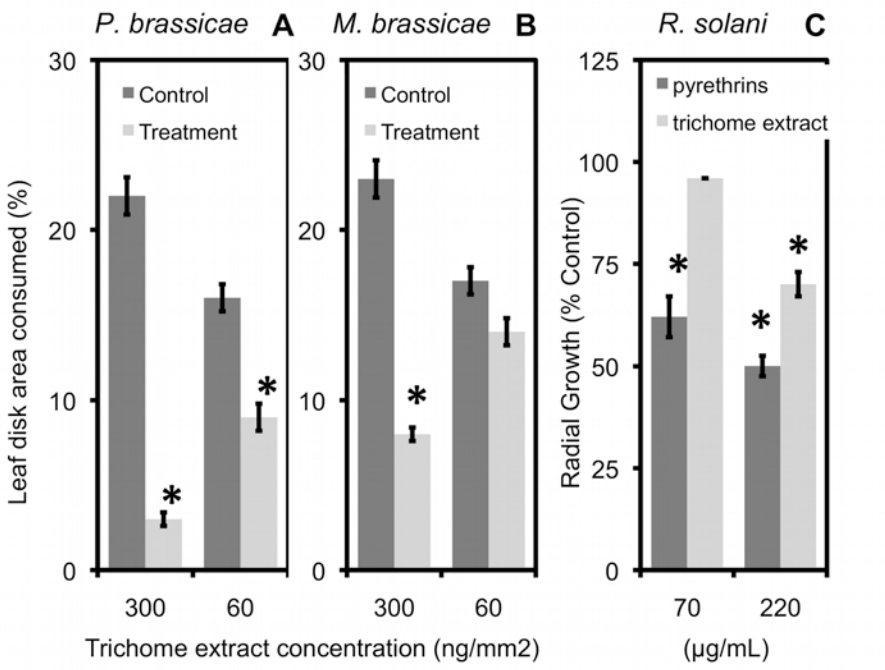

Figure 8: Pyrethrum Seed Trichome Content is Insect Antifeedant and Pyrethrins have Antifungal Properties. Percentage of cabbage leaf disk area consumed by the specialist caterpillar *Pieris brassicae* L. (A) and the generalist caterpillar *Mamestra brassicae* L. (B) in relation to the surface concentration of trichome extract (void of pyrethrins). Replication n=20. Panel C: *Rhizoctonia solani* radial growth reduction given as percentage of control for two tested concentrations (70 and 220 µg/mL) of trichome compounds and pyrethrin oil. Replication n-=3. (*) Indicate significant differences between treatment and control ($p < 0.05$; Student's t-test). Error bars represent standard errors.

Discussion

Terpene Metabolites of Pyrethrum Seed Trichomes are Selectively Transported in Apical and Basipetal Directions

The current state of the art is that glandular trichomes store mono- and sesquiterpene compounds in the subcuticular cavity just above the apical cells, or emit them into the headspace [29,30]. Here, we show that specific terpenoid metabolites produced in glandular trichomes can also be selectively secreted and sequestered in basipetal direction. More specifically, we demonstrate that a trichome selectively secretes sesquiterpenoids in apical direction and monoterpenoids in basipetal direction (Figure 7).

Previous research by Brodelius and co-workers [34,35] has provided evidence that sesquiterpene and monoterpene production may to some extent be divided between apical and subapical cells of *Artemisia annua* glandular trichomes. The evidence so far suggests that artemisinin biosynthesis genes are expressed in both layers [35]. However, germacrene A synthase (germacrene A is a precursor of STLs in pyrethrum) has only been detected in chloroplast-free apical cells [35], and 1-deoxy-D-xylulose-5-phosphate reductoisomerase (DXR) participating in the plastidial methyl erythritol phosphate (MEP) pathway and linalool synthase (LS) were amplified only from the chloroplast-containing sub-apical cells [34,35]. *A. annua* trichomes have an additional stalk cell layer compared to pyrethrum glandular trichomes, but otherwise they are phenotypically very similar [31]. To understand the mechanism of selective secretion of STLs and pyrethrins we, therefore, propose that STLs of pyrethrum are mainly produced in the apical cell layer without chloroplasts, allowing immediate secretion into the subcuticular space, whereas monoterpenoid pyrethrin precursors are produced by the subapical cells with chloroplasts. CDS in pyrethrin biosynthesis is dependent on the MEP pathway [8], and is localized in chloroplasts [10]. Secretion of pyrethrin precursors from these subapical cells may be blocked in apical direction, and result in default secretion in basipetal direction, or, alternatively, there may be an active process involving specific proteins that transport monoterpenoid precursors of pyrethrins in basipetal direction. Duke [31] studied very similar glandular trichomes of *Artemisia annua* and showed by TEM that osmiophilic (=lipid) material is produced profusely by chloroplast-containing subapical cells, and is present between plasma membranes and cell walls that do not border the subcuticular space. This supports the contention that the subapical cells of biseriate capitate glandular trichomes from Asteraceae are able to secrete osmium-colored products (most likely terpenoids) into the intercellular space, even though it does not prove basipetal transport occurs.

Further research is needed to confirm this task division of apical and subapical cells in sesquiterpenoid and monoterpenoid production and secretion, and to understand which transport mechanisms are operating. That work must also answer the question how TcGLIP forms pyrethrin esters in the pericarp, from where it recruits the lipid alcohols, and whether this occurs intracellularly or in the apoplast as recently proposed [12]. The C-terminus of TcGLIP ends with the peptide NDEL which is a well-known endoplasmic reticulum retention signal [36]. It is possible, therefore, that this enzyme is in fact retained in the ER, and that the C-terminal GFP fusions have resulted in the artefact secretion into the apoplast [12].

The biosynthesis of monoterpenoids that do accumulate in trichomes was described best for peppermint [30]. Peppermint accumulates large quantities of monoterpenoids in peltate glandular trichomes, which consist of one basal cell, one stalk cell, eight glandular secretory cells and a sub-cuticular oil storage cavity. Trafficking of monoterpenoids occurs between plastid, ER and mitochondria for several oxidation steps, but like sesquiterpenes they are stored in the apical subcuticular cavity immediately contacting the secretory cells [30]. The biosynthesis and transformation of the monoterpene indole alkaloid vinblastine in *Catharanthus roseus,* and of several benzylisoquinoline-derived alkaloids in opium poppy are organized differently. There, the biosynthesis does not occur in trichomes, and is mediated by secretion and uptake of intermediates among at least three cell types. They move from the internal phloem cells or phloem parenchyma, to the epidermis or cells surrounding the laticifers, and, for their final modification and storage, to laticifers and idioblasts [30]. Similar intercellular transport mechanisms of monoterpenoid intermediates may also occur in the biosynthesis of pyrethrins, where it is necessary, for example, that chrysanthemic acid is activated first by Coenzyme A for TcGLIP to produce pyrethrins.

Translocation to the intercellular space of leaves and achenes may be an adaptation to accumulate larger pyrethrin quantities, or to preserve the bioactivity of these compounds, as pyrethrins are sensitive to UV degradation [37,38]. Additionally, the mode of action of pyrethrins depends on physical contact with the insect adult, larva or embryo [39]. This may occur more effectively if the toxin is present intercellularly in leaves than when contained in surface glands [39]. Yet, such a mechanism requires that the surrounding cells are tolerant to these compounds, which at high concentrations is not the case for many terpenoids [40]. Ultimately, however, the significance of a potentially common mechanism of basipetal secretion is, that secretory trichomes may participate in the synthesis of compounds, that have diverse physiological and defensive functions in the organism also elsewhere outside the trichomes.

A Novel Pathway of Immunizing Seedlings

Our results show that the biosynthesis of pyrethrins starts in the trichomes present on the pericarp of the achenes. Intermediates are exported and utilized in the pericarp to synthesize pyrethrins which accumulate in intercellular spaces, and are absorbed by the desiccating embryo. Upon germination these embryo-stored pyrethrins allow the recruitment by seedlings of 100-fold higher concentrations of pyrethrins than normally found in leaves. This seed trichome-mediated pathway of immunization of seedlings has not been described before..Cotyledons of pyrethrum completely lack trichomes, and, thus, without investing itself in defence at all, the uptake by the embryo of pyrethrins from maternal pericarp tissues guarantees a very high level of protection of seedlings [39]. Similar chemical seedling defences derived from maternal tissues are based on e.g. phenolics [41], alkaloids [42], cyanogenic glucosides [43] or glucosinolates [44]. Those secondary metaboliltes are not formed in trichomes, but are also directly stored in cotyledons or endosperm, already at the embryonic stage. Whether pyrethrin absorption is a passive or an active process remains to be investigated, by for example testing pyrethrin absorption by embryos of other plant species or by overexpressing putative transporter genes in model organisms such as yeast or *Xenopus* oocytes [44].

Roles of Pyrethrins and Trichome Oil in Plant Defense

Secondary metabolites from pyrethrum stored in trichomes or in leaves and seeds provide leaves and seeds with chemical protection against attacking insect herbivores and pathogens, and potentially result in fitness benefits [39,45,46]. Chemical analysis of pyrethrum leaves and seeds showed that glandular trichomes present on the ovaries have a more complex composition than the glandular trichomes of leaves (Figure 3). The differences may reflect functional adaptations to specific herbivores and pathogens of leaves and seeds (this report), or even the surrounding competing flora by inhibiting root growth [23]. For individual compounds it was demonstrated that they may have specific biological activities, but these activities cannot yet be generalized to an ecological level, and work is needed to study the effects of genetic variation for these traits [24]. Susurluk and colleagues [47] showed that sesquiterpene lactones from *Tanacetum cadmeum* ssp. *cadmeum* aerial plant parts and *Tanacetum corymbosum* ssp. *cinereum* flowers are effective antifeedants for the generalist larvae of *Spodoptera littoralis*. Goren et al. [48] studied the flowers of *Tanacetum argenteum* subsp. *argenteum* (Asteraceae) and isolated a sesquiterpene lactone which also showed antifeedant activity against the neonate larvae of *S. littoralis*. We show that the larvae of the Brassicaceae specialist *P. brassicae* are more sensitive to trichome oil of pyrethrum than the larvae of the generalist *M. brassicae* that also feed on Asteraceae. It is known that specialist herbivores have evolved enzyme

systems to detoxify specific plant compounds associated with their specific diet, whereas generalist herbivores are able to produce enzymes to detoxify a wider range of substrates [49,50,51]. Moreover, particularly *M. brassicae* is known to feed on *Helianthus annuus* (Asteraceae), which produces sesquiterpene lactones that act as antifeedants for other insects [52]. This may explain why *M. brassicae* is less sensitive to the *T. cinerariifolium* trichome compounds applied on cabbage leaves than the cabbage specialist *P. brassicae*.
We observed that upon germination STLs were largely gone from the husks. Presumably, they were dissolved in the germination solution. This argues in favour of roles as antibiotics of soil-borne pathogens or inhibitors of root growth of competing flora as was described for pyrethrum lactones at concentrations of 5 ppm [23], whereas on leaves the antifeedant activity against insects may be more relevant. Surprisingly, pyrethrins were quite potently active to reduce mycelial growth rates of *R. solani,* which is a major root rot and wilt disease of pyrethrum [33]. We were unable to test higher concentrations as the ethanol solvent concentration would then become toxic itself. However, in seedling tissues pyrethrins are present at concentrations 100-fold higher than tested by us, and sensitive pathogens might be stopped during the invasion process. The finding that pyrethrins are potent inhibitors of the plant pathogen *R. solani,* indeed points to a possible dual role for the high concentrations of pyrethrins in seedlings against both pathogens and insects, and *R. solani* might be an important selective pressure to maintain high levels of pyrethrins in seeds. The pathogen may still attack other plant parts as the seedling fresh weight concentrations of 1.5-2% (w/w) are about 70-100-fold higher compared to normal concentrations in leaves. Also here, studies are needed to verify these roles, and the existing high genetic variation for the content of pyrethrins in seeds and leaves could be used for this.
In conclusion, glandular trichomes have been widely studied although so far they are only known to synthesize apically stored, or head-space emitted products. Our findings introduce the need for an extension of the current model of the role and function of glandular trichomes: Trichomes can also secrete products into the intercellular space of sub-epidermal tissues, and these can even immunize seedlings, allowing roles in tissues which are unable to produce such compounds themselves

Materials and Methods

Plant material

T. cinerariifolium seeds were originally obtained from Honghe Senju Biology Co. Ltd. (Kunming, Yunnan, China) [53], and propagated without selection at PRI. Before chemical characterization seeds were selected by sorting and sieving. Plants were grown in the

field in a sandy soil, supplied with a regular fertilizer regime. After an initial flush plants were flowering continuously from June until the first frost in November. Samples were taken from flowers between the months of June and September

Cryo-Scanning Electron Microscopy

Whole pyrethrum disc florets and transverse cut sections of disc florets and embryos isolated from mature seeds were mounted on a brass cylindrical sample holder with TBS (Tissue Freezing Medium EMS, Washington, PA, USA) for imaging of their morphology. All samples were first frozen in liquid nitrogen. To prepare cuttings, florets were placed in a cryo-ultramicrotome (Reichert Ultracut E/FC4D) and cut at a specimen temperature of -100 °C with glass and diamond knives, (Histo no trough, 8mm 45 °C, Drukker International, The Netherlands). The planed samples as well as the whole flowers were placed in a dedicated cryo-preparation chamber (CT 1500 HF, Oxford instruments, UK), freeze dried for 3 min at -90 °C at $1x10^{-4}$ Pa to remove ice contamination from the surface, and subsequently sputtered with a layer of 10 nm Pt. The samples were cryo-transferred into the field emission scanning microscope (JEOL 6300F, Japan) on the sample stage at -190 °C. All images were recorded digitally (Orion, 6 E.L.I. sprl, Belgium) at a scan rate of 100 sec (full frame) and a size of 2528 x 2030, 8 bit [54].

Extraction and analysis of trichome and ovary contents

Seed secretory trichome content was isolated by a brief extraction in 100% chloroform ($CHCl_3$) as described by Duke et al. (1994) [55]. 1 ml of solvent were pipetted into a glass vial containing 100 mg of dry seeds and vortexed for 30 sec. The solvent was carefully removed from the vial and dehydrated by passing through a Pasteur pipette filled with 2 cm Na_2SO_4. Leaf trichome contents, on the other hand, were isolated by submerging a fresh leaf of known weight in 5 ml $CHCl_3$ for 15 sec, followed by a similar dehydration step on sodium sulphate.

In order to get sufficient trichome oil for the insect bioassays, 5 g of *T. cinerariifolium* seeds were extracted for 30 sec in 35 ml $CHCl_3$. After filtering and drying through a 60 ml glass funnel with a glass fritted disc filled with Na_2SO_4, the clear extract was collected in a 250 ml glass evaporation balloon and distilled off with a rotary evaporator (Büchl Rotavapor-R; Switzerland) under vacuum at 150-200 mbar. The solution was reduced to 1 ml, split into three equal volumes and evaporated to complete dryness (10-20 mg) under nitrogen flow.

Ovary glandular trichomes were isolated from fresh stage-3 flowers of plant genotype 10, collected from the PRI pyrethrum field. For the isolation of ovaries, ray florets were removed and the composite flower head was cut into half. Corollas of disc florets were

abscised at the calyx. The remaining ovaries were then isolated by cutting the achenes from the receptacle and immediate collection in a 2 ml Eppendorf tube filled with liquid nitrogen. Parts of these ovaries (400 mg) were used for trichome isolation. Trichomes were removed from the ovaries with 4 vortex pulses of 1 min and 1 min cooling rest between pulses in liquid nitrogen. Frozen ovaries were spread on a pre-cooled 150 µm mesh mounted over a pre-cooled 50 ml screw-capped tube The trichomes that passed through the mesh were collected at the bottom of the tube and later used for oil extraction with chloroform or RNA isolation with TriPure (Roche). Ovaries without trichomes that remained on the mesh and intact ovaries were collected and stored at -80 ºC until further use.

Frozen isolated trichomes from plant genotype 10 and also from an assorted mixture of genotypes collected from the field, were extracted independently with 1 mL of the appropriate solvent, followed by homogenization with a glass potter and 5-min sonication. Intact ovaries, ovaries without trichomes, intact seeds, seeds after chloroform dipping, intact leaves, and seedlings were flash frozen in liquid nitrogen and ground to a fine powder. The content of the tissues (100 mg) was extracted with 1 ml chloroform, followed by 30 sec of vortexing and 5 min sonication. The extracts were centrifuged for 5 min at 3500 rpm, dehydrated using anhydrous Na_2SO_4 and analyzed by gas chromatography-mass spectrometry (GC-MS) and by gas chromatography- flame ionization detector (GC-FID). Quantification of constituents isolated from trichomes, seeds and leaves was done by relating peak areas to two reference curves, one of parthenolide for the quantification of STLs, and one of pyrethrum oil, for the quantification of pyrethrin esters.

Instrumentation

The GC-MS and GC-FID measurements were conducted on Agilent 7890A gas chromatographs consisting of a 7683 series autosampler, 7683B series injector, and for the mass spectrometer a 5975C inert MSD with triple-axis detector. The detector temperature for the GC-FID was 340^{o}C. Control of the equipment, data acquisition, processing, and management of chromatographic information were performed by the Agilent Enhanced ChemStation E.02.00.493 software. A Zebron ZB-5MS GC13 capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness; Phenomenex, USA) with 5 m guard column was employed for both chromatographic analyses. The injector temperature of the GC intruments was set at 250°C and helium was the carrier gas with a column flow rate of 1.0 ml/min. The injection volume was 1 µl and samples were injected in splitless mode. The oven temperature was held at 45°C for 2 min and programmed to 300°C at 15°C/min, the final temperature was held for 4 min. Total run time per sample was 23 min. The mass spectrometer was operated in the electron ionisation mode (70 eV) with an ion source

temperature of 230°C. The detector was switched on after 4.5 min solvent delay and the full mass-range mode was used for the analyses of the samples with a mass to charge ratio range (m/z) from 45–250 atomic mass units (amu), and a scan time of 0.2 sec and an inter-scan delay of 0.1 sec. If not described otherwise, samples were prepared in $CHCl_3$ and diluted 5x before injection. Constituents of the essential oil were identified by comparing their mass spectra with those of the reference library, the NIST 08 mass spectral database.

Trichome quantification and oil content determination of trichomes and seeds

A number of freshly collected and one-year-old seeds were placed under a Zeiss fluorescence microscope and the number of trichomes was counted. The average density of trichomes per seed or seed surface area was determined by scanning images of seeds using the ImageJ software version 1.40 g (National Institute of Health, United States, http://rsb.info.nih.gov). The average density of trichomes on leaves was determined by counting the number of trichomes on the top and on the bottom of 17 mm diameter young and old leaf discs.

To estimate the amount of compounds found in trichomes and ovaries by GC-MS and GC-FID, the sum of their respective peak areas was correlated to a standard curve that were generated using different dilutions of a 1mg/mL parthenolide stock (0x, 2x, 4x, and 6x) and a series of standard curves generated using three different dilution of pyrethrum oil (0x, 2x and 4x) containing a mixture of the six pyrethrin esters. Independent calibration curves of the different esters were used to estimate their respective amounts in the samples. Based on the average number of trichomes per seed (650 ± 10 for freshly collected seeds) and the number of seeds used in the extraction (100 seeds per mL), the amount of trichome compounds per seed and per trichome were calculated.

Insect dual-choice assays

Trichome oil constituents were tested for their ability to act as insect deterrents. For insect assays a chloroform extract of 15 mg trichome components was evaporated and redissolved in 100 µl methanol and diluted in demineralized water with 0.2 % (v/v) Tween-80 to the appropriate concentration. Tween-80 was added to suspend insoluble fractions and to facilitate spreading on hydrophobic leaf discs. Control solutions contained methanol, Tween80, and demineralized water in the same ratios. Leaf discs of 1.7 cm diameter were cut from cabbage leaves using a cork borer. Six leaf discs were placed in each Petri dish holding a moist filter paper. 10 µl of either the diluted trichome extract or control solution, were pipetted onto a leaf disc in an alternating manner and dispersed

with a saturated paintbrush. The Petri dishes were left open for 20 min to allow the leaf discs to dry.

One fifth instar (L5) caterpillar of the specialist *Pieris brassicae*. (Lepidoptera: Pieridae) or the generalist *Mamestra brassicae* (Lepidoptera: Noctuidae) was gently placed in each Petri dish and left to feed for 3 h. Two concentrations, 60 ng/mm², and 300 ng/mm², of trichome oleoresin were tested for both *M. brassicae* and *P. brassicae.* Results were scored by scanning the remaining leaf disc areas and analyzing the differences in size using the image software ImageJ. The consumed areas were calculated for treatment and control,discs and a paired Student's t-test was used to assess differences in consumption of control and pyrethrin-treated leaf discs. Experiments were done in 20 replicates

Insect rearing

The insect herbivores were maintained on Brussels sprouts plants (*B. oleracea* var. gemmifera L. cultivar Cyrus) in acclimatized rooms at 20–22 °C, 50–70% relative humidity and a 16/8 h light/dark photoperiod. *M. brassicae* moths were offered only filter paper as oviposition substrate, without contact to cabbage plants.

Fungal growth inhibition assays.

Pyrethrin oil (70 % pyrethrins plus 1 % Butylated Hydroxy-Toluene (BHT), gift from Honghe Senju Biological Co. Ltd) and trichome oil constituents were tested for their ability to inhibit the growth of *Rhizoctonia solani* AG2.2IIIB. For the inhibition assays the trichome extract (15 mg) and the pyrethrum oil (2 M) was dissolved in 1000 µl EtOH to achieve a 50 mM stock concentration (calculated using MW: 306 of β-cyclopyrethrosin), which were further diluted in R2A agar (BD Difco) to their final concentration (0, 0.2, 0.6 and 2 mM). Control stock solution contained either EtOH or EtOH/BHT in the same ratios. All media were transferred in 3 mL quantities to six-wells microtiterplates. Each well was inoculated with a 5 mm agar disk containing densely grown *R. solani* mycelium and incubated at 20 °C in the dark. Growth was monitored during six days and the radial growth was registered after 1, 2, 3 and 6 days. All tests were performed in triplicate.

Expression analysis

For RNA extraction plant tissue was homogenized by adding one pre-cooled grinding ball to each 2 ml Eppendorf containing 50-100 mg of frozen plant tissue and using a pre-cooled Mikro-disembrator II (Braun; Germany) for 1 min at maximum speed. After careful removal of the beads, RNA was isolated using TriPure (Roche), purified using RNeasy Mini Kit (Qiagen), and transcribed into cDNA using TaqMan Reverse Transcription reagents (Applied Biosystems) according to the manufacture's instructions.

RT-qPCR was used to study the expression of Chrysanthemyl diphosphate synthase (*CDS*), farnesyl diphosphate synthase (*FDS1*) and GDSL lipase (*TcGLIP*) in cDNA derived from different tissues. Gene specific primers were design using Beacon Designer Software (TciCDS-F: 5'-CATCTTCTGGACCTCTTCAATGAG-3', TciCDS-R: 5'-GTACTGAACAATCCGACGGTTAAG-3', TciFDS1-F: 5'-GGTTGGTATGATTGCTGCGAAC-3', TciFDS1-R: 5'-TGAACAGGTCAACAAGATCCAC-3', TciTcGLIP-F: 5'-TTGAGAACTAAGGCAACCTGTAGG-3', TciTcGLIP-R: 5'-ACCTCTTGTCTGAGCACATATAAGC-3'). *T. cinerariifolium* GAPDH gene (TciGAPDH-F: 5'- AGACGAGTTTCACAAAGTTG-3' and TciGAPDH-R '5-AGGAATCTGAAGGCAAGC-3') was used for normalization. PCR reactions were prepared in duplicate by mixing in a 500 µL tube, 22.5 µL iQ SYBR green supermix 2x (Biorad), 4.5 µL sense primer (3 µM), 4.5 µL antisense primer (3 µM), 11.5 µL deionized water, and 2 µL cDNA template. After vortexing, each mix was distributed into two wells in 20 µL amounts. Quantification of the transcript level was performed in a in an MyiQ iCycler system (Bio-Rad Laboratories, USA) using a three-step program, which included (i) enzyme-activation at 95 °C for 3 min, (ii) 40 cycles of 95 °C for 10 sec, 60 °C for 30 sec, and (iii) 95 °C for 1 min, from 65 °C to 95 °C for 10 sec for dissociation curve analysis. At the end of each run, amplified products were sequenced to verify their identity. Relative expression values were calculated using the efficiency δCt method [56]. Average efficiencies of the primers were 2.04 for GAPDH, 2.02 for CDS, 2.08 for FDS1 and 1.91 for TcGLIP.

Germination assay

For germination assays, 2 g of *T. cinerariifolium* seeds were placed in a 50 ml polypropylene tube containing 40 ml of tap water and hydro-primed by incubating for 30 min at 27 ºC in a water bath. After removing the water, seeds were spread onto 8.5 cm diameter Petri dishes containing moist cotton wool covered with a 9 cm diameter Whatman Qualitative 1 filter paper. Seeds were incubated in the dark at 4 ºC for 3 days to synchronize germination. After the cold period, seeds were transferred to room temperature and kept in the dark until germination commenced. Once the first seeds started to germinate, plates were transferred to the light.

Twenty-five fully developed seedlings, 25 husks, and 25 primed seeds were collected in a 2 ml Eppendorf, flash frozen in liquid nitrogen and ground to a fine powder by using a pre-cooled Mikro-disembrator II (Braun; Germany) for 1 min at maximum speed. The whole content of the tube was extracted with 1 ml 100 % chloroform ($CHCl_3$) and analyzed for pyrethrin content by GC-MS according to the protocol previously described. In order to determine whether pyrethrins are found on the outside of seedlings, prior to being frozen they were subjected to a washing step consisting of dipping the 25 seedlings for 10 sec in

a 4 mL glass vial containing 2 ml 100% chloroform ($CHCl_3$). The solution was finally brought to 1ml by evaporating the excess solvent under a nitrogen flow. The procedure was repeated 3 times (3 independent replicates).

Enzymatic assay

Protein extracts were prepared from seedlings, ovaries with trichomes, ovaries without trichomes and trichomes. Ovaries without trichomes and trichomes were prepared as previously described. Two hundred fifty grams of frozen material and the trichomes isolated from 250mg of ovaries were homogenized by adding a pre-cooled grinding ball to each tube and using a pre-cooled Mikro-dismembrator II (Braun, Germany) for three minutes at maximum speed. The plant material was re-suspended in 500 µL of extraction buffer containing 200mM Tris-HCl (pH8), 15mM β-mercaptoethanol. 1.5% polyvinylpyrrolidone 40 and 30% glycerol. The slurry was incubated at 4^{o}C for 20 min in a rotator. After incubation, samples were centrifuged two times for 10 min at 17.000xg at 4^{o}C. After the second centrifugation step the supernatant was immediately used for the enzymatic assay.

For the enzymatic assay, 100 µL of supernatant was incubated with 100 µL of DMAPP (3.3mM in Ethanol) and 400 µL buffer (15mM MOPSO pH7.5, 12.5% (w/w) glycerol, 1mM ascorbic acid, 0.001% (v/v) Tween20, 1mM MgCl2, and 2mM DTT) overnight at 30oC with shaking. Control assay were prepared in the same way but replacing the 100 µL DMAPP by 100 µL ethanol. After incubation samples were subjected to phosphatase treatment by adding 100 µL Glycine (30mM), 25 µL ZnCl2 (100mM), and 3.5 µL phosphatase, incubating for 1h at 37oC. After adding 250mg NaCl samples were extracted with 1mL ethyl acetate. Followed by 5min centrifugation at 1200xg, the organic phase was dried using a anhydrous Na2SO4 glass-wool plugged column and analyzed on a GC-MS instrument as described before. Protein determination of soluble enzymes was done by Bradford assay (BioRad) according to the manufacturer's instructions.

Pyrethrin content determination of pericarp and embryo

Embryos were separated from the pericarp of ovaries from flower developmental stages 6 and 7 and seeds. Fifteen frozen ovaries of each stage and 15 fully developed seeds were cut open with the help of a scalpel and the embryo was carefully separated and removed. Embryos, the remaining pericarp tissues and intact ovaries or seeds were flash frozen in liquid nitrogen, ground to a fine powder, extracted with 1mL CHCl3, and analyzed by GC-MS according to the protocol described above. The isolation procedure was repeated three times with the exception of developmental stage 6, from which embryos were still very small and difficult to isolate,

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Supplemental data

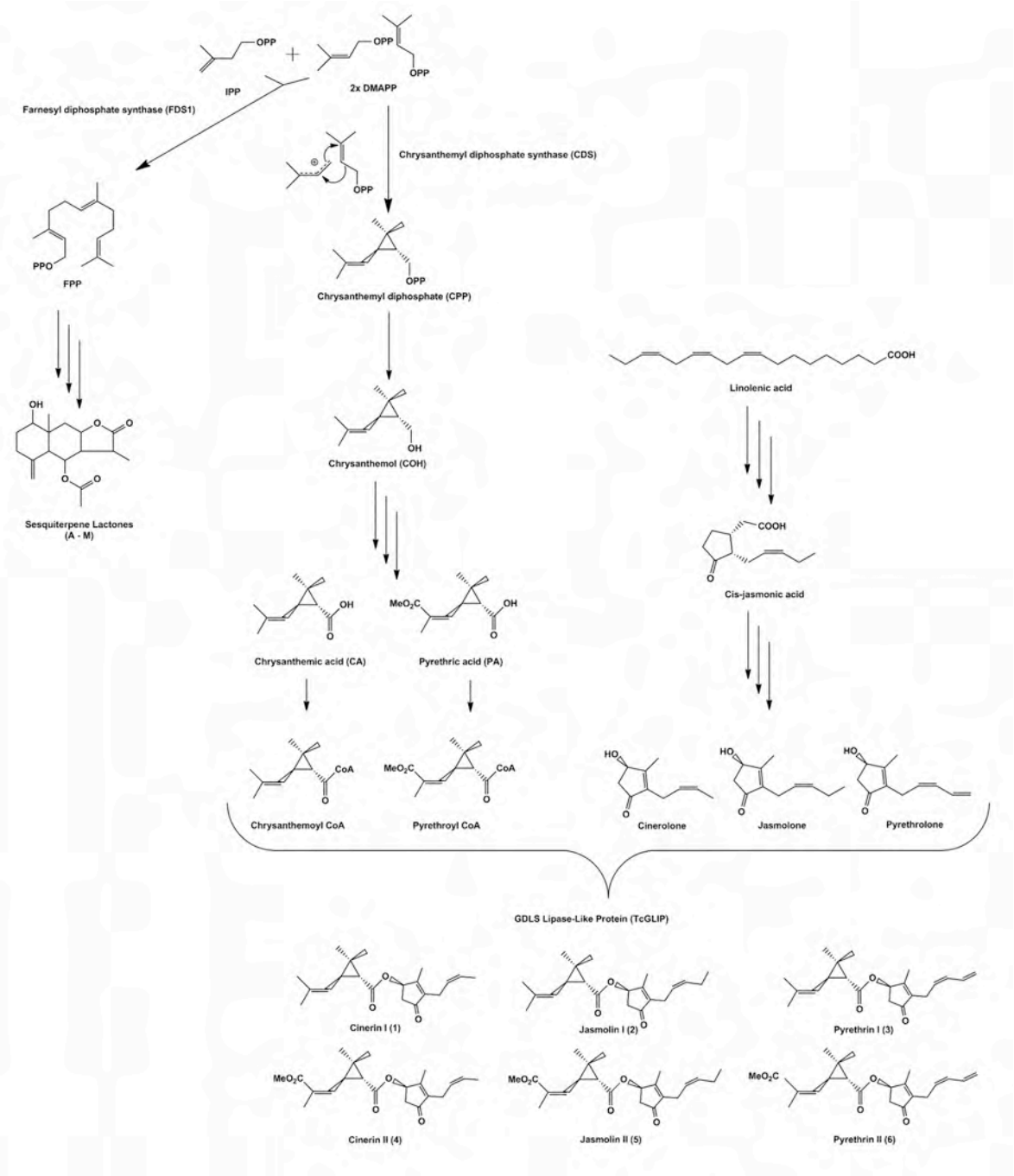


Figure S1: Biosynthesis of pyrethrins and sesquiterpene lactones

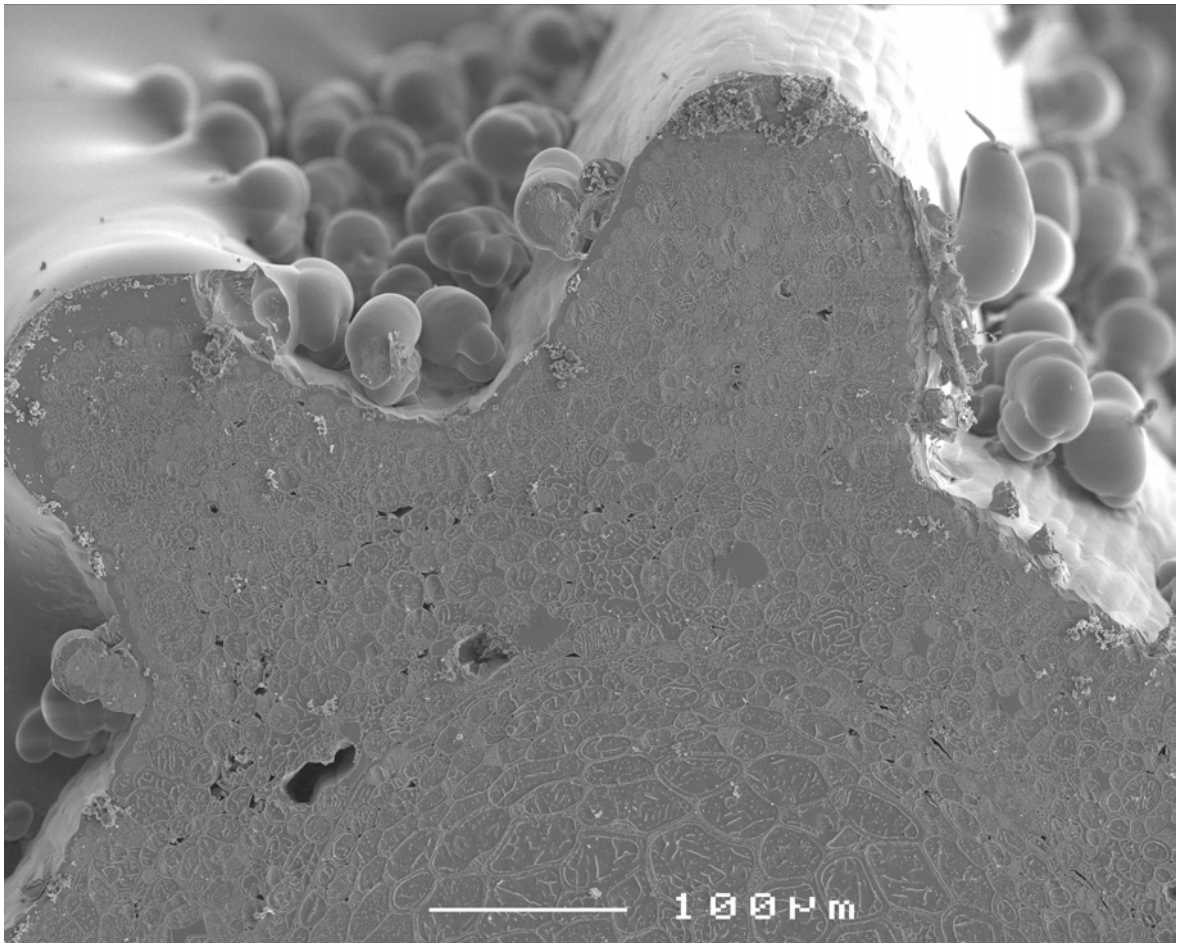
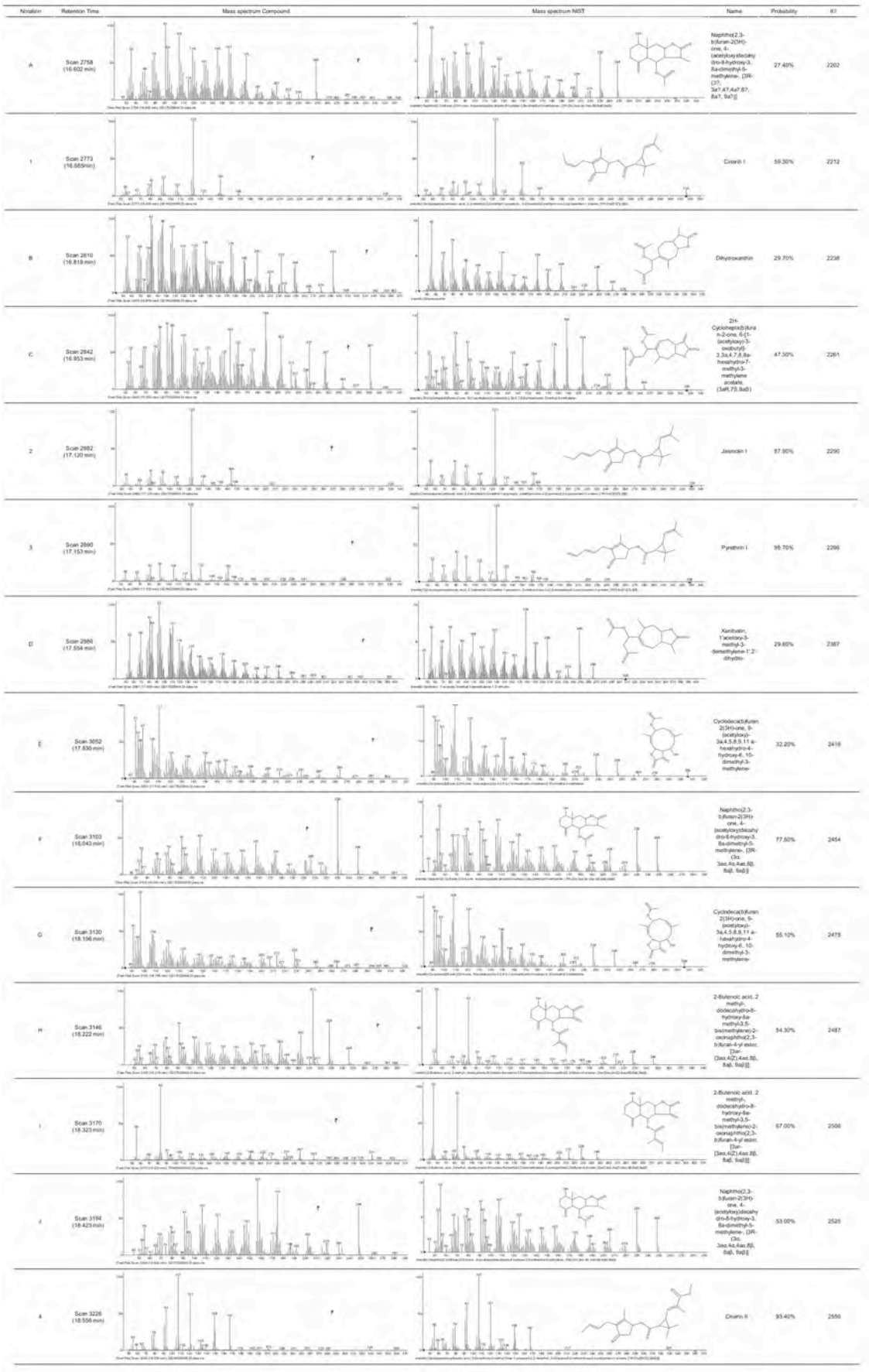


Figure S2: CryoSEM image of a cross section of an achene that was used to draw Figure 7.

Table S1. List of compounds detected by GC-MS



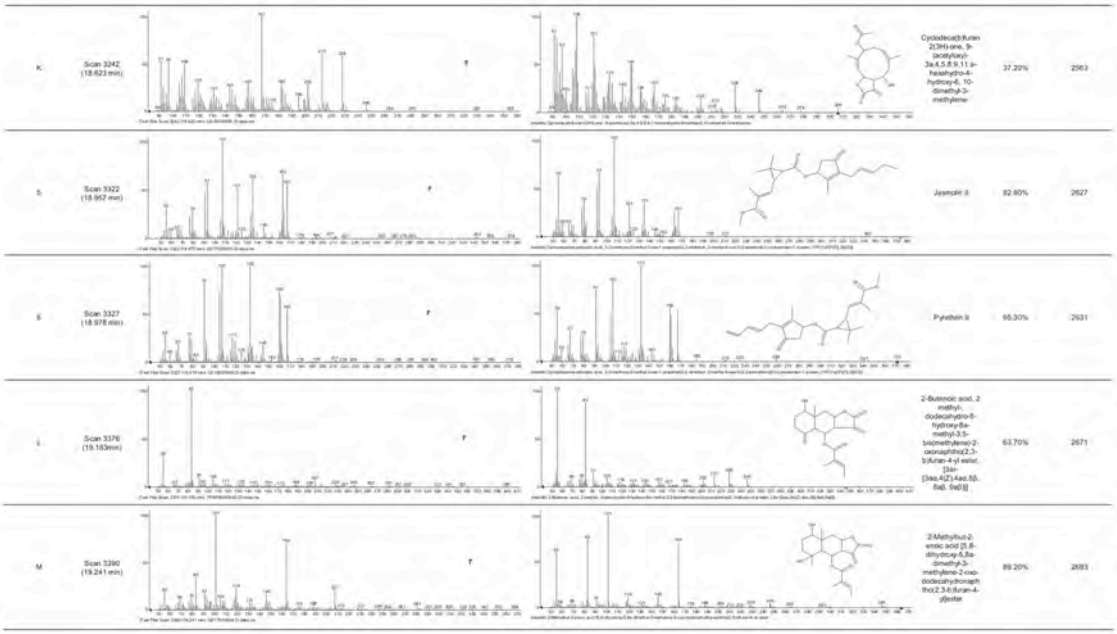

K
Scan 3242
(18.623 min)
Cyclodeca[b]furan-
2(3H)-one, 9-
(acetyloxy)-
3a,4,5,6,9,11-a-
hexahydro-4-
hydroxy-6, 10-
dimethyl-3-
methylene-
37.20%
2563
L
Scan 3322
(18.957 min)
Jasmolin II
82.60%
2627
E
Scan 3327
(18.978 min)
Pyrethrin II
95.30%
2631
J
Scan 3376
(19.183min)
2-Butenoic acid, 2
methyl-,
dodecahydro-6-
hydroxy-8a-
methyl-3,5-
bis(methylene)-2-
oxonaphtho(2,3-
b)furan-4-yl ester,
[3ar-
[3aa,4(Z),4aa,5b,
8ab, 9a]]]
63.70%
2671
M
Scan 3390
(19.241 min)
2-Methylbut-2-
enoic acid [5,8-
dihydroxy-6,8a-
dimethyl-3-
methylene-2-oxo-
dodecahydronaph
tho[2,3-b]furan-4-
yl]ester
89.20%
2693

Table S2: Non-normalized and normalized RT-qPCR results for 4 biosynthetic genes and the GAPDH gene (used for normalization)

	Ovaries with trichomes	Ovaries without trichomes	Trichomes	Leaves	Seedlings
Ct values (uncorrected with GAPDH)					
GAPDH	17.14	16.62	17.59	17.00	18.26
CDS	19.20	20.74	15.57	20.99	31.00
TcGLIP	19.76	17.76	24.02	18.88	21.25
FDS	18.75	19.45	16.75	19.39	20.4
Ct SD values (SD, uncorrected with GAPDH)					
GAPDH	0.31	0.35	0.29	0.45	0.37
CDS	0.21	0.20	0.50	0.50	1.01
TcGLIP	0.22	0.09	0.35	0.45	0.54
FDS	0.04	0.11	0.16	0.28	0.33
Percentage values relative to the highest expressed gene (not corrected with GAPDH)					
GAPDH	33.7	48.3	24.7	37.1	15.5
CDS	8.1	2.8	100.0	2.3	0.0
TcGLIP	5.5	21.9	0.3	10.1	2.0
FDS	11.0	6.8	44.1	7.1	3.5
Percentage error (STE, uncorrected with GAPDH)					
GAPDH	4.7	7.7	3.2	7.8	2.6
CDS	0.7	0.2	23.9	0.6	0.0
TcGLIP	0.5	0.8	0.0	2.1	0.5
FDS	0.2	0.3	3.0	0.9	0.5
Percentage values relative per gene (not corrected with GAPDH)					
GAPDH	69.7	100.0	51.1	76.8	32.1
CDS	8.1	2.8	100.0	2.3	0.0
TcGLIP	25.0	100.0	1.3	46.0	8.9
FDS	25.0	15.4	100.0	16.0	8.0
Percentage error (STE, uncorrected with GAPDH)					
GAPDH	9.7	15.9	6.6	16.2	5.4
CDS	0.7	0.2	23.9	0.6	0.0
TcGLIP	2.4	3.7	0.2	9.7	2.3
FDS	0.4	0.7	6.8	2.0	1.2
Percentage values relative to the highest expressed gene (corrected with GAPDH)					
GAPDH	24.7	24.7	24.7	24.7	24.7
CDS	5.9	1.4	100.0	1.6	0.0
TcGLIP	4.0	11.2	0.3	6.7	3.1
FDS	8.1	3.5	44.1	4.7	5.6
Percentage error (STE, corrected with GAPDH)					
GAPDH	3.4	3.9	3.2	5.2	4.2
CDS	0.5	0.1	23.9	0.4	0.0
TcGLIP	0.4	0.4	0.0	1.4	0.8
FDS	0.1	0.2	3.0	0.6	0.8
Percentage values relative per gene (corrected with GAPDH)					
GAPDH	100.0	100.0	100.0	100.0	100.0
CDS	5.9	1.4	100.0	1.6	0.0
TcGLIP	35.8	100.0	2.6	59.9	27.7
FDS	18.3	7.9	100.0	10.7	12.7
Percentage error (STE, corrected with GAPDH)					
GAPDH	13.8	15.9	12.9	21.1	16.9
CDS	0.5	0.1	23.9	0.4	0.0
TcGLIP	3.4	3.7	0.4	12.7	7.3
FDS	0.3	0.4	6.8	1.3	1.9

Table S3: Content of pyrethrin biosynthesis related compounds in ovaries and trichomes in terms of specific masses and relative to ovaries with less trichomes before and after normalization with the content of STL (measure for trichome quantities).

	mass	specific mass peaks			standard error of specific mass peaks		
		O+	O-	T	O+	O-	T
Chrysanthemol (COH)	**123**	13,707	4,398	41,200	6,405	1,126	14,916
Chrysanthemic acid (CA)	**123**	146,786	107,356	139,779	36,959	4,396	30,307
Cinerin I (C1)	**123**	19,529,560	22,255,871	662,826	3,114,186	513,384	40,493
Jasmolin I (J1)	**123**	9,098,617	7,838,073	210,668	1,518,776	237,640	14,255
Pyrethrin I (P1)	**123**	4,662,062	4,548,377	133,909	159,101	269,554	14,750
Cinerin II (C2)	**133**	1,692,842	2,108,493	52,890	252,861	20,433	2,895
Pyrethrin II Jasmolin II (P/J2)	**133**	3,103,531	3,505,555	114,003	136,800	112,014	22,493
Dihydro-β-cyclo-pyrethosin (peak E)	**84**	5,240,300	1,826,885	9,613,170	705,367	95,580	1,957,667
		relative % mass peaks			STE relative % mass peaks		
		O+	O-	T	O+	O-	T
Chrysanthemol (COH)	**123**	312	100	936.72	146	26	339.13
Chrysanthemic acid (CA)	**123**	137	100	130.20	34	4	28.23
Cinerin I (C1)	**123**	88	100	2.98	14	2	0.18
Jasmolin I (J1)	**123**	116	100	2.69	19	3	0.18
Pyrethrin I (P1)	**123**	102	100	2.94	3	6	0.32
Cinerin II (C2)	**133**	80	100	2.51	12	1	0.14
Pyrethrin II Jasmolin II (P/J2)	**133**	89	100	3.25	4	3	0.64
Dihydro-β-cyclo-pyrethosin (peak E)	**84**	287	100	526.21	39	5	107.16
		relative % normalized for STL			STE relative % normalized for STL		
		O+	O-	T	O+	O-	T
Chrysanthemol (COH)	**123**	109	100	178.01	51	26	64.45
Chrysanthemic acid (CA)	**123**	48	100	24.74	12	4	5.36
Cinerin I (C1)	**123**	31	100	0.57	5	2	0.03
Jasmolin I (J1)	**123**	40	100	0.51	7	3	0.03
Pyrethrin I (P1)	**123**	36	100	0.56	1	6	0.06
Cinerin II (C2)	**133**	28	100	0.48	4	1	0.03
Pyrethrin II Jasmolin II (P/J2)	**133**	31	100	0.62	1	3	0.12
Dihydro-β-cyclo-pyrethosin (peak E)	**84**	100	100	100.00	13	5	20.36

Table S4. Peak areas based on specific masses (blue panels) of chrysanthemic acid, STL peak E and the different pyrethrin esters in husk extracts, seedling washes and seedling extracts relative to intact seeds (100%, yellow panels).

	Average specific mass				STE average specific mass			
	Seed	Husk	Wash	Seedling	Seed	Husk	Wash	Seedling
CA	6874350	1682126	35985	284801	667373	138626	6552	16777
Peak E	884562	79720			31874	22519		
CI	6115784	558022	249916	7757030	894003	92851	27612	183378
CII	390053	63153	37203	535508	54339	8561	8917	24568
JI/PI	11241149	2244962	617091	10192002	1218054	208433	49623	1305371
JII/PII	2103551	142430	214486	3586957	196549	30822	29716	13279
	Relative content compared to seeds (%)				STE of the relative content compared to seeds (%)			
	Seed	Husk	Wash	Seedling	Seed	Husk	Wash	Seedling
CA	100.0	24.5	0.5	4.1	9.7	2.0	0.1	0.2
Peak E	100.0	9.0	0.0	0.0	3.6	2.5	0.0	0.0
CI	100.0	9.1	4.1	126.8	14.6	1.5	0.5	3.0
CII	100.0	16.2	9.5	137.3	13.9	2.2	2.3	6.3
JI/PI	100.0	20.0	5.5	90.7	10.8	1.9	0.4	11.6
JII/PII	100.0	6.8	10.2	170.5	9.3	1.5	1.4	0.6

Chapter 3

A single Cytochrome P450 Enzyme Catalyzes the Formation of Chrysanthemic Acid from Chrysanthemol in Pyrethrin Biosynthesis

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Abstract

The daisy flowers of pyrethrum, *Tanacetum cinerariifolium*, accumulate a group of potent insecticidal metabolites called pyrethrins. Pyrethrins are esters of an alcohol and an acid moiety derived from the oxylipin and monoterpene pathways. The first committed step in the biosynthesis of the acid moiety leading to the formation of chrysanthemyl diphosphate (CPP) is catalyzed by chrysanthemyl diphosphate synthase (*CDS*). After the formation of chrysanthemol (COH) two oxidative steps are postulated to be required for the formation of the acid precursor of pyrethrins, chrysanthemic acid (CA). Theoretically, these two reactions could be catalyzed by one or two cytochrome P450s and/or dehydrogenases. In pyrethrum EST sequence data, a number of genes encoding cytochrome P450 (CYP) enzymes were developmentally expressed in a pattern similar to the *TcCDS* gene, and specifically in CA-producing glandular trichomes. Experiments with yeast microsomes allowed the selection of two enzymes capable of converting COH into chrysanthemal. When leaves of *Nicotiana tabacum* stably transformed with the *TcCDS* gene were analyzed, COH emissions were accompanied by low levels of non-volatile CA. However, after agro-infiltration with all *CYP* genes candidates, only *Ct21854* resulted in strongly reduced COH emissions. This did not affect the steady state level of CA, but leaves agro-infiltrated with *Ct21854* showed significantly higher amounts of a CA conjugate. Co-infiltration of *Nicotiana benthamiana* leaves with *TcCDS* and *Ct21854* confirmed that only *Ct21854* is a chrysanthemic acid synthase *(TcCYP71BZ1),* which efficiently converts COH into CA.In tobacco, CA subsequently conjugates to a compound putatively identified as 1-2(2,3-dihydro-2-furyl)-4-(thien-2-yl)but-1-en-3-yne.

Introduction

Pyrethrum (*Tanacetum cinerariifolium*) accumulates pyrethrins in the achenes of flowers [1-3] and in leaves [4]. These defense chemicals are neurotoxins that exhibit high contact toxicity against insect herbivores. Pyrethrins are transferred, as the flowers develop, from the maternal tissues of young achenes, to the embryo, and finally protect the emerging seedling [5].Pyrethrins are esters of either chrysanthemic or pyrethric acid with rethrolones (Figure 1). The acid and alcohol moieties of pyrethrins derive from distinct pathways. The alcohol portions pyrethrolone, cinerolone, or jasmolone are linolenic acid derivatives, and share the pathway with jasmonic acid. The acid moieties are monoterpenes with a cyclopropane ring. The enzyme catalyzing the formation of the cyclopropane ring, chrysanthemyl diphosphate synthase (TcCDS), has been studied extensively. TcCDS catalyzes the condensation of two molecules of dimethylallyl pyrophosphate (Figure 2 I) to produce chrysanthemyl diphosphate (Figure 2 II), a monoterpene with a non-head-to-tail or irregular c1'-2-3 linkage between isoprenoid units [6]. Only recently *TcGLIP*, an acyltransferase shown to be a member of the GDSL lipase family, has been purified, cloned and demonstrated to be responsible for the esterification of (1R,3R)-chrysanthemoyl-CoA and (S)-pyrethrolone [7]. The rest of the biosynthetic pathway, and enzymes to dephosphorylate and oxidize chrysanthemol (Figure 2 III), have yet to be identified.

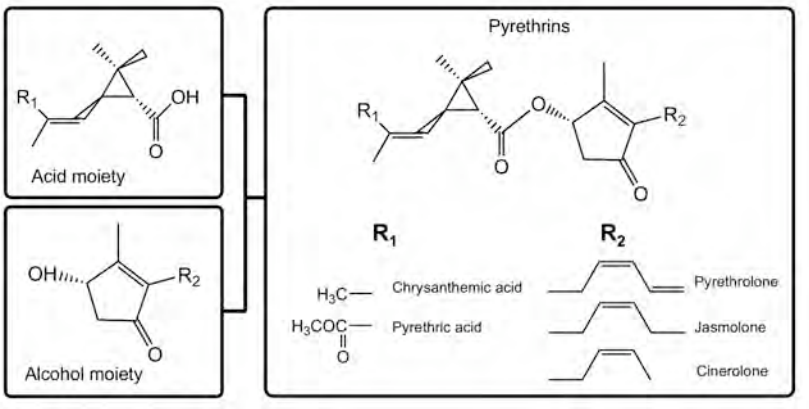

Figure 1: Natural pyrethrin ester insecticides from *Tanacetum cinerariifolium*. The monoterpene acid moieties, chrysanthemic acid or pyrethric acid (R1), form esters with pyrethrolone, jasmolone or cinerolone (R2) yielding six different in total.

In the oxidation of COH, two dehydrogenases, a combination of a dehydrogenase and a P450, or one or two P450s may be involved. In plants, cytochrome P450s are membrane-bound heme-containing proteins which have been implicated in a variety of oxidative reactions in plant secondary metabolism, such as the synthesis of lignin phenolics,

membrane sterols, phyto-alexins and terpenoids [8]. In terpenoid pathways, P450 mono-oxygenases are involved in the oxidation of many different types of terpene backbones [9-12] and best known for their roles in the biosynthesis of sesquiterpenoids [13-15]. Artemisinic acid, the immediate precursor of the antimalarial drug artimisinin, was demonstrated to be derived from a three-step oxidation of amorpha-4-11-diene, catalyzed by a single cytochrome P450 mono-oxygenase, *CYP71AV1* [16]. For the biosynthesis of menthol, a monoterpenoid found in *Mentha* spp., the hydroxylation of (-)-limonene was demonstrated to depend on two regio-specific P450 limonene hydroxylases, (-)-limonene-3-hydroxylase and (-)-limonene-6-hydroxylase [9,17]. Dehydrogenases, on the other hand, also play a role in terpene metabolism [18-20]. During the biosynthesis of menthol, following the hydroxylation of limonene, a dehydrogenase was demonstrated to oxidize (-)-trans-isopiperitenol to (-)-isopiperitenone in peppermint [21]. Similarly, dehydrogenases also play a role in the oxidation of aldehydes to their corresponding carboxylic acid, in a reaction that involves the reduction of NAD^+ and/or $NADP^+$.

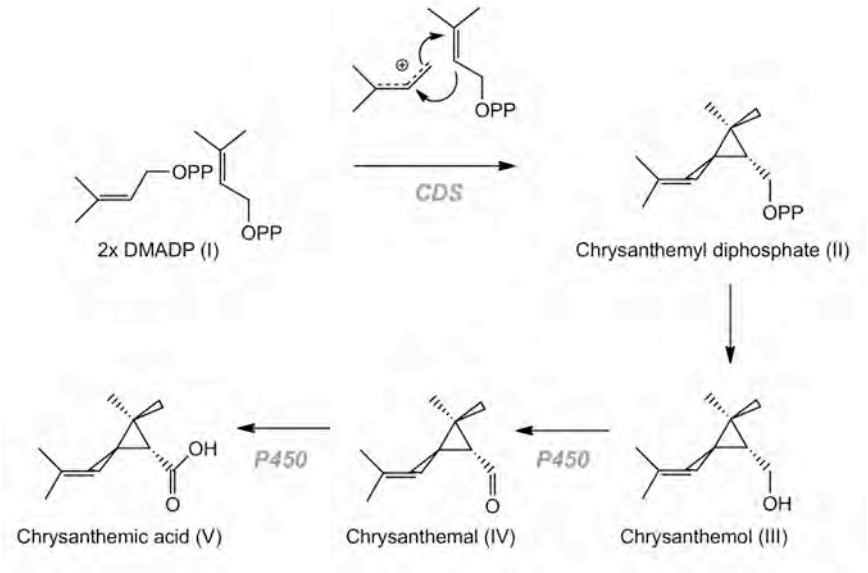

Figure 2: Proposed biosynthetic pathway of the acid moiety of pyrethrins. In grey the identity of the enzyme involved in the first demonstrated dedicated step (CDS) and the proposed cytochrome P450s catalysing one or the two consecutive steps leading to chrysanthemic acid.

In our previous work we demonstrated that pyrethrum trichomes are the site of biosynthesis of COH, which is further modified to CA, before being exported out of the trichomes to the pericarp, where it serves as substrate for pyrethrin biosynthesis [5]. Given this observation and the potential role of single or multiple cytochrome P450s in the oxidative reactions leading to CA, the aim of this study was to clone trichome-specific pyrethrum *CYP* genes, and to test their ability to oxidize chrysanthemol into an aldehyde or acid, using both yeast microsomal preparations and plant expression hosts.

Results

Phylogenetic analysis of 12 trichome expressed *CYP* genes

Strongly expressed *CYP* genes to be evaluated as potential candidates to catalyze one or two of the consecutive oxidative reactions of the COH substrate in pyrethrin biosynthesis were obtained by keyword interrogation of an annotated local trichome EST contig database. Based on contig length in the trichome library (> 1kb), among 26 candidate contigs seven full-length (> 1.5kb) and five partial (1 to1.5kb) *CYP* genes were selected as possibly involved in the formation of chrysanthemal and/or chrysanthemic acid from chrysanthemol.

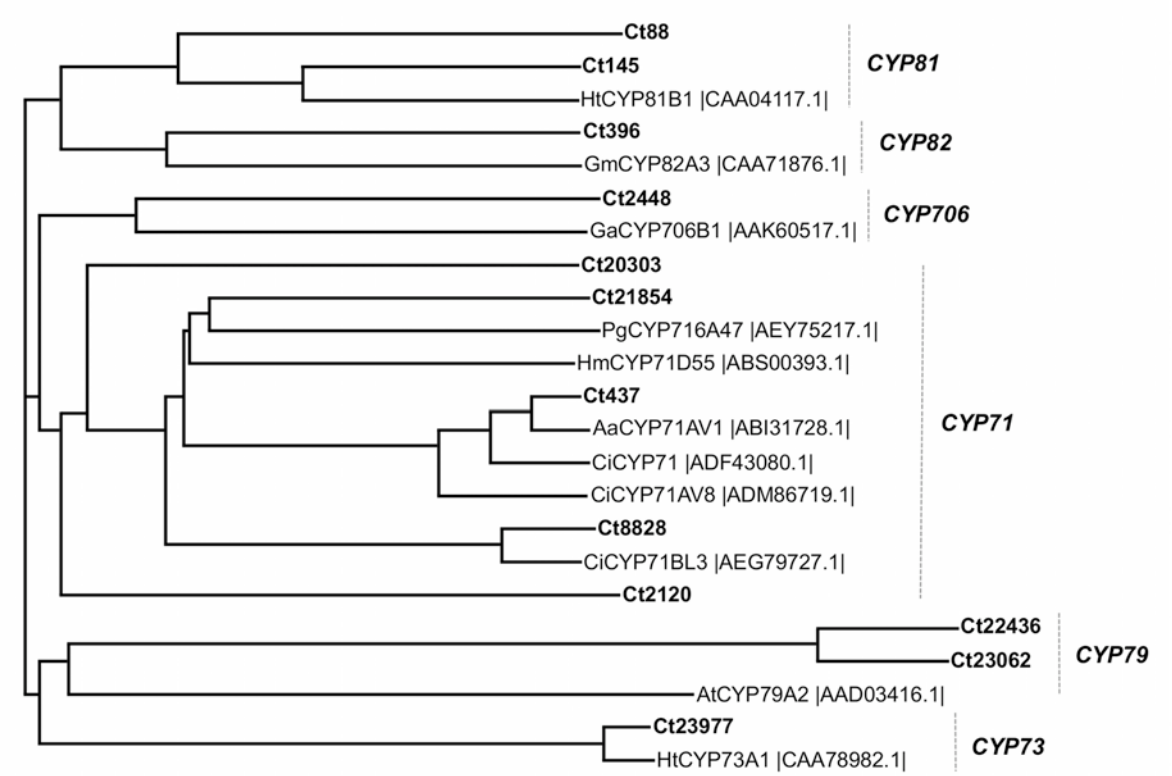

Figure 3: Phylogenetic tree constructed based on the basis of the deduced amino acid sequences of the 12 putative pyrethrum CYP genes and other known related plant CYPs. The tree was constructed by Neighbour-Joining method using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). The species abbreviations are Aa, *Artimisia annua*; Pg, *Penax ginseng*; Ci, *Cichorium intybus*; Hm, *Hyoscyamus muticus*; At, *Arabidopsis thaliana*; Ht, *Helianthus tuberosus*; Ga, *Gossypium arboretum*; Gm, *Glycine max*. (Sequences, primers used, and relevant protein alignments are shown in the Supplemental data, Table S1, Figure S1, and Figure S2).

Full-length genes were obtained (Table S1) and analysis of the phylogenetic relationships between 12 *CYP* genes from pyrethrum and other plant *CYP*s (Figure 3) revealed that

Ct20303 (KC441531), *Ct21854* (KC441525), *Ct437* (KC441527), *Ct8828* (KC441528) and *Ct2120* (KC441530) were most closely related and showed close amino acid sequence similarity with the CYP71 family genes that are known to be involved in terpene biosynthesis. *Ct23977* (KC441536) shows significant amino acid sequence similarity to the *Helianthus tuberosus* CYP73A1 (cinnamate 4-hydroxylase) involved in the phenylpropanoid pathway [22]. Both *Ct22436* (KC441533) and *Ct23062* (KC441537), group together with *Arabidopsis thaliana* CYP79A2, thought to be involved in the biosynthesis of cyanogenic glucosides and glucosinolates [23]. *Ct2448* (KC441534) displays similarity to a mono-oxygenase (CYP706B1), which catalyses the conversion of (+)-δ-cadinene into 8-hydroxy-(+)-δ-cadinene during cotton sesquiterpene biosynthesis [24]. *Ct396* (KC441535) shows sequence similarity to an elicitor-induced cytochrome P450 (CYP82A3) presumably involved in the biosynthesis of glyceollins, the major phytoalexins of soybean [25]. Finally *Ct88* (KC441532) and *Ct145* (KC441529) are phylogenetically related to a *Helianthus tuberosus* CYP81B1, involved in the in-chain hydroxylation of medium chain saturated fatty acids [26].

Functional characterization of pyrethrum cytochrome P450s using yeast microsomal preparations.

The full-length sequences of all twelve putative cytochrome P450s isolated from pyrethrum (*Tanacetum cinerariifolium)* were cloned into a yeast expression vector. To examine the ability of these genes to catalyze the conversion of chrysanthemol to chrysanthemal (Figure 4C) or the two consecutive oxidizing steps leading to chrysanthemic acid, microsomal fractions from WAT11 yeast expressing the twelve *CYP* genes were incubated with chrysanthemyl alcohol for 24 h at 30 ^{o}C. Gas chromatography–mass spectrometry (GC/MS) analysis of the extracted products revealed that except for *Ct2120* and *Ct23977*, all microsomal preparations including the one prepared with the empty vector were able to convert chrysanthemol (Figure 4A) into a product with a GC retention time of 8.22 min and a mass spectrum (Figure 4Ba) that matches the mass spectrum of chrysanthemal (Figure 4Bb). Yeast transformed with *Ct2120* and *Ct23977* were unable to grow properly, resulting in poor microsomal preparations (despite corrections for pellet size), which could explain the inability to find chrysanthemal in the bioassay performed with these two fractions. Although chrysanthemal was found in all assays, only microsomal fractions prepared with *Ct437* and *Ct21854* were repeatedly able to produce about three times more chrysanthemal than the other cytochrome P450s.

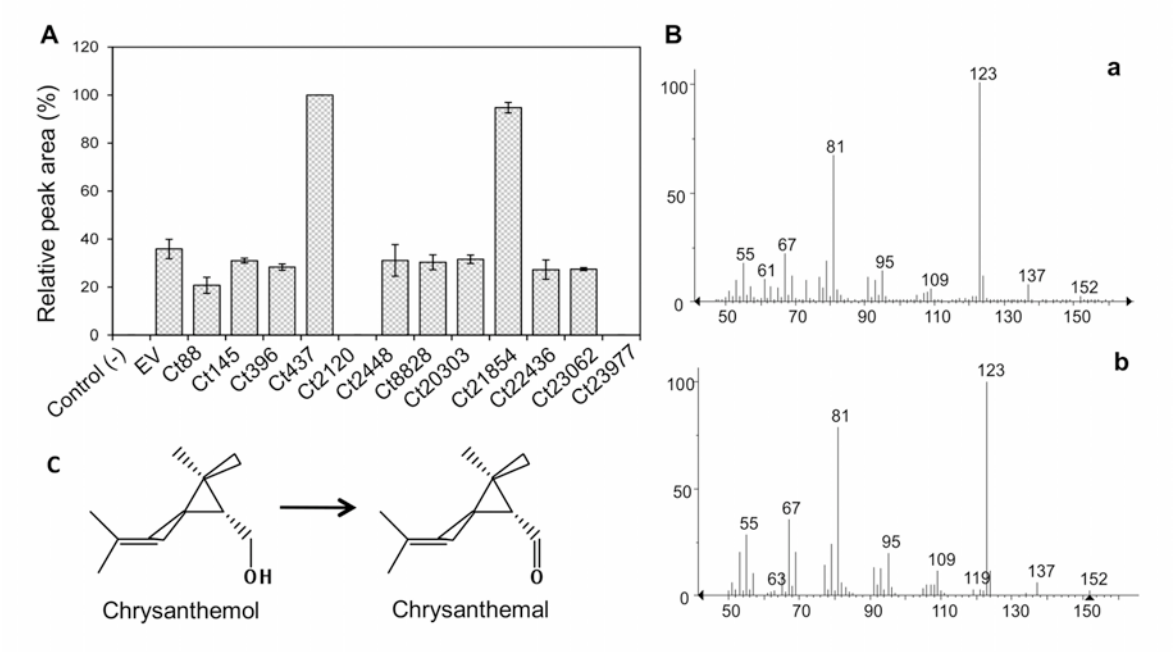

Figure 4: Chrysanthemal production by yeast microsomes overexpressing pyrethrum CYP genes**.** Relative peak areas of chrysanthemal extracted from microsome assays prepared from yeast transformed with the indicated CYP genes and Empty Vector (EV) control (A). Mass spectra of the peak at retention time 8.22 min (a), and chrysanthemal standard (b). Proposed conversion of chrysanthemol to chrysanthemal (C). Error bars represent SD (n=2)

Expression of *Ct437* and *Ct21854* in *N. tabacum TcCDS* stable transformants and *N. benthamiana* plants

The two genes showing chrysanthemal production *in vitro*, *Ct437* and *Ct21854*, and the extra gene of the same CYP71 family (Figure 3), which we were not able to characterize *in vitro*, *Ct2120*, were sub-cloned into pBin binary plant expression vectors and transformed into *Agrobacterium*. Thermo-desorption GC/MS (TD-GC/MS) of the headspace of *N. tabacum TcCDS* stable transformants, agro-infiltrated with these genes, revealed that among the three genes tested, only *Ct21854* was able to significantly (*t*-test, p=0.037) reduce chrysanthemol emission rates by half (Figure 5D) compared to the *TcCDS* control (A), *Ct437* (B), and *Ct2120* (C), while there was no appearance of any new product. Thus, it seemed that in the context of a plant cell only one of the two genes able to produce higher concentrations of chrysanthemal *in vitro*, could specifically take chrysanthemol and convert it into a non-volatile product, not detected in the headspace.

The presence of free chrysanthemic acid was analyzed using UPLC-MRM MS of aqueous methanol extracts prepared from the same *N. tabacum* agro-infiltrated leaves, and *N. benthamiana* leaves co-infiltrated with *Agrobacterium* cultures with *TcCDS* in combination with *Ct2120* or *Ct21854*. Except for *N. benthamiana* infiltrated with the empty vector

(*pBin*), free chrysanthemic acid was detected in all *N. tabacum* and *N. benthamiana* extracts at an average concentration of 8 $ng.g^{-1}$ fresh weight.

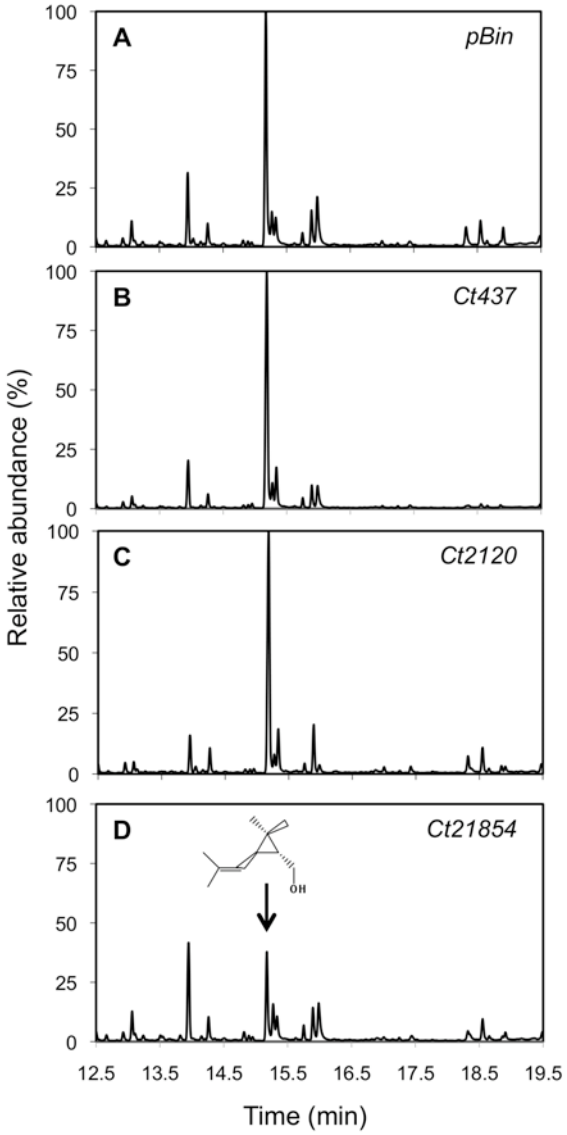

Figure 5: Total ion chromatograms from TD-GC/MS analyses of the headspace of *N. tabacum TcCDS* stable transformants agro-infiltrated with *Agrobacterium tumefaciens* expressing pBin (A), Ct437 (B), Ct2120 (C), and Ct21854 (D). The arrow indicates the chrysanthemol peak at a retention time of 15.23min. Chrysanthemol emissions were significantly (*t*-test, P=0.037) reduced (n=3).

In order to investigate whether any other metabolites were being produced, the methanol extracts previously prepared from the *N. tabacum* and *N. benthamiana* agro-infiltrated leaves, were analyzed using LC-QTOF-MS. Comparison of the chromatograms (Figure 6A) and the corresponding relative peak areas (Figure 6B) showed a 2 times higher concentration of a peak representing a putative CA-conjugate eluting at 12.68 min in *N. tabacum* leaves infiltrated with *Ct21854* compared to leaves infiltrated with the empty

vector. Leaves infiltrated with either *Ct437* or *Ct2120* also showed the presence of the CA-conjugate but at concentrations comparable to those found for the *TcCDS* control (Figure 6B). Similarly, *N. benthamiana* co-infiltrated with *TcCDS* in combination with *Ct21854* showed a concentration of the CA-conjugate that was 6 times higher than the concentrations found in leaves infiltrated with only *TcCDS.* The concentrations of CA-conjugate in leaves co-infiltrated with *TcCDS* in combination with *Ct2120*, were similar to those found for *TcCDS* alone. The compound was not detected in *N. benthamiana* leaves agro-infiltrated with the empty vector control (Figure 6B)

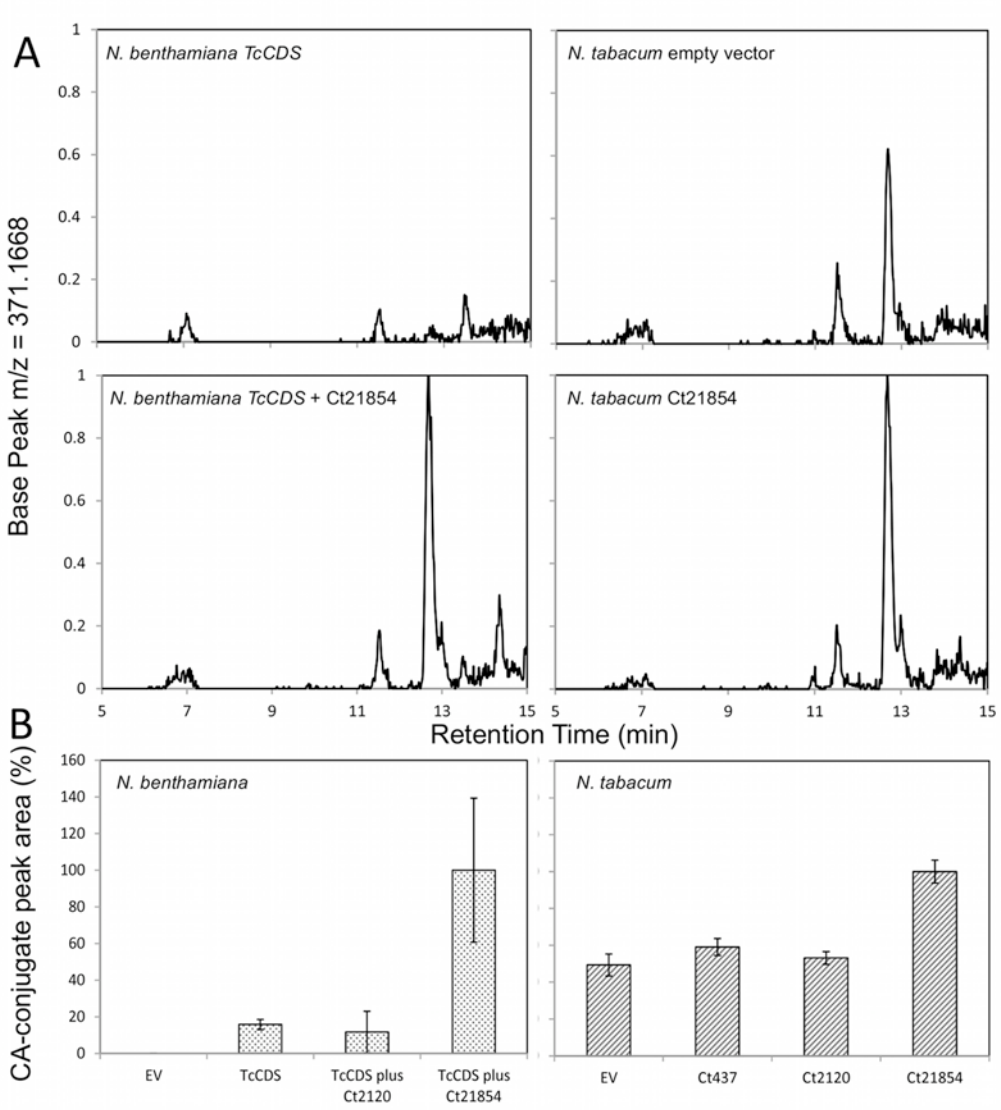

Figure 6: LC-MS analysis of the production of chrysanthemic acid conjugate ($[m/z]^+$= 371, RT: 12.68min). A, LC-QTOF/MS chromatograms of extracts of *N. benthamiana* agro-infiltrated with *TcCDS* and *TcCDS* plus *Ct21854* and *N. tabacum* agroinfiltrated with empty vector and *Ct21854*. B, Relative peak areas of chrysanthemic acid conjugate found for the different treatments in *N. tabacum* and *N. benthamiana*. Error bars represent SD (n=3).

Chrysanthemic acid conjugates

In order to demonstrate that the compound appearing in higher concentrations in the *N. tabacum* leaves infiltrated with *Ct21854* and in the *N. benthamiana* leaves co-infiltrated with *TcCDS* and *Ct21854*, is a chrysanthemic acid conjugate, samples were analyzed by HPLC-PDA-Orbitrap-FT-MS in positive mode. Within the fragments of 371.1668 (parent ion of peak at 16.01min, a 0.9858 ppm deviation from the elemental formula $C_{22}H_{27}O_3S$), we detected an ion with a mass of 169.1221 which co-elutes with mass 371.1668 (Figure 7A/B), and deviates 1.2150 ppm from the elemental formula of chrysanthemic acid ($C_{10}H_{17}O_2$), that elutes at 32.25 min (Figure 7C). Therefore indicating that Ct21854 encodes a cytochrome P450 enzyme capable of converting chrysanthemol into chrysanthemic acid and which was designated as CYP71BZ1 [27]. We also detected a second ion with a mass of 202.0448, a 0.4063 ppm deviation from the elemental formula of $C_{12}H_{10}OS$. This compound, with an elemental formula of $C_{12}H_{10}OS$, and an accurate mass of 202.04535 was putatively identified using the dictionary of natural products (http://dnp.chemnetbase.com) and using an accuracy of 3 ppm, as 1-2(2,3-dihydro-2-furyl)-4-(thien-2-yl)but-1-en-3-yne ($[m/z]^+$= 202.04523) (Figure 7B).

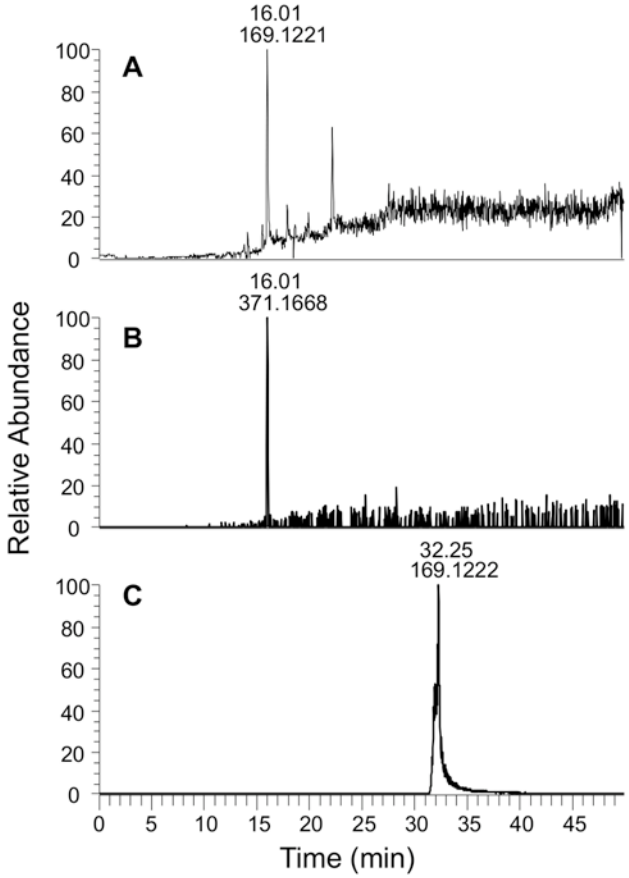

Figure 7: HPLC-PDAESI-MS analysis of chrysanthemic acid and its conjugate . LC-Orbitrap FT-MS chromatograms of [m/z]+=169.1221 (A), $[m/z]^+$=371.1668 in extracts of *N. benthamiana* leaves

agro-infiltrated with *TcCDS* and *Ct21854* (B), and $[m/z]^+$=169.1221 for the trans-chrysanthemic acid standard (C).

Tissue specific and developmentally regulated expression of *CYP* genes

Chrysanthemyl diphosphate synthase (*CDS*) is involved in the formation of chrysanthemyl diphosphate (CPP) and constitutes one of two pathway genes so far identified. Expression of *TcCDS* in the different flower developmental stages shows a relationship with pyrethrin accumulation, and, therefore, their biosynthesis [5]. We hypothesised that *CYP* genes putatively involved in the subsequent steps leading to chrysanthemic acid, would follow the pattern of expression of the *TcCDS* gene in flower development and be trichome-specific.

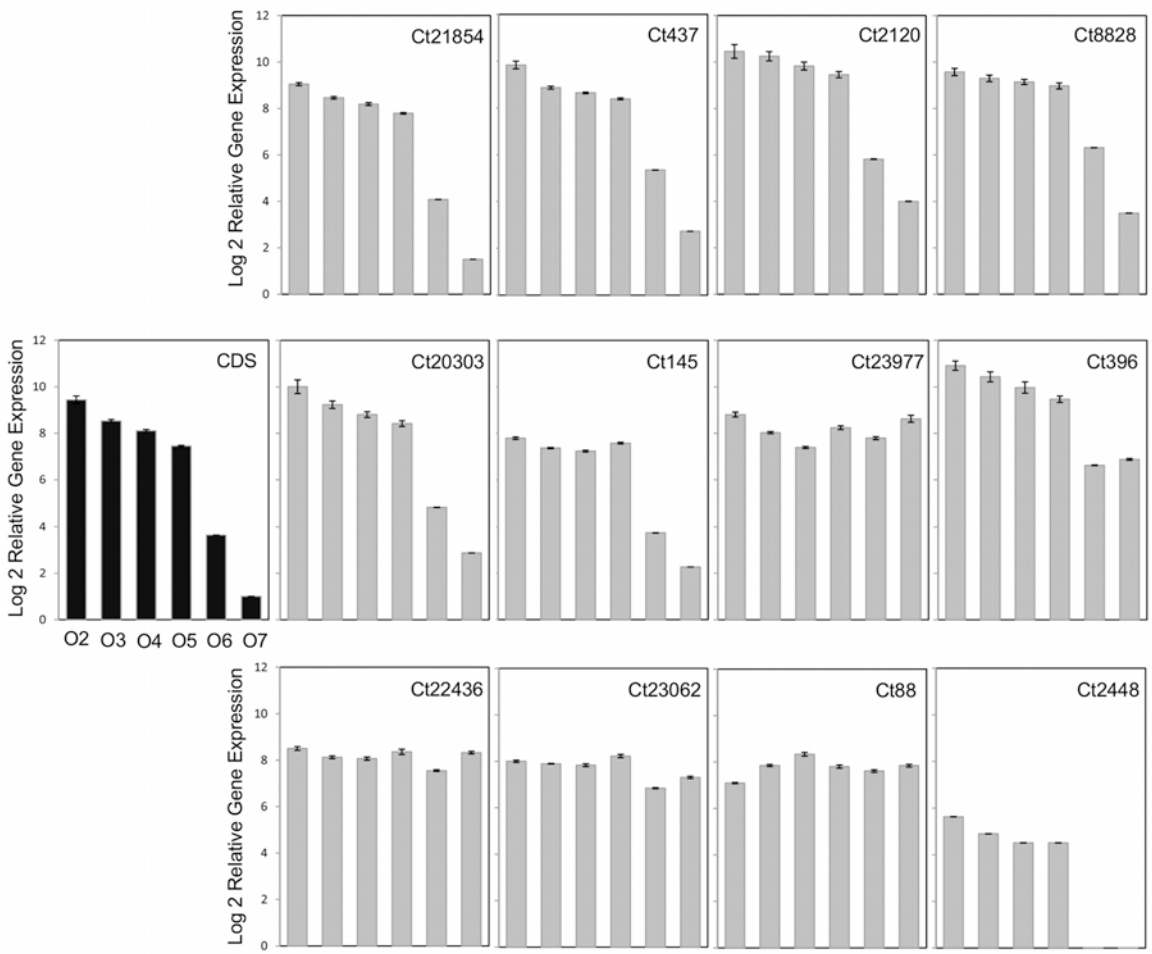

Figure 8: RTqPCR analysis of the 12 *CYP* genes and the *TcCDS* gene in ovaries isolated from flowers at different developmental stages. Error bars represent SD between two technical replicates.

Thus, transcriptional activity of the 12 *CYP* genes was monitored using RTqPCR. Figure 8 shows that all the *CYP* genes that are phylogenetically related to genes belonging to the CYP71 family (*Ct21854*, *Ct437*, *Ct2120*, *Ct20303*, and *Ct8828*), showed a pattern of

expression congruent with the *TcCDS* genes. The expression of all the other *CYP* genes remained rather constant along the different flower developmental stages, except *Ct145* and *Ct2448,* which are more similar to the CYP71 family genes.

Earlier we established that chrysanthemic acid is exclusively produced in glandular trichomes, and that *TcCDS* expression is completely absent in seedlings, which are devoid of trichomes. Here, we investigated which of the two most interesting candidates showed an expression most similar to the expression of the *TcCDS* gene. Figure 9, shows that although both candidates were highly expressed in the trichomes, *Ct437* also showed some expression in the seedlings, which were devoid of trichomes, while *Ct21854* expression was completely absent in the seedlings, similar to what was observed for the *TcCDS* gene.

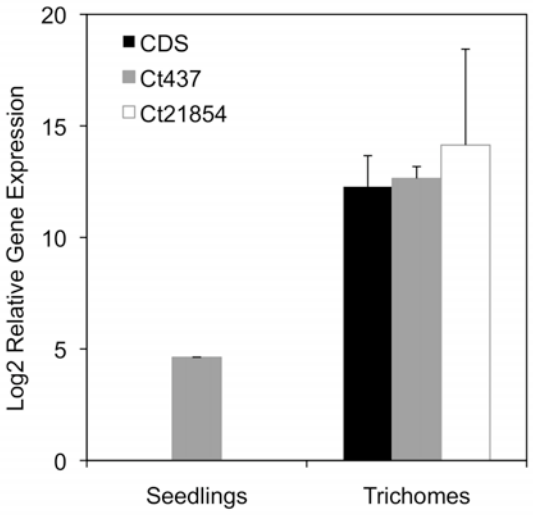

Figure 9: RTqPCR analysis of Ct437, Ct21854 and the *TcCDS* gene in trichomes and seedlings. Error bars represent $_{SD}$ (n=3).

Discussion

Biochemical characterization of *CYP71BZ1* as a chrysanthemic acid synthase

Many reports have shown that the highest concentrations of pyrethrins exist in the ovaries of pyrethrum flowers [1-3]. In our previous study we demonstrated that the acid portion of pyrethrins is exclusively synthesized in the trichomes on the ovaries [5].. These findings, and the possible role of cytochrome P450 enzymes in catalyzing the steps after the formation of chrysanthemol, led us to hypothesize that genes putatively involved in pyrethrin biosynthesis should dominate EST sequences isolated from trichome libraries. Among the 64 trichome contigs that were homologous to *CYP* genes, the 12 most

abundant cDNAs were isolated. In yeast, among the 12 *CYP* genes tested, *Ct437* and *Ct21854* showed significant production of chrysanthemal from chrysanthemol. These two genes were further characterized *in planta.* The disappearance of the endogenous chrysanthemol from the headspace of plants agro-infiltrated with *Ct21854* compared to plants agro-infiltrated with either *Ct437*, *Ct2120* or the empty vector control, suggested that among the two enzymes showing chysanthemal production *in vitro*, *in vivo* only *Ct21854* was capable of converting chrysanthemol into a product, that was not detected by GC/MS headspace analysis. This suggested chrysanthemal, chrysanthemic acid or a derivative as potential products of this reaction *in planta*. The conversion of chrysanthemol into chrysanthemic acid was subsequently demonstrated to be catalyzed by the same cytochrome P450 enzyme, when conjugation products of chrysanthemic acid were clearly identified in two different tobacco species. Although there was some non-specific conversion of chrysanthemol into chrysanthemic acid, likely due to endogenous oxidizing enzymes, the amount of chrysanthemic acid conjugate found in *N. tabacum* and *N. benthamiana* leaves agro-infiltrated with *Ct21854* was in both cases significantly increased. The ability of CYP enzymes to perform two consecutive oxidations has been reported multiple times for other cytochrome P450 enzymes [12,28].

The nature of the conjugate $C_{22}H_{27}O_3S$ we could not solve, but likely consists of chrysanthemic acid conjugated to 1-2(2,3-dihydro-2-furyl)-4-(thien-2-yl)but-1-en-3-yne. The latter compound has been isolated from *Chrysanthemum macrotum* [29], but never reported in *Nicotiana* spp. Others have also found conjugates of terpene acids, but usually those are glycosides. Transient expression of the genes leading to the formation of artemisinic acid in *N. benthamiana* leaves, for example, led to the formation of an artemisinic acid conjugate, artemisinic acid-12-β-diglucoside [30]. In addition, Yang et al. 2011 found that maize plants transformed with geraniol synthase produced geranoyl-6-O-malonyl- β-D-glucopyranoside, a malonyl-glucoside conjugate of geranic acid [31].

Transcriptional and phylogenetic analysis of CYP71BZ1

Pyrethrin accumulation during flower development depends on transcriptional regulation of the genes involved in the biosynthetic pathway [5].The first committed step in the biosynthesis of the monoterpene moiety of pyrethrin esters is catalyzed by chrysanthemyl diphosphate synthase (CDS) [6], which shows an initial high expression during stages 2 to 4 in which the concentration of pyrethrins increases, followed by a down-regulation in stages 5 to 7 as the concentration of pyrethrins reaches a maximum. All the gene candidates of the CYP71 family, showed a pattern of expression that matched with the pattern of expression of *TcCDS* and this feature was therefore not helpful in discriminating these enzymes (Figure 7). In our previous study, we also demonstrated that the synthesis

of chrysanthemic acid occurrs exclusively in trichomes, and the expression of *TcCDS* was high in trichome tissue and absent in tissues devoid of trichomes, such as seedlings [5]. All three candidate genes tested *in planta* showed a high level of expression in trichomes, but only *Ct21854* (CYP71BZ1) completely lacked expression in seedlings. This trichome localization and high similarity in gene expression between *TcCDS* and *Ct21854* (CYP71BZ1) is additional evidence that *Ct21854* (CYP71BZ1 is the enzyme catalizingt the conversion of chrysanthemol into the corresponding acid during pyrethrin biosynthesis.

The phylogenetic analysis of the three candidate genes shows they belong to the CYP71 family, widely known for their role in terpene metabolism. The two most relevant candidates, *Ct437* and *Ct21854* belonged to two distinct subgroups. The *Ct21854* includes a recently characterized ginseng cytochrome P450 (*PgCYP716A47*) involved in the hydroxylation of dammarenediol-II at C_{12} to give protopanaxadiol [32] and a cytochrome P450 (*HmCYP71D55*), premnaspirodiene oxygenase (HPO) that catalyzes the hydroxylation and subsequent oxidation to the ketone at C_2 of the spirene substrate [33]. *Ct437*, on the other hand, groups with the *Artemisia annua* amorpha-4,11-diene oxidase (*AaCYP71AV1*) [16] and two chicory cytochrome P450 mono-oxygenases, germacrene A oxidase (*CiCYP71*) [12] and valencene oxidase (*CiCYP71AV8*) [13]. These three enzymes perform oxidative reactions on the allylic C_{12} position of the substrate. The similarity of *Ct437* to this group of enzymes, and the fact that it can take chrysanthemol *in vitro*, but not *in vivo*, suggests that chrysanthemol is probably not its natural substrate and may represent a more “primitive” enzyme with broader substrate specificity, as reported for two of the enzymes belonging to this group [12,13].

Materials and Methods

Isolation of putative *CYP* genes from the trichome EST library.

An expressed sequence tag (EST) database of three cDNA libraries derived from pyrethrum leaves, ovaries and trichomes were produced using the GS FLX Titanium platform. At least 3 µg of RNA of each tissue was submitted to Vertis Biotechnologie AG (Germany) to be sequenced with 454 sequencing technology. The cDNAs from 3 different tissues, obtained with random hexamer primers, were labeled with different adapters to generate 3 cDNA libraries. The libraries were normalized and then sequenced in a single 454 run generating 281,264 reads from the ovary library (60% of total reads), 87,226 reads from the trichome library (19 % of total reads) and 98,672 reads from the leaf library (21 % of total reads). After adapter clipping, reads were discarded if the length of the reads were lower than 60 nt. For each library, less than 3% of reads were discarded. The

remaining reads were clustered and assembled into contigs using CLC Main Workbench (CLCBio, Denmark). In every library, about 40-50 % reads could not be assembled into contigs and were left as singlets. Since all the plant material was harvested from the same plant, the reads from different libraries were pooled together for assembly. In this way, 458,726 high quality reads from all 3 libraries were incorporated into 27,314 contigs leaving 31.6 % (144,825) reads as singlets. The average length of contigs was 411 bp.
Using an in-house bioinformatics facility, potential gene function of the resulting contigs was identified by pairwise comparison against the global database of annotated genes, saving the 50 best hits for each contig into a local database. *CYP* genes to be evaluated as putative candidates to catalyze the missing steps in pyrethrin biosynthesis were obtained by keyword interrogation of the local database using P450 as keyword. For each contig the number of contributing ESTs from each of the three libraries was known. Among the 64 contigs thus found in the trichome library, only those composed of multiple trichome ESTs were selected for further characterization. These criteria resulted in five full-length and seven partial *CYP* candidate genes. SMART RACE cDNA amplification kit (Clontech, USA) was used to obtain the 5' and 3'-region of the partial genes, according to the manufacturer's instructions. 5'-RACE and 3'-RACE products were cloned into pGEMT-easy vector (Promega, USA) and sequenced. Assembly of sequencing products using SeqMan software resulted in full-length cDNA sequences that were deposited in genebank (Supplemental Table S1). The sequence for Ct21854 was also submitted to David Nelson's cytochrome P450 homepage (http://drnelson.uthsc.edu/cytochromeP450.html) and was assignated the name of CYP71BZ1 [27].

Plasmid construction and expression in yeast

The twelve full-length pyrethrum cytochrome P450's were amplified from cDNA of trichomes and ovaries using high fidelity Phusion polymerase (Finnzymes) with the addition of *Not*I/*Pac*I restriction sites. The amplified products were digested by *Not*I/*Pac*I and cloned into the pYEDP60k plasmid. Plasmids containing the cytochrome P450's were transformed into the WAT11 yeast strain [34]. Yeast clones containing the plasmids were selected on synthetic dextrose (SD) minimal medium (0.67 % Difco Yeast Nitrogen Base Medium without amino acids, 2 % D-Glucose, and 2 % Agar) supplemented with amino acids, but omitting uracil and adenine sulphate for auxotrophic selection of transformants.

Microsomal preparations

Microsomes were prepared from medium scale expression cultures of transformed yeast lines, including an empty vector control, by published procedures [35].

Yeast cell cultures were grown 36 hours at 30 ^{o}C with shaking (250 rpm) in 250 mL flasks containing 50 mL SGI medium (0.7 % Difco yeast nitrogen base medium without amino acids, 2 % D-Glucose, 0.1 % Bactocasamino acids, 20 mg/L tryptophane). All 50 mL was used to inoculate 1L flasks containing 250 mL YPL medium (1 %Yeast extract, 1 % Bactopeptone, 2 % D-galactose). The induction was allowed to proceed for 24 hours at 30^{o}C with moderate shaking (200 rpm). The induced culture was collected in 300 mL centrifuge bottle and chilled on ice for 20 min. Cells were harvested by centrifugation at 4,900 x *g* for 10 min using a Sorvall RC 3C Plus bucket centrifuge. The pelleted cells were re-suspended in 100 mL of extraction buffer (50 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.6 M sorbitol and 10 mM β-mercaptoethanol) and incubated for 10 min at room temperature. After repeating this centrifugation step, the pellet was washed three times with extraction buffer. Cells were re-suspended in 5 mL extraction buffer without β-mercaptoethanol and transferred into a 50 mL blue-capped tube. About 25 mL of glass beads (450-500 µm) were added to the solution and over a period of 20 min the cells were shaken for 10 min in the cold room, using five intervals of 2 min shaking and 2 min rest on ice. The supernatant above the beads was removed using a glass Pasteur pipette and collected into a 25 mL sterile centrifuge bottle. After centrifuging at 10,500 x *g* for 10 min using a Sorvall RC 3C Plus bucket, the pellet was discarded and the resulting supernatant was centrifuged at 195,000 x g in a Beckman L7 Ultracentrifuge, model L7-80, for at least 2 hours. The pellet was re-suspended by using a tight-fitting chilled glass Tenbroeck homogenizer in 4 mL chilled 50 mM Tris-HCl buffer pH 7.5 containing 1 mM EDTA and 20 % (v/v) glycerol. Yeast microsomes were then aliquoted and stored at –80°C until further use.

In vitro enzymatic activity assay

Seventy-two microliters of microsome preparation was diluted in 408 µL assay buffer (288 µL MQ, 20 µL 1 M KPi buffer pH 7.5, and 100 µL 10 mM NADPH). Ten microliters of a 100 mM solution of chrysanthemyl alcohol (Sigma, 194654) in DMSO was added and the assay was incubated with mild agitation at 30 ^{o}C for 24 h. Each incubation was acidified with 40 µL of 3 M HCl. Subsequently, the reaction products were extracted three times with 1 mL ethyl acetate, vortexing shortly and centrifuging each time for 10 min at 1200 rpm. The combined layers were dried on an anhydrous Na_2SO_4 glass column, concentrated to 1mL and analyzed on an Agilent GC-MS instrument.

GC-MS analysis of in-vitro assays

A gas chromatograph (7809A, Agilent, USA) equipped with a 30 m × 0.25 mm, 0.25 mm film thickness column (ZB-5, Phenomenex) using helium as carrier gas at flow rate of 1

mL min^{-1} was used for GC-MS analysis. Splitless mode was used for the injector with the inlet temperature set to 250 °C. The initial oven temperature was 45 °C for 1 min, and was increased to 300 °C after 1 min at a rate of 10 °C min^{-1} and held for 5 min at 300 °C. The GC was coupled to a Triple-Axis detector (5975C, Agilent). Chrysanthemal was identified by comparing its retention time and mass spectra with that of d a standard that was prepared by stirring for 72 h at room temperature 10 mM chrysanthemol with 100 mM MnO_2 (Merk, Germany) in dichloromethane (DCM).

CDS* plasmid construction and stable transformation of *Nicotiana tabacum.

The complete cDNA of *TcCDS* was placed under the control of chrysanthemum RbcS1 promoter.*Agrobacterium tumefaciens* strain AGL-0 harboring the binary vector was used to transform the tobacco plants as previously described [36]. Transgenic plants were grown in a greenhouse at 25 ± 2 °C under 18/6 h light/dark photoperiod. All positive plantlets of T0 transgenic lines were analyzed by GC-MS to check the presence of chrysanthemol in the headspace. Plant line11 had the least severe obvious slower growing phenotype. The F1 rapid-growing control phenotypes were removed, and the remaining *TcCDS* transgenic plants were used for agroinfiltration.

Plasmid construction for transient expression in *Nicotiana benthamiana* and *N. tabacum TcCDS* stable transformants.

For expression in *N. benthamiana* and *N. tabacum*, *CYP* genes and the *TcCDS* gene were cloned into a modified ImpactVector1.1 (http://www.impactvector.com/) to express them under the control of the Rubisco promoter (RBC). The vector was modified into a Gateway donor vector to allow subcloning of the expression cassette into pBINPLUS [37]. An LR reaction (Gateway-LR Clonase TM II) was carried out to subclone each gene into an acceptor pBinPlus binary [38] vector between the right and left borders of the T-DNA for plant transformation**.**

Transient expression in wild type *Nicotiana benthamiana* and transgenic *Nicotiana tabacum TcCDS* lines

A. tumefaciens infiltration (agro-infiltration) was performed according to the description of van Herpen et al [30]. *A. tumefaciens* batches were grown at 28 ºC at 220 rpm for 24 h in YEP media with kanamycin (50 mg L^{-1}) and rifampicin (50 mg L^{-1}). Cells were harvested by centrifugation for 20 min at 4000 x g and 20^{o}C and then resuspended in 10 mM MES buffer containing 10 mM $MgCl_2$ and 100 µM acetosyringone (4'-hydroxy-3', 5'-dimethoxyacetophenone, Sigma) to a final OD_{600} of ~ 0.5, followed by incubation at room temperature under gentle shaking at 50 rpm for 150 min. For the co-infiltration of *N.*

benthamiana leaves equal volumes of *Agrobacterium* batches were mixed. Mixtures or single batches were infiltrated into leaves of 3-weeks-old *N. benthamiana* and 6-weeks-old *N. tabacum TcCDS* plants 12 leaves developmental stage) by pressing a 1 mL plastic syringe without metal needle against the abaxial side of the leaf and slowly injecting the bacterium suspension into the leaf. *N. tabacum* and *N. benthamiana* plants were grown from seeds on soil in the greenhouse with a minimum of 16 h light. Day temperatures were approximately 28 ºC, night temperatures 25 ºC. After agro-infiltration the plants were grown under greenhouse conditions for another 4 to 5 days and then harvested for analysis.

Headspace collection and thermodesorption GC-MS

Volatile collection from agro-infiltrated *TcCDS N. tabacum*, leaves and GC-MS analysis were performed according to van Herpen et al. [30]. Steel sorbent cartridges (89 mm 429 × 6.4 mm O.D.; Markes) containing Tenax were used for volatile collection. Cartridges were conditioned at 280°C for 40 min under a nitrogen flow of 20 psi in a TC-20 multi-tube conditioner and were capped airtight until use. *TcCDS N. tabacum* leaves were sampled, placed on water in a small vial and were enclosed in a 2.5L glass container closed with a Teflon-lined lid equipped with an inlet and an outlet, and placed in a climate room at 21 °C and a light intensity of 120 µmol m^{-2} s^{-1} at the level of the plant. To trap the leaf-produced volatiles, air was sucked through the containers with a flow rate of 90 mL min^{-1} for 4 hours and led through one cartridge. A second cartridge was used to purify the incoming air. Sample cartridges were dried for 15 min at room temperature with a nitrogen flow of 20 psi before GC-MS analysis on a Thermo Trace GC Ultra connected to a Thermo Trace DSQ quadruple mass spectrometer (Thermo Fisher Scientific, USA). Cartridges were placed in an automated thermodesorption unit (Ultra; Markes, Llantrisant) in which they were flushed with helium at 20 mL min^{-1} for 3 min to remove moisture and oxygen just before thermodesorption. The volatiles were desorbed by heating of the cartridges at 250 °C for 5 min with a helium flow of 50 mL min^{-1}. The compounds released were trapped on an electrically cooled sorbent trap (Unity; Markes, Llantrisant) at a temperature of 30 °C. Subsequently, the trapped volatiles were injected on the analytical column (ZB-5MSI, 30 m × 0.25 mm ID, 1.0 µm – film thickness, Zebron, Phenomenex) with a split of 1:4 by ballistic heating of the cold trap to 280 °C (40 °C sec^{-1}) for 3 min. The temperature program of the GC started at 40 °C (3 min hold) and rose 10 °C min^{-1} to 280 °C (2 min hold). The column effluent was ionized by electron impact (EI) ionization at 70 eV. Mass scanning was done from 33 to 280 m/z with a scan time of 4.2 scans s^{-1}. Xcalibur software (Thermo, USA) was used to identify the eluted compounds by comparing the mass spectra with those of the reference library, the NIST 08 mass spectral database.

LC-QTOF-MS analysis

Non-volatile metabolites were analyzed by LC–QTOF-MS (liquid chromatography, coupled to quadrupole time-of-flight mass spectrometry) according to a protocol for untargeted metabolomics of plant tissues [39]. For agro-infiltrated *N. benthamiana* and *N. tabacum*, 100 mg infiltrated leaf from each treatment was ground in liquid nitrogen and extracted with 300 µl methanol : formic acid (1000:1, v/v). After brief vortexing and sonication for 15 min, the extracts were centrifuged for 5 min at 3500 rpm and filtered through a 0.2 µm inorganic membrane filter (RC4, Sartorius, Germany). A Waters Alliance 2795 HPLC connected to a Waters 2996 PDA detector and subsequently a QTOF Ultima V4.00.00 mass spectrometer (Waters, MS technologies, UK) operating in positive ionization mode was used. An analytical column (Luna 3 µ C18/2 100A; 2.0 × 150 mm; Phenomenex, USA) attached to a C18 pre-column (2.0×4 mm; Phenomenex, USA) was used. Degassed eluent A [ultra-pure water: formic acid (1000:1, v/v)] and eluent B [acetonitrile : formic acid (1000:1, v/v)] were used at a flow rate of 0.19 mL min^{-1}. The gradient of the HPLC started at 5 % eluent B and increased linearly to 95 % eluent B in 45 min, after which the column was washed and equilibrated for 15 min before the next injection.

Masses were recorded between *m/z* 80 and *m/z* 1000; leucine enkaphalin ([M-H]−=554.2620) was used as a lock mass for on-line accurate mass correction.

Chrysanthemic acid detection and quantification by UPLC- MRM- MS

Targeted analysis of CA in agro-infiltrated *N. benthamiana* and *N. tabacum* leaves was performed by comparing retention times and mass transitions with that of a (+)-*trans*-chrysanthemic acid standard (18509, Sigma-Aldrich) using ultraperformance liquid chromatography (UPLC) coupled to MS/MS essentially as described by Kohlen et al. [40] with some modifications. A Waters Xevo tandem quadrupole mass spectrometer equipped with an electrospray ionization source and coupled to an Acquity UPLC system (Waters) was used for analysis. Chromatographic separation was obtained on an Acquity UPLC BEH C18 column (100 × 2.1 mm, 1.7 µm; Waters) by applying a water/acetonitrile gradient to the column, starting from 5% (v/v) acetonitrile in water for 1.25 min and rising to 50% (v/v) acetonitrile in water in 3.25 min, followed by an increase to 90% (v/v) acetonitrile in water in 5.0 min, which was maintained for 0.75 min before returning to 5% acetonitrile in water using a 0.15 min gradient, prior to the next run. Finally, the column was equilibrated for 2.85 min using this solvent composition. Operation temperature and flow rate of the column were 50°C and 0.5 mL min^{-1}, respectively. Injection volume was 20 µL. The mass spectrometer was operated in positive electrospray ionization mode.

Cone and desolvation gas flows were set to 50 and 1,000 L h^{-1}, respectively. The capillary voltage was set at 3.0 kV, the source temperature at 150°C, and the desolvation temperature at 650 °C. Argon was used for fragmentation by collision-induced dissociation in the ScanWave collision cell. Multiple reaction monitoring (MRM) was used for quantification of chrysanthemic acid in aqueous extracts (50:50, water: extract 100mg/300 µL MeOH: formic acid) of agro-infiltrated *N. benthamiana* and *N. tabacum* leaves by comparing retention times and MRM mass transitions with that of a chrysanthemic acid standard. MRM transitions were optimized for chrysanthemic acid using the Waters IntelliStart MS Console.

Chrysanthemic acid conjugate analysis by HPLC-PDA-Orbitrap-FT-MS

Chrysanthemic acid conjugates in aqueous extracts of agro-infiltrated *N. benthamiana* and *N. tabacum* leaves were analyzed by HPLC-PDA-ESI-MS according to Van der Hooft *et al*. [41] with minor modifications. The system consisted of an Accela HPLC system with an Accela photodiode array (PDA) detector and autosampler, connected to a LTQ/Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific) that was equipped with an ESI source. Sample injection volume was 20 µl and chromatographic separation took place on a Luna C18(2) analytical column, using a binary eluent solvent system of degassed ultrapure water and acetonitrile, both containing 0.1 % v/v FA (solvent A and B, respectively), at a flow rate of 0.19 mL/min and column temperature at 40 °C. The HPLC gradient started at 5% B and linearly increased to 75 % B in 45 min. In addition, 15 minutes of washing and equilibration preceded each next injection. Subsequently, the accurate mass of the generated molecular fragments were recorded using a Orbitrap fourier-transform mass spectrometer (FT-MS).

Expression analysis

For RNA extraction plant tissue was homogenized by adding one pre-cooled grinding ball to each 2 ml Eppendorf vial containing 50-100 mg of frozen plant tissue and using a pre-cooled Mikro-disembrator II (Braun; Germany) for 1 min at maximum speed. After careful removal of the beads, RNA was isolated using TriPure (Roche) and transcribed into cDNA using TaqMan Reverse Transcription reagents (Applied Biosystems) according to the manufacture's instructions.

RT-qPCR was used to study the expression of the twelve candidate *CYP* genes, the 9 dehydrogenase ESTs and the Chrysanthemyl diphosphate synthase (*CDS*) gene, in cDNA derived from ovaries at different flower developmental stages, trichomes and seedlings. Gene-specific primers were designed using Primer3Plus

(http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (Supplemental Table S1).

The *T. cinerariifolium* GAPDH gene [5] was used for normalization. PCR reactions were prepared in duplicate by mixing in a 500 µL tube, 22.5 µL iQ SYBR green supermix 2x (Biorad), 4.5 µL sense primer (3 µM), 4.5 µL antisense primer (3 µM), 11.5 µL deionized water, and 2 µL cDNA template. After vortexing, each mix was distributed into two wells in 20 µL amounts. Quantification of the transcript level was performed in an MyiQ iCycler system (Bio-Rad Laboratories, USA) using a three-step program, which included (i) enzyme-activation at 95 °C for 3 min, (ii) 40 cycles of 95 °C for 10 sec, 60 °C for 30 sec, and (iii) 95 °C for 1 min, from 65 °C to 95 °C for 10 sec for dissociation curve analysis. At the end of each run, amplified products were sequenced to verify their identity. Relative expression values were calculated using the efficiency δCt method.

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Supplemental data

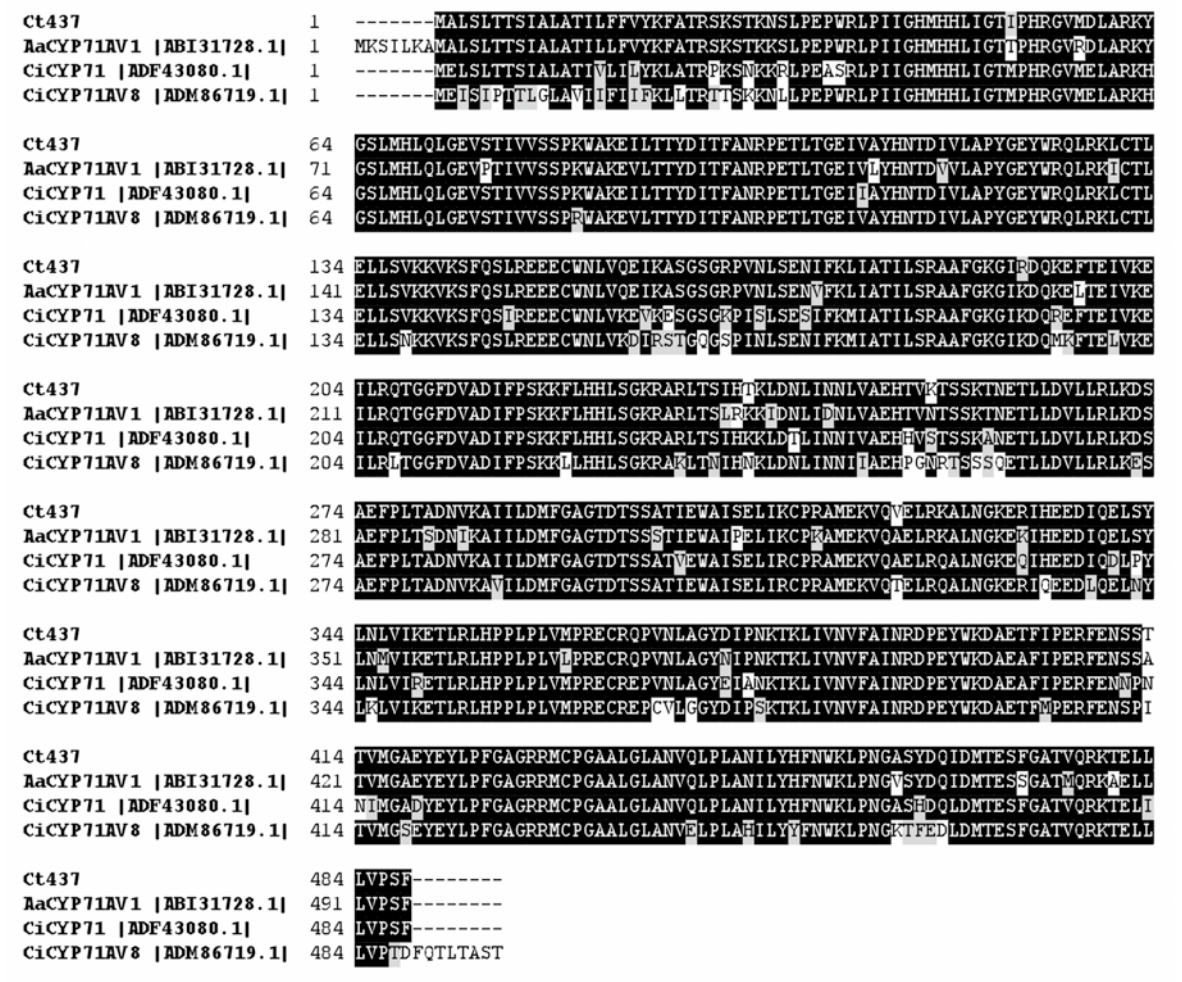

Figure S1: Sequence alignment based on the deduced amino acid sequence of the pyrethrum Ct437, the *Artemisia annua* CYP71AV1 and the *Cichorium intybus* CYP71 and CYP71AV8. The alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2).

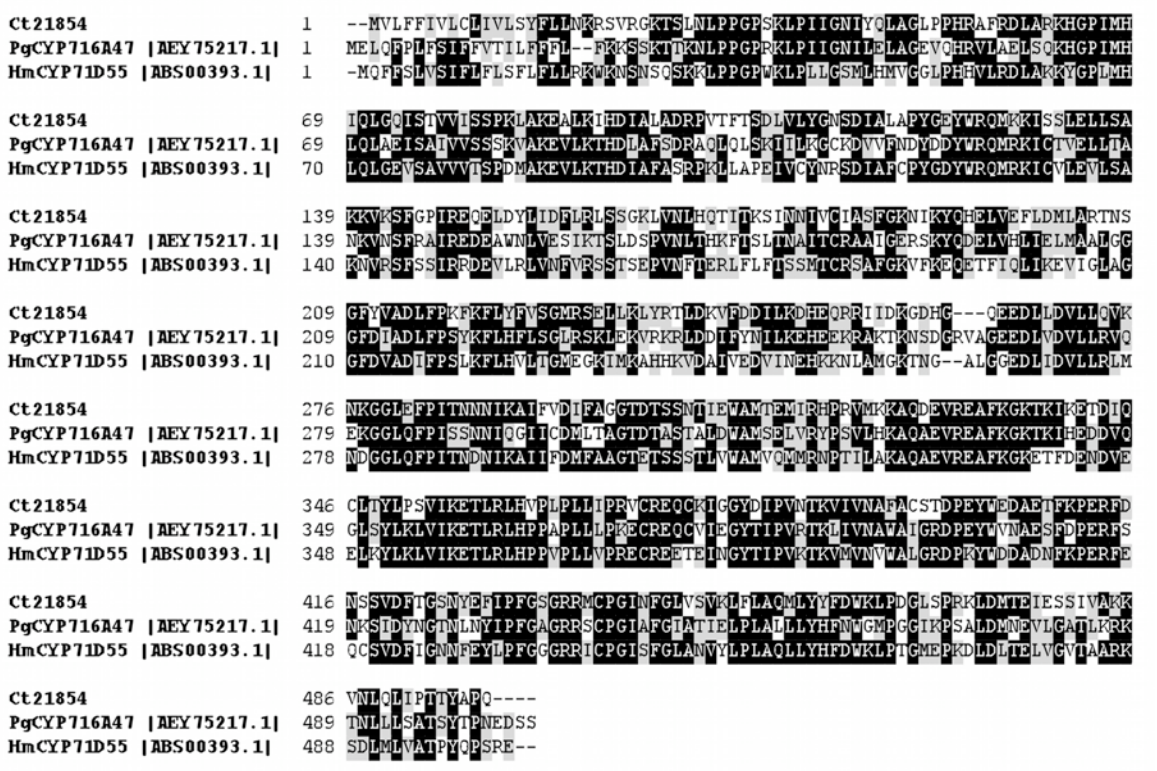

Figure S2: Sequence alignment based on the deduced amino acid sequence of the pyrethrum Ct21854, the *Panax ginseng* CYP716AV47 and the *Hyoscyamus muticus* CYP71D55. The alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2).

Table S1: CYP cDNA, deduced protein sequence and primers used.

Identifier	Sequence
Ct145 **(KC441529)**	**cDNA** ATGGATTATCTCTTCGTCACACTCATTCTCCTCTTGGTCTCATTTCTTATCGCCACCGTA TTCCGCCACAAATTCTCCAAACTTCCTCCAACTGTCTTCCCAACCATTCCGATAATCGG CCACCTTTATCTCTTGAAGCAACCCCTCCATAGAACCTTCGCTAAACTCACGGCCAAAC ATGGTCCAATACTACTATTGCGCTTTGGGTCACGCAACGTCCTAGTGCTCTCTTCTCCT TCCCTAGCCGAAGAATGCTTCACTAAGAACGACATCATCTTTGCAAACCGCCCTCGCT TGCTTGCTGGCAAGATTCTTGGTTACAACTTCACTAGCCTTGGTTTAGCACCATATGGG GACCATTGGCGCAACCTGCGTCGTATATCTGCCATCGAGGTCTTTTCTTCTCAGCGTA TGCAACAGTTTTCTAGTATACGTGTTGATGAAGCAAGACTACTGGTAAAAAAACTGGTG TCCGAGAGTTCTAAGCCAGTGAACTTGCATTCGGTATTTGATGATTTGATAGTTAATGT GATTATGAGGATAATCTCGGGCAAGAGATATTTTGATGGCGATAGTGATTTGGAGGAA GAAGGGAAAGTGCTTCAGCAGATAGTTAAGGACACGTTTTTAATCAGCAAAGCTTCGA ATTTGGGTGATTCGTTACCTTTTTTACGATGGTTAGGAGTTAAAGGGCTAGAAAAGAAG TTAATTTCGTTGCAGGAAACAAGAAATAACTTCTTTCAAGGGTTAATCAATCAACTTAGA AAAGTTGAAAATAAGAAGCAAAACTTGATTCATGTGTTGTTGCACTTACAAGATACAGA TCCAGATTACTACACCGATGAAATGATTAAAAGTTTTGTACTAAATCTGTTAGCAGCTG GTGTTGATACTTCTAGTACAACTATGGAATGGGCTTTTTCACTATTGCTCAACCATACA CATGTTTTAAAGAAGGCACAAAAAGAAATCGACAATCATGTGGGCAAAGACCGCTTTG TTGATGAATCGGACATGGCCAACTTACCATATATTCGTTGCATAATAAACGAGACTTTG AGAATGTACCCTGCTGTTCCACTACTAGTACCACACGCGTCATCAGATGATTGTGTTGT CGGAGGCTATCATGTCCCAGGTGAGACCATGCTATTGGTGAATCAATGGGCTATACAG CGTGACCCCAACGTGTGGAGTGAGCCTGAAAGGTTCTACCCAGAGAGGTTTGAAAGG CTTGAAGGAACAAGAGATGGGTACAAGTTAATGCCATTTGGGTCCGGAAGGAGGAGTT GTCCGGGGGAAGGATTAGCGATACGTGTGATGGGGTTGGCCATAGGGTTGCTTATAC AATGCTTTGATTGGGAGCGGGTTAGTGAAGAGATGGTTGATATGACAGAAGGTTATGG GATGACCATGCCAAAGGCTCAACCTTTGGTAGCTAAGTGTATACCACGCCAAATAACT CGCAAGTTAAAAATATGA **Protein** MDYLFVTLILLLVSFLIATVFRHKFSKLPPTVFPTIPIIGHLYLLKQPLHRTFAKLTAKHGPILLL RFGSRNVLVLSSPSLAEECFTKNDIIFANRPRLLAGKILGYNFTSLGLAPYGDHWRNLRRIS AIEVFSSQRMQQFSSIRVDEARLLVKKLVSESSKPVNLHSVFDDLIVNVIMRIISGKRYFDGD SDLEEEGKVLQQIVKDTFLISKASNLGDSLPFLRWLGVKGLEKKLISLQETRNNFFQGLINQ LRKVENKKQNLIHVLLHLQDTDPDYYTDEMIKSFVLNLLAAGVDTSSTTMEWAFSLLLNHT HVLKKAQKEIDNHVGKDRFVDESDMANLPYIRCIINETLRMYPAVPLLVPHASSDDCVVGG YHVPGETMLLVNQWAIQRDPNVWSEPERFYPERFERLEGTRDGYKLMPFGSGRRSCPG EGLAIRVMGLAIGLLIQCFDWERVSEEMVDMTEGYGMTMPKAQPLVAKCIPRQITRKLKI. **RTqPCR** **F-**GGCCAAACATGGTCCAATAC **R-**TGAAGCATTCTTCGGCTAGG **NotI/PacI restriction primers** **F-**GCA**GCGGCCGC**ATGGATTATCTCTTC **R-**TCG**TTAATTAA**TCATATTTTTAACTTG

Ct437 **(KC441527)**	**cDNA** ATGGCACTCTCACTGACCACTTCCATTGCTCTTGCCACGATCCTCTTCTTCGTTTACAA GTTCGCTACTCGTTCCAAATCCACAAAAAACAGCCTTCCTGAGCCATGGCGACTTCCC ATTATTGGTCACATGCATCACTTGATTGGTACAATACCACATCGTGGGGTTATGGATTT AGCCAGAAAGTATGGATCTTTGATGCATTTACAGCTTGGTGAAGTTTCAACAATCGTGG TGTCATCTCCGAAATGGGCTAAAGAGATTTTGACAACGTACGACATTACCTTTGCTAAC AGGCCCGAGACTTTAACCGGTGAGATTGTTGCGTATCACAATACGGATATTGTTCTTG CACCTTATGGTGAATACTGGAGGCAATTACGTAAACTTTGCACATTGGAGCTTTTGAGT GTTAAGAAAGTAAAGTCATTTCAGTCGCTTCGTGAAGAGGAGTGTTGGAATTTAGTTCA AGAGATTAAAGCTTCAGGTTCAGGGAGACCGGTTAACCTTTCAGAGAACATTTTCAAGT TGATTGCAACCATACTTAGTAGAGCCGCGTTTGGAAAAGGAATCAGGGACCAGAAAGA GTTTACGGAGATTGTGAAAGAGATATTGAGGCAAACTGGTGGTTTTGATGTGGCAGAT ATCTTTCCTTCGAAGAAATTTCTTCATCATCTTTCGGGCAAGAGAGCTAGGTTAACTAG CATTCACACGAAGCTCGATAATTTAATCAATAACCTTGTTGCTGAGCATACTGTTAAAA CCTCAAGTAAAACTAACGAGACACTCCTTGATGTTCTTCTAAGGCTCAAAGATAGTGCT GAATTCCCGTTAACAGCTGATAACGTTAAAGCTATCATTTTGGATATGTTTGGAGCAGG CACAGACACTTCGTCAGCCACAATCGAATGGGCGATTTCAGAACTCATAAAGTGTCCG AGAGCGATGGAGAAAGTACAAGTGGAACTGAGGAAAGCACTTAACGGAAAAGAAAGG ATCCATGAGGAAGACATTCAAGAACTAAGCTACTTAAACTTGGTAATCAAAGAAACATT AAGGTTGCACCCTCCACTACCCTTGGTTATGCCAAGAGAGTGCCGCCAACCAGTCAAT TTGGCTGGATACGACATACCCAATAAGACCAAACTTATTGTCAACGTCTTTGCTATAAA TAGGGACCCGGAATACTGGAAAGACGCTGAAACTTTCATCCCCGAACGATTTGAAAAT AGTTCTACAACTGTCATGGGTGCAGAATACGAGTATCTTCCGTTTGGAGCTGGGAGAA GGATGTGTCCTGGAGCCGCACTTGGTTTAGCCAACGTGCAGCTCCCGCTTGCTAATAT ACTGTATCATTTCAACTGGAAACTCCCCAATGGTGCGAGCTATGATCAGATCGACATG ACCGAGAGCTTTGGAGCCACGGTTCAAAGAAAGACTGAGTTGTTACTCGTACCAAGTT TCTAG **Protein** MALSLTTSIALATILFFVYKFATRSKSTKNSLPEPWRLPIIGHMHHLIGTIPHRGVMDLARKY GSLMHLQLGEVSTIVVSSPKWAKEILTTYDITFANRPETLTGEIVAYHNTDIVLAPYGEYWR QLRKLCTLELLSVKKVKSFQSLREEECWNLVQEIKASGSGRPVNLSENIFKLIATILSRAAFG KGIRDQKEFTEIVKEILRQTGGFDVADIFPSKKFLHHLSGKRARLTSIHTKLDNLINNLVAEHT VKTSSKTNETLLDVLLRLKDSAEFPLTADNVKAIILDMFGAGTDTSSATIEWAISELIKCPRA MEKVQVELRKALNGKERIHEEDIQELSYLNLVIKETLRLHPPLPLVMPRECRQPVNLAGYDI PNKTKLIVNVFAINRDPEYWKDAETFIPERFENSSTTVMGAEYEYLPFGAGRRMCPGAALG LANVQLPLANILYHFNWKLPNGASYDQIDMTESFGATVQRKTELLLVPSF. **RTqPCR** **F-**CAAGAGAGTGCCGCCAACC **R-**TCAGCGTCTTTCCAGTATTCCG **NotI/PacI restriction primers** **F-**CGA**GCGGCCGC**ATGGCACTCTCAC **R-**TCG**TTAATTAA**CTAGAAACTTGGTAC

Ct8828 **(KC441528)**	**cNDA** ATGGAGCCCTTCACGATCTTCTCCCTCGTTGTGGCTTCACTCGTTCTCTTTGCATATTG GGCACTCCTTGCGCCGAACACCTCCAAGAACTTGCCACCAGGGCCACCGAAACTACC AATAATTGGAAACATACACCAACTTAAAAGCCCAACTCCGCATCGAGTTCTTAGAGAAC TAGCCAAGAAATATGGCCCCATCATGCACCTACAACTCGGCCAAGTCTCTACGGTTGT CGTGTCCACGCCTAGACTAGCTCAAGAGATCATGAAGACCAACGATATCAGCTTTGCA GATAGACCAACCACTACAACCTCCCAAATATTCTTTTACAAAGCTCAAGATATCGGGTG GGCTCCTTATGGCGAATACTGGAGGCAAATGAAGAAGATTTGTACGTTGGAGCTTCTT AGTGCCAAAAAAGTGCGATCTTTTAGCTCTATAAGAGAAGAGGAGCTTACTAGAATTCG TAAAATTCTTGAATCCAAAGCTGGAACACCAATCAACTATACGGAAATGACTATAGAGA TGGTGAACAATGTGATATGTAAGGCTACTTTGGGAGATTGTTGTAAAGACCAGGCGTT ATTGATTGAACTATTGTATGATGTGTTGAAAACGTTAAGCGCGTTCAATCTAGCATCTTA TTATCCTCGTCTACAGTTTCTTAATGTGATTTCCGGGAAGAAAGCTAAGTGGTTGAAGA TGCAGAAAAAGCTTGATGACATCATGGAAGATATCTTGAAGGAACATAGAGCTAAAGG AAGAGCAAAGAACAGTGATCAAGAAGATCTAGTTGATGTTCTTTTGAGGGTCAAAGAG ACCGGTGGACTTGATATCGATGTCACGGATGAACATGTTAAGGCCGTTGTTTTGGATA TGCTAACTGCTGGGACCGATACCTCTTCAACAACACTTGAATGGGCGATGACTGAGCT CATGAGGAACCCTGATATGATGAAGAGAGCACAAGAAGAAGTGAGATCAGTGGTGAAA GGAGAACATGTTACCGAAACAGACTTGCAAAGCTTACATTACCTCAAATTGATAGTAAA GGAAACAATGAGGTTACATGCTCCTACACCTTTATTAGTCCCCAGAGAATGCCGACAA GATTGTAACGTTGATGGATATGATATTCCTGCCAAGACCAAGGTACTCGTAAACGCAT GGGCTTGTGGAGTCGATCCTGGTAGTTGGGAAAATCCTGAAAGCTTCATCCCGGAGC GATTTAAAAATTGTCCCATTAACTTCATGGGTGCAGACTTCCAGTACATTCCTTTTGGT GCTGGCCGAAGAATTTGTCCTGGACTTACATTTGGACTTAGCATGGTTGAATACCCAC TCGCACATTTCTTGTATCATTTTGATTGGAAGCTTCCAAACGGAATGAAACCGCATGAG CTTGATATCACAGAAATCACAACGATTTCGACATCACTCAAACATCACTTAAAGATCGT ACCCTTCCCCAAGTCCTCATTAGCAAAGTAA **Protein** MEPFTIFSLVVASLVLFAYWALLAPNTSKNLPPGPPKLPIIGNIHQLKSPTPHRVLRELAKKY GPIMHLQLGQVSTVVVSTPRLAQEIMKTNDISFADRPTTTTSQIFFYKAQDIGWAPYGEYW RQMKKICTLELLSAKKVRSFSSIREEELTRIRKILESKAGTPINYTEMTIEMVNNVICKATLGD CCKDQALLIELLYDVLKTLSAFNLASYYPRLQFLNVISGKKAKWLKMQKKLDDIMEDILKEH RAKGRAKNSDQEDLVDVLLRVKETGGLDIDVTDEHVKAVVLDMLTAGTDTSSTTLEWAMT ELMRNPDMMKRAQEEVRSVVKGEHVTETDLQSLHYLKLIVKETMRLHAPTPLLVPRECRQ DCNVDGYDIPAKTKVLVNAWACGVDPGSWENPESFIPERFKNCPINFMGADFQYIPFGAG RRICPGLTFGLSMVEYPLAHFLYHFDWKLPNGMKPHELDITEITTISTSLKHHLKIVPFPKSS LAK. **RTqPCR primers** **F-**AACTTCATGGGTGCAGACTTCC **R-**CAAGCTCATGCGGTTTCATTCC **NotI/PacI restriction primers** **F-**CGA**GCGGCCGC**ATGGAGCCCTTCAC **R-**TCG**TTAATTAA**TTACTTTGCTAATG

Ct2120 **(KC441530)**	**cDNA** ATGGATTCATCACCCTTATGGTTTCTTCTAACAGTTACAGCCCTTACCTTATCATGCATC TATATATATACTATACGAAGGAAGAGCTCAGCCATGGCTGCCCGTAAGCTTCCACCAA GCCCACCTGGGCTTCCAATAATCGGGAACATGCATCAAGTGCTAGCAGGTGAAAGCAT ATATAAAACCCTATGGAATCTCTCCCAGAAATACGGTCCGGCTATGCTTCTTCATTTTG GAAGCCAACAAATTCTTCTCATTTCTTCAAGTGAAATGGCTAACCAGGTCTTGAAAACT AACGATCATGAGCTGTCTACGCGTCCACAATCGAAGGCTGCCAAGCGAATGACGTACA ATTACTTGGATGTTTCATTTTCACCTAGTGATGATCATTGGAGGGACATGCGCAAGGTC CTCGTATCTGAATTATTGATTGGTAAGAGAAATAAAATGTTTAAGAACAAGTTGGAGAT CGAGATGGATGGTTTCGTTCGTTCACTCTTATCGCAACCCTCAGGAACCATAGTTAACT TAGACGATGCACTCTATTCTCTACTAGAGGATGTTACGTGCTTGGTTGCATTTGGGAAG AGCCATAGGGGCAAGACCTTTAATGGTAAAAATTTGAAAGAGATATTTAATGAAACAAT TGTAATGCTTGGTGGTTCATTAGCAGATTTTTTTCCAATAGTTGGGCCGATTTTGGACG AGTTAAGAGGGTGGAATCGTAGATTAGAGAAGTCTTTTAGTGATGCTGATGGATTTCTT CAAATGCTTATTGATGAGCATTTTGAACATAATGCAACCAATAAAACTGACGATGAGAA ATGTTTTGTTGATGACTGCATTTCTCGGTTAACTAATGACGAAATTAAAGCAGTCTTGAT GAATGTACTTAATGGAGCAATTGACACAAGCTCTACAACCGCGGTGTGGGCAATGTCT GAGATCGTGAAAAGTCCTAGAATTATGGAAAAGTTGCAGAAGGAAATCCGAAGCTGTG TTGGAACAAAATCGAAGGTACATGAATCAGATATTGCCAAGATGGCATACTTGAAAATG GTGGTGAAGGAGACACTAAGATTGCACGGACCTGCCTCATTTTTGATCGGACGAGAAT GTGTAAGCCATTGTAAAGTTGGCGGATATGACGTCTACCCAGGAACTAAAATCATGAT CAATGTGTGGGGAATAGGAAGGGATTCACGAACATGGAAAGAGCGGCCAAATGAGTT TTGGCCCGAGCGGTTTGAAAATTTTAAGTTCGATTTTCTAGGAAAACACTGCGAGATGA TCCCATTTGGAGGGGGCAGGAGAGCATGCCCGGGGTATAACTCGGGTATTGCAACTG TGGAATTTATCCTTGCAAACCTTCTTTATGTGTTTGATTGGGAAGTACCTGGGGGCGTA AAAAACCAAGATCTGGACATGGAAGAGTATGGCTCCTGGCATATTGATAGGAAAACAC CTCTTTGCCTTGTTCCCAAAGAGCACCAAGGGAAGGACTAA **Protein** MDSSPLWFLLTVTALTLSCIYIYTIRRKSSAMAARKLPPSPPGLPIIGNMHQVLAGESIYKTL WNLSQKYGPAMLLHFGSQQILLISSSEMANQVLKTNDHELSTRPQSKAAKRMTYNYLDVS FSPSDDHWRDMRKVLVSELLIGKRNKMFKNKLEIEMDGFVRSLLSQPSGTIVNLDDALYSL LEDVTCLVAFGKSHRGKTFNGKNLKEIFNETIVMLGGSLADFFPIVGPILDELRGWNRRLEK SFSDADGFLQMLIDEHFEHNATNKTDDEKCFVDDCISRLTNDEIKAVLMNVLNGAIDTSSTT AVWAMSEIVKSPRIMEKLQKEIRSCVGTKSKVHESDIAKMAYLKMVVKETLRLHGPASFLIG RECVSHCKVGGYDVYPGTKIMINVWGIGRDSRTWKERPNEFWPERFENFKFDFLGKHCE MIPFGGGRRACPGYNSGIATVEFILANLLYVFDWEVPGGVKNQDLDMEEYGSWHIDRKTP LCLVPKEHQGKD. **RTqPCR** **F-**TTATCGCAACCCTCAGGAAC **R-**CTATGGCTCTTCCCAAATGC **NotI/PacI restriction primers** **F-**GCA**GCGGCCGC**ATGGATTCATCACC **R-**TCG**TTAATTAA**TTAGTCCTTCCCTTG

Ct21854 **(KC441525)**	**cDNA** ATGGTCTTGTTTTTCATTGTTTTGTGTCTCATCGTCCTCTCTTACTTTCTACTCAACAAG AGATCGGTCAGAGGGAAAACATCTCTAAACCTGCCACCAGGGCCATCAAAGCTACCTA TAATCGGAAACATATACCAGTTAGCTGGATTACCTCCACATCGTGCATTTAGAGACTTA GCACGAAAGCATGGCCCAATCATGCACATCCAACTTGGCCAAATTTCAACAGTCGTCA TTTCATCACCAAAACTAGCAAAAGAGGCACTTAAAATCCATGATATTGCCCTTGCAGAC CGACCCGTTACCTTTACGAGCGATCTCGTATTATATGGAAACAGTGATATAGCTCTTGC TCCCTATGGAGAATATTGGAGACAAATGAAAAAGATTTCTTCTTTGGAACTACTTAGTG CCAAAAAGGTTAAATCATTTGGTCCTATTAGAGAACAAGAGCTTGACTATCTTATAGATT TCCTCCGATTATCTTCAGGGAAGCTTGTAAATTTACACCAAACCATCACTAAATCAATTA ATAACATTGTTTGTATAGCTTCCTTTGGAAAAAATATTAAATACCAACATGAACTCGTAG AGTTTTTAGATATGTTGGCAAGAACAAACTCTGGATTTTATGTGGCGGATCTTTTCCCG AAGTTTAAGTTTCTATACTTTGTCTCTGGAATGAGATCAGAGTTATTGAAACTCTATAGG ACCCTTGACAAGGTATTCGATGACATCTTAAAGGATCACGAACAACGTCGTATAATAGA CAAAGGTGATCACGGGCAAGAGGAAGATCTTCTTGATGTACTTCTTCAAGTAAAAAATA AAGGTGGTCTCGAGTTTCCCATCACCAACAACAACATTAAAGCCATATTTGTAGACATA TTTGCTGGAGGAACTGATACATCTTCAAATACAATTGAATGGGCAATGACAGAAATGAT AAGGCATCCGAGGGTGATGAAGAAGGCACAAGATGAAGTAAGAGAAGCCTTCAAAGG TAAGACAAAAATCAAAGAGACAGACATACAATGTTTAACGTATTTACCATCGGTAATTAA GGAAACTTTACGGCTTCACGTCCCACTCCCTTTGTTAATTCCACGTGTATGTAGGGAG CAATGCAAAATTGGTGGTTATGACATACCCGTTAATACCAAAGTCATTGTTAATGCTTT CGCATGTTCAACTGATCCTGAATATTGGGAGGACGCAGAGACCTTTAAGCCAGAGAGA TTTGACAATAGTTCAGTTGACTTCACAGGATCTAATTACGAGTTCATACCATTTGGGTC AGGAAGGAGAATGTGCCCAGGTATCAATTTTGGTTTAGTATCAGTCAAGCTTTTCCTTG CTCAAATGCTGTACTATTTCGATTGGAAACTCCCTGATGGACTAAGTCCTAGGAAACTT GATATGACGGAGATTGAGAGCTCAATAGTAGCAAAAAAGGTTAACTTACAGTTGATTCC TACAACTTATGCTCCCCAATAA **Protein** MVLFFIVLCLIVLSYFLLNKRSVRGKTSLNLPPGPSKLPIIGNIYQLAGLPPHRAFRDLARKH GPIMHIQLGQISTVVISSPKLAKEALKIHDIALADRPVTFTSDLVLYGNSDIALAPYGEYWRQ MKKISSLELLSAKKVKSFGPIREQELDYLIDFLRLSSGKLVNLHQTITKSINNIVCIASFGKNIK YQHELVEFLDMLARTNSGFYVADLFPKFKFLYFVSGMRSELLKLYRTLDKVFDDILKDHEQ RRIIDKGDHGQEEDLLDVLLQVKNKGGLEFPITNNNIKAIFVDIFAGGTDTSSNTIEWAMTEM IRHPRVMKKAQDEVREAFKGKTKIKETDIQCLTYLPSVIKETLRLHVPLPLLIPRVCREQCKI GGYDIPVNTKVIVNAFACSTDPEYWEDAETFKPERFDNSSVDFTGSNYEFIPFGSGRRMC PGINFGLVSVKLFLAQMLYYFDWKLPDGLSPRKLDMTEIESSIVAKKVNLQLIPTTYAPQ. **RTqPCR** **F-**TTGGGTCAGGAAGGAGAATG **R-**CATCAGGGAGTTTCCAATCG **NotI/PacI restriction primers** **F-**GCA**GCGGCCGC**ATGGTCTTGTTTTTC **R-**TCG**TTAATTAA**TTATTGGGGAGCATAAG

Ct20303 **(KC441531)**	**cDNA** ATGTTTTCTTCCTCCTTTGAGACTTTGATCCTCTCTTTTGTGTCACTCTTCGTTATCATG ATCTTTATCCGTTCCAAATGGATTTCATCAAATTCTAAGATGGCGAAAAACTTACCTCCA TCTCCATCTGGGCTTCCGATTATTGGAAACCTCCACCAACTAGGGTTTACACCTCATGA ATCCCTTAGAACATTAGCTCATAAATATGGTTCACTCATGTTGATTCATCTAGGAAGTGT TCCCGTCATTGTAGCTTCTTCTGCTGAAGCGGCCAAAGAAATCATGAAAACTCATGATC TGATATTTTCAAATAGGCCTAAAATGAATATTGCAAGCATAGTTACTTTTGATGCTAAAA TTGTGGCGTTGTCTCCTTATGGTGAACATTGGAGACAATCAAAGAGCGTATATCTTCTC AACCTCCTAAGTGCGAAAAGAGTTCAGTCATTTCGTCACGTAAGAGAAGACGAGATGA ATCAAATGCTTGATATAATTGGAAATAGTTGCGGTTCTGAAATTGACTTAAGCAAAAGC ATCACGTCACTCACAAACGATGTAGTATGCCGGGTAGCTTTCGGGAAGAAATATTACG AGGATTCGTTCAAGGACCTCATGAAGGAGTTATTTGACATGTTTGCTTTCTTTAGTGTT GGAAATTATGTTCCATCACTTGCATGGGTAGATCGATTGAGCGGCTTGGAAGGAAAGG CAAAAAAAATTGCTAAAAAACTTGATGCGATTTTCGATGGCATCGTCAAACAACATGAG ACTAGGAGCAATGAAAGTAAGACAAATCAAGACATCGTTGACATATTACTAGAAACTCA AAGAGAACAAGCAAGAGCAGGCACCCCGTTTCATAGAGATACGGTTAAAGCTCTAACA CAAGAAATGTTTGTCGGTGGGACGGATACTACAACTACAGCTATAGAGTGGGCAATCT CTGAGGTAATAAAGCATCCAAGAGTAACGAAGAAACTCCAGCAAGAACTAGACGAAAT AGCCCAAGGAAGACAAAGAATCACAGAAGAAGATTTGGAGAACGCGCAACACCCATA CTTAGACGCCGTATTAAAAGAGAGTATGCGATTGCATATTCCCCTTCCACTAATTGTTC CTCGAGAAGCAACACAAGATGTCAAAGTAATGGGTTATGACATCGCAGCAGGTACACA AGTGGTTATCAATGCGTGGATGATTGCACGAGATCCCACGATATGGAAMGATGCGGAT GAGTTTAAGCCTGAGAGGTTCCTAGACACTGATATCGACTACAAAGGTCTACACTTTGA ACTTCTTCCGTTTGGTGCTGGCAGAAGAGGGTGCCCAGGGATTCAGTTTGCTATGTCT GTTAACAAGCTTGCTTTAGCAAATTTGGTATACAAATTTGACTTCAAACTTCCAAATGGA CAGAGCGTAGAACAATTAGACATGACTGATTCTCCTGGTATTACTCTCAGTAAAAAGYA TCCTCTGCTAGTAATACCAACTGCTCGAGTTTAG **Protein** MFSSSFETLILSFVSLFVIMIFIRSKWISSNSKMAKNLPPSPSGLPIIGNLHQLGFTPHESLRT LAHKYGSLMLIHLGSVPVIVASSAEAAKEIMKTHDLIFSNRPKMNIASIVTFDAKIVALSPYGE HWRQSKSVYLLNLLSAKRVQSFRHVREDEMNQMLDIIGNSCGSEIDLSKSITSLTNDVVCR VAFGKKYYEDSFKDLMKELFDMFAFFSVGNYVPSLAWVDRLSGLEGKAKKIAKKLDAIFDG IVKQHETRSNESKTNQDIVDILLETQREQARAGTPFHRDTVKALTQEMFVGGTDTTTAIE WAISEVIKHPRVTKKLQQELDEIAQGRQRITEEDLENAQHPYLDAVLKESMRLHIPLPLIVPR EATQDVKVMGYDIAAGTQVVINAWMIARDPTIWXDADEFKPERFLDTDIDYKGLHFELLPF GAGRRGCPGIQFAMSVNKLALANLVYKFDFKLPNGQSVEQLDMTDSPGITLSKKXPLLVIP TARV **RTqPCR** **F-**GCAAAAGCATCACGTCACTC **R-**TGAGGTCCTTGAACGAATCC **NotI/PacI restriction primers** **F-**GCA**GCGGCCGC**ATGTTTTCTTCCTCCTTTG **R-**TCG**TTAATTAA**CTAAACTCGAGCAGTTGG

Ct88 **(KC441532)**	**cDNA** ATGGAAGCCCTTCATTTCTTCATCTTGTTTCTAGGCTTAGCCTGGTCCATATTTCACRG ATATGTCATTCCAAAGATGAAAAATCTTCCACCAACTCCATTTTCACCCTTATCATTAAT AGGCTACCTTTGCTTCTACAAGCAGCAACCACTCCACCGAACTCTCTCACAAATCGCC TCGCGATACGGCCCTATTTTGCTACTCCAGTTTGGCTGCAAACGAGTCCTCTTAGTTTC TTCCTCATTAGCCGCGGAGGAACTGTTTGCCAAAAATGATAAGGTATTTGCATACCGA CCTAAGTTGATTACAGGAAAGGAATTTGGATGTAATTATACCGGCCTTGCTTCGGCGC CACATGGTGCTCATTGGCGTCACCTACGTCGTGTTTCAAGCCTTGAAATTCTCCCATTT CATCGTTTGCCAGGCCAAGATGGCTCAATTGCTGATGAGGTGAAGCTGTTGCTAGGCC GGCTTTTTCATACCGAGAAGGAAATAGTAGAGATGAAATCTGTCTTTCTAGACCTTGTG TTTGACATGATGATGAAGATGTTCGCTGGAAAACGGTATGACGAAAAGAAAAGGACAA CAGAGGATGACCAGATCAAGTCAACTTCATTGAAAGATTATGTGATCCGTTCATTTAGG ATTACCACGGGAGAGCCTGATGTAGCGTATTTTATGCCAATATTAAAGTCTCTGGGACT GAGAGGCTTGGAGCGAAAGTGCAACGAGTTGCAGAAAAAAGGAGATGCGCTCATGGA CAGCTTAATTGAAGATGTAAGGAAAAAGATACCAGAGTTTTCTATTGGATCAGGGGAG ACGGAAGAAAAAGTGATAGAATTATTGTTGGCTAGACAGAAGGATGATCCTAAACGTTA CAGTGATGAAACTATCAGGGGACTGTTGCTCGGTTTATTAACAGCAGGTACTACAACA TCGGCAAGTATACTGGAATGGGCATTTTCACTTTTGCTGAACCATCCAGAAATTCTACA TAAAGCACAAAGTGAGATCGATAATCATGTTGGCAACAATCGTTTCTTAGAGGAGTCAG ACATAGAGCATCTTCCTTATCTTCGTTGCATTGTTAAGGAGACACTGCGATTATACCCA AGTGCACCTTTACTTGTTCCTCACGAGTCGTCAAAGGACTGTAAAGTTGGAGGGTATC TTGTACCTAAAGAAACCATGCTAATGGTGAATGTTTGGGATATTCAAAATGATCCTAAT ATATGGGCTGAACCAACAAAGTTCAGTCCAGAGAGATTTAGGGAGATTGTAGACGAAA GAGATGGATTTAAATTGATGCCCTTTGGATATGGAAGGAGGAGTTGTCCTGGGAAGCA CATGGCGGTGCGGGTGATCACTTTCGCTTTGGGCTCACTAATACATTGCTTTAAATGG GAAAGGATTAGTGAGGAGATGGTTAACTTGACTGAACAAACAGGCCTTGCTTTGCTCA AAGCACAACATTTGATGGCTAAATGTAGCGCGCGTCGTACAATGGTGAAGTTACTACG TCAAGTTTGA **Protein** MEALHFFILFLGLAWSIFHXYVIPKMKNLPPTPFSPLSLIGYLCFYKQQPLHRTLSQIASRYG PILLLQFGCKRVLLVSSSLAAEELFAKNDKVFAYRPKLITGKEFGCNYTGLASAPHGAHWR HLRRVSSLEILPFHRLPGQDGSIADEVKLLLGRLFHTEKEIVEMKSVFLDLVFDMMMKMFA GKRYDEKKRTTEDDQIKSTSLKDYVIRSFRITTGEPDVAYFMPILKSLGLRGLERKCNELQK KGDALMDSLIEDVRKKIPEFSIGSGETEEKVIELLLARQKDDPKRYSDETIRGLLLGLLTAGT TTSASILEWAFSLLLNHPEILHKAQSEIDNHVGNNRFLEESDIEHLPYLRCIVKETLRLYPSA PLLVPHESSKDCKVGGYLVPKETMLMVNVWDIQNDPNIWAEPTKFSPERFREIVDERDGF KLMPFGYGRRSCPGKHMAVRVITFALGSLIHCFKWERISEEMVNLTEQTGLALLKAQHLMA KCSARRTMVKLLRQV. **RTqPCR** **F-**AGCAAGGCCTGTTTGTTCAG **R-**GGTGATCACTTTCGCTTTGG **NotI/PacI restriction primers** **F-**GCA**GCGGCCGC**ATGGAAGCCCTTCATTTC **R-**TCG**TTAATTAA**TCAAACTTGACGTAGTAAC

Ct22436 **(KC441533)**	**cDNA** ATGACGGCGTTACTTAACGGCAAAGTATTCGACTCATCAAACACATCCGAAACGAATAT TCCGTTAGCCATGATTATGGAGAATCGTGARCTTTTGATGATTTTAACGACTTCAGTTG CGGTTTTGATCGGATGCGTTGTGGTGCTTGTTTGGAGACGGTCGTCGTCGTCGAAGAA AGTTGAGGCTCCGGTGATCGTTGTGCCGAAGAGAGTGACGGAGGAGGAGGTTGATGA TGGTAGGAAGAARGTTACTGTGTTTTTTGGAACTCAGACTGGTACTGCTGAAGGTTTTG CTAAGGCRCTTGTTGAAGAAGCTAAAGCGAGATATGAAAAGGCGGTGTTTAAAGTGAT TGATTTGGATGATTACGCTGCTGAGGATGATGAGTATGAGGAGAAGTTAAAGAAAGAA TCTCTTGCTTTTTTCTTTTTAGCAACAWATGGAGRTGGTGAGCCGACAGATAATGCTGC TAGATTTTATAAATGGTTTACCGAGGGAGAAGARAGAGGTGAATGGCTAGAAAAGCTT CAATACGGAGTGTTTGGACTTGGTAACAGACAGTATGAGCATTTCAACAAGATTGCGA AGGTGGTCGATGATAAACTTGTGGAACAGGGTGCAAAGCGTCTTGTTCCTGTTGGCAT GGGAGATGACGATCAATGTATTGAAGATGACTTCACTGCATGGAAAGAGTTGGTGTGG CCCGAGTTGGATCAGTTACTTCGTGATGAGGATGATACATCTGTTGCCACTCCATACA CAGCTGCTGTTGCAGAATACCGTGTTGTGTTTCATGACAAACCAGACACATACGATGA GGATCAACTGACAAATGGCCATGCAGTTCATGATGCTCAACATCCATGCAGATCCAAT GTCGCTGTCAAAAAGGAACTCCATTCCCCTGAATCTGACCGGTCTTGCACTCATTTGG AATTTGATATATCTAATACTGGATTATCGTATGAAACCGGGGACCATGTTGGAGTCTAC TGTGAGAATCTAAGTGAAGTTGTGGACGAAGCTGAAAAATTAATAGGTTTACCGCCAC ACACTTATTTCTCGGTACACACTGATAACGAAGACGGGACACCACTTGGTGGAGCCTC TTTGCCACCTCCTTTCCCTCCATGCACTTTAAGAAAAGCATTGGCTTCCTATGCTGATG TCTTGAGCTCTCCCAAAAAGTCAGCTTTGCTTGCTTTAGCTGCTCATGCTACTGATACC ACTGAAGCTGATAGACTGAAATTTCTTGCGTCTCCTGCTGGAAAGGATGAATATGCTCA GTGGATAGTTGCAAGCCAGAGAAGTCTCCTTGAGGTCATGGAGGCATTCCCATCAGCT AAGCCTCCACTTGGTGTCTTTTTTGCATCTGTCGCCCCACGCTTGCAGCCGAGATACT ATTCTATTTCTTCTTCCCCAAAGGATTCATGTAACTTGTGCATTAGTGTATGA **Protein** MTALLNGKVFDSSNTSETNIPLAMIMENRELLMILTTSVAVLIGCVVVLVWRRSSSSKKVEA PVIVVPKRVTEEEVDDGRKKVTVFFGTQTGTAEGFAKALVEEAKARYEKAVFKVIDLDDYA AEDDEYEEKLKKESLAFFFLATXGXGEPTDNAARFYKWFTEGEERGEWLEKLQYGVFGL GNRQYEHFNKIAKVVDDKLVEQGAKRLVPVGMGDDDQCIEDDFTAWKELVWPELDQLLR DEDDTSVATPYTAAVAEYRVVFHDKPDTYDEDQLTNGHAVHDAQHPCRSNVAVKKELHS PESDRSCTHLEFDISNTGLSYETGDHVGVYCENLSEVVDEAEKLIGLPPHTYFSVHTDNED GTPLGGASLPPPFPPCTLRKALASYADVLSSPKKSALLALAAHATDTTEADRLKFLASPAG KDEYAQWIVASQRSLLEVMEAFPSAKPPLGVFFASVAPRLQPRYYSISSSPKDSCNLCISV. **RTqPCR** **F-**CCTGGGCCAATCATGATAAC **R-**GAAAGCCAGGATTGCAGTTG **NotI/PacI restriction primers** **F-**GCA**GCGGCCGC**ATGCAAAACTCCGACAAG **R-**TCG**TTAATTAA**TCATACACTAATGCACAAG

Ct2448 **(KC441534)**	**cDNA** ATGGCTGCAAACAGCCATAGCTCATTGGGGGAGGAAGCGAGCGCCAACAAAATCGAG GTTGCTCTTGCAGTCCTTGTTGGTCTCCTCGCAATGTTAACTATTTCATGGTATAAAAG GTCAAATTGGAGCACGGGAAAAGGCACACCTCCGTTGCCTCCAGGTCCAAAAGGTCT ACCAATTCTGGGATACCTTCCATTTCTTAGTCCTAACTTGCACCATGAATTTGCCAAAAT GGCTAATCAGTATGGTCCCATTTTCAAGCTCTACCTTGGAAGCAAGCTTCACATCGTG GTAAACACCGCGGACCTAGCCAAGGTTGTAACTGGTGAGCAAGACGAGAGCTTTGCT AACCGCGATCCACATATTGCTGGACTAACCGCAAGTTATGGTGCAAGCGATGTAGCAT GGCAAAACAACAACGCCAATAGGCGTAAMTTACGTAAAGTTTTAGTGCATGAAGTCCT TAGCAACAAGAACCTTGAAGCTTCACATGCCTATCGTCGAAGTGAAGTCAGAAAGACC ATAAAATATGTCCATGATATGATTGGTACAGCAGTTGACATTAACGAGGTATCTTTCTC AACGGTCTTGAATATTTTAACACACATAGTGTGGGGGAAAGGTTTTGTCGAAGGCGCA AAATACCCTGATCTGGCAGCTGATATACGTAAAGTGGTATTAGCAATTGTTGAGACCG CAGAAGGGCTAAACCTCTCTGATTTTTTCCCGATGCTTGCGAGGTTTGATTTCCAAGGT GTCGAGCATAGAATGAAGACGCAAGTTAAGAAGTTCGACTACATTTTCGAGACTACTAT CGAGGAGAGAACCAGCTCCAAGTCCGAAATGTCCGAAGAGACAGTTAAGCAAGAAGG AAGGAAAGATTTTTTACAGATCTTATTAGAGCTTCTAGATCAAAACACCGCGACATCAA TTAACATGACTCAATTAAAGGCGCTTGTCGTGGATATCTTTCTTGGTGGAACAGATGCA ACTTCAGCCATGACAGAATGGGCAATGACAGAGATTTTACGATATCCAAAGGTAATGA AAAAGGTGCAAGATGAATTAGCTGAAGTTGTAGGGTTAAACAACATTGTCGAAGAATC CCATCTCCCACAATTGAAGTACCTCGATGCAGTGTTTAAAGAAACATTCCGTTTACATA CCCCACTACCTTTTCTACTCCCGCGAACCCCAGATAAATCCTGTGTGGTCGGTGGATA CACAGTCCCAAAGGGTGCTACAGTCTTTTTGAATGTATGGGCGATACAAAGGGATCCT CAAAATTGGGCGAATCCATCTGAATTCAACCCTGAGAGGTTCTTGAACAACAAAGGGT CAGAGAAATGGGACTATAGTGGGACAAATTCTACGTATTTTCCATTTGGGTCTGGTAGA AGAAGGTGCCCAGGAATCCCCTTAGGAGAGAAAATGATGATGCATATTTTGGCTTCGT TGATGCACTCATTTGATTGGAGCTTGCCAAAAGGCGAAGAGCTTGACCTTTCTGATAAA TTTGGCATTGCAATGAAGAAGAAAATGCCACTGGTAGTTATCCCGTCTCCAAGATTGA GCGATTTAAGCCTTTACTCGTGA **Protein** MAANSHSSLGEEASANKIEVALAVLVGLLAMLTISWYKRSNWSTGKGTPPLPPGPKGLPIL GYLPFLSPNLHHEFAKMANQYGPIFKLYLGSKLHIVVNTADLAKVVTGEQDESFANRDPHIA GLTASYGASDVAWQNNNANRRXLRKVLVHEVLSNKNLEASHAYRRSEVRKTIKYVHDMIG TAVDINEVSFSTVLNILTHIVWGKGFVEGAKYPDLAADIRKVVLAIVETAEGLNLSDFFPMLA RFDFQGVEHRMKTQVKKFDYIFETTIEERTSSKSEMSEETVKQEGRKDFLQILLELLDQNT ATSINMTQLKALVVDIFLGGTDATSAMTEWAMTEILRYPKVMKKVQDELAEVVGLNNIVEE SHLPQLKYLDAVFKETFRLHTPLPFLLPRTPDKSCVVGGYTVPKGATVFLNVWAIQRDPQN WANPSEFNPERFLNNKGSEKWDYSGTNSTYFPFGSGRRRCPGIPLGEKMMMHILASLMH SFDWSLPKGEELDLSDKFGIAMKKKMPLVVIPSPRLSDLSLYS. **RTqPCR** **F-**CAGGATTTATCTGGGGTTCG **R-**TGTCGAAGAATCCCATCTCC **NotI/PacI restriction primers** **F-**GCA**GCGGCCGC**ATGGCTGCAAACAGCCAT **R-**TCG**TTAATTAA**TCACGAGTAAAGGCTTAA

Ct396 **(KC441535)**	**cDNA** ATGGAGTTAACAACTCTAGCAACATTCTTATTTGTTCTTGTAATAGCTTTCATGATCCTG AACAAAATGAGGGTAAACAGATCCAAGCACAACGCACCGCCTCGAGCAAAAGGCGCA TGGCCTATAATCGGACACCTTCATCTTGTAGCCGGATCTCGACCACGTCAACATGTTTT AGGTGACTGGGCAGACAAATATGGGCCTATTTTCACCATCAARCTTGGTTTCCACCAA GCTTTGGTTGTTAGTAGTACAGAGATAGCTAAAGAGTGCTTTACCACAAACGATAAAGT GTTTGCAAGTCGACCCAAGGCAAAGGCAGTAGAGATCATGGGCTATAACTATGCCATG TTCGGTCTAGCTCCATATGGAGATTACTGGCGACAAGAGCGCAAGATTATCACACTCG AGGTTCTCTCTCCACGCCGAGTTGAGATGCTAGGACATGTTCGAGAATCAGAGATTAG AGCGTCCTTGAAAGATATCCACGTGGCTTGGGAAAATAACAAAGAGAATGAAGGTTCG AATATGGTAAAGGTGGATATGAAACAATGGTTTGGTACTTTGGTACTAAACATTGTAGT TAGAGTTATTTCAGGAAAAAGATTTCCAGCCAATGATGAAGAAGGTGTTCATTTTCAAA ATGTGTTAAGGAAAAATGTCGTGTTAATGGGCGCCTTTGTGGCGTCAGATTACATTCCA TATATTGATCGTTTTGACTTGGGAGGATATCAGAAAGAAATGAAGTTAGTATGGAAAGA ACTGGACGACATCATGGAGGGATGGTTACAGGAGCGCAGGAGAGAAATGGAGTCGG GCCAACAACTCGAAAGAAACCAAGCCTTCATGGATGTCTTGATTTCTAGCCTACAAGG TGCCCCCAAAGAGGATTTCTCCGATTTTGACCATGACACAGTTATCAAAGCCTCGTGT CTGGAACTAATAACTGCTGGATTGGACACGACATCTGTAACTCTAACGTGGGTGTTAT GTTTGTTGCTCAATAATCCAAAAGCATTAAGAACTGTACAAGAAGAAATTGATGAGCAT GTCGGAAGAGATAGACCAGTGGAGGAATCAGACATGAAAAACTTACTCTACCTTGGAG CCGTCATCAAAGAAACAATGCGCTTGTACCCGGCAGCTCCTCTAGCCGTTCCTCACGA GTCTACGGAAGATTGTGTTGTGAGTGGCTACAACGTCCCCAAAGGCACACGTTTGTTA ATAAATCTCTGGAAATTGCACCGTGATCCAAATATTTGGTCAGATCCTGAAGAGTTTAA GCCAGAACGATTTATGACAACTAACAAGGATATTGATGTCAAAGGGAGACATTACGATT TGCTCCCATTTGGAAGTGGTAGAAGAATGTGCCCTGGTATATACTTTGCCCTACAGGC TATGCATTTAACATTAGCAACTCTGATACAACAGTTCGAGCTCGTAAAACCAACTGATG AACAAATTGATATGAGTGAGCGCTCAGGACTAACTACAAGCAAAGCGACGCCACTTGA AGTCCTTCTTTCCCCTCGTTCAGTACCCCTCAATACGTAA **Protein** MELTTLATFLFVLVIAFMILNKMRVNRSKHNAPPRAKGAWPIIGHLHLVAGSRPRQHVLGD WADKYGPIFTIKLGFHQALVVSSTEIAKECFTTNDKVFASRPKAKAVEIMGYNYAMFGLAPY GDYWRQERKIITLEVLSPRRVEMLGHVRESEIRASLKDIHVAWENNKENEGSNMVKVDMK QWFGTLVLNIVVRVISGKRFPANDEEGVHFQNVLRKNVVLMGAFVASDYIPYIDRFDLGGY QKEMKLVWKELDDIMEGWLQERRREMESGQQLERNQAFMDVLISSLQGAPKEDFSDFD HDTVIKASCLELITAGLDTTSVTLTWVLCLLLNNPKALRTVQEEIDEHVGRDRPVEESDMKN LLYLGAVIKETMRLYPAAPLAVPHESTEDCVVSGYNVPKGTRLLINLWKLHRDPNIWSDPE EFKPERFMTTNKDIDVKGRHYDLLPFGSGRRMCPGIYFALQAMHLTLATLIQQFELVKPTD EQIDMSERSGLTTSKATPLEVLLSPRSVPLNT. **RTqPCR** **F-**TCTCTTCCGACATGCTCATC **R-**TAACTGCTGGATTGGACACG **NotI/PacI restriction primers** **F-**GCA**GCGGCCGC**ATGGAGTTAACAACTCTAG **R-**TCG**TTAATTAA**TTACGTATTGAGGGGTAC

Ct23977 **(KC441536)**	**cDNA** ATGGATCTTCTCCTTTTGGAGAAAACCCTTTTGGGTCTCTTAGCAGCCATTTTAGGAGC AATATTTATTTCCAAGTTACGCGGTAAACGTTTCAAGCTCCCACCAGGGCCAATTCCAG TACCTATTTTCGGGAACTGGCTCCAAGTGGGAGATGATTTAAACCACCGTAACTTAACT GATCTCGCCAAGAAATTTGGCGAGATCTTTCTGTTACGCATGGGACAACGCAACCTAG TTGTTGTGTCCTCCCCTGATCTTGCTAAGGATGTTTTACATACACAAGGTGTCGAGTTC GGTTCACGTACTCGAAACGTTGTGTTTGATATATTTACGGGTAAAGGACAAGATATGGT GTTTACGGTTTATGGTGAACATTGGAGGAAGATGCGTAGGATTATGACTGTCCCGTTTT TTACTAACAAAGTGGTCCAACAATACAGATTCGGATGGGAAGCAGAGGCAGCAGCAGT TGTGGAAGATGTGAAGAAGAATCCTGCATCTGCAACAGAAGGTACCGTGCTAAGAAGA AGACTACAATTGATGATGTACAACAACATGTTCAGGATTATGTTTGACAGAAGGTTTGA GAGTGAGGATGATCCTTTGTTCTTGAAGCTCAAGGCTTTGAATGGTGAAAGGAGTCGA TTAGCACAAAGTTTCGAATACAATTATGGCGATTTTATCCCCATCTTGAGGCCGTTTTT GAGAGGTTATTTGAAGTTGTGTAAGGAAGTTAAGGAGAAGAGGTTGCAACTGTTTAAG GATTACTTTGTTGATGAAAGGAGGAAGTTGGGAAGCACCAAAAGCCTGGATAACAACC AAATCAAATGTGCAATTGATCATATCCTTGAAGCAAAGGACAAGGGAGAAATCAACGA GGACAATGTTCTTTACATTGTTGAGAATATCAACGTTGCAGCAATTGAGACAACCTTGT GGTCTATCGAATGGGGTATTGCCGAGCTTGTGAACCACCCTGAGATCCAAGCCAAACT AAGGCACGAGCTTGACACTAAGCTTGGACCAGGAGTACAAGTCACTGAGCCCGACAT CCAGAATCTTCCCTACCTCCAAGCTGTAGTTAAGGAAACTCTTCGCCTTAGAATGGCC ATCCCTCTTCTAGTCCCACACATGAACCTACATGATGCCAAGCTTGGTGGCTTTGACAT TCCAGCTGAGAGCAAGATCTTGGTCAACGCCTGGTGGCTCGCTAACAATCCCGACCA ATGGAAAAAACCTGAGGAGTTTAGACCCGAGAGATTCTTAGAAGAGGAAAGTAAAGTT GATGCTAACGGGAATGATTTCAGGTACTTGCCCTTTGGAGTTGGGAGGAGGAGTTGTC CTGGTATTATACTTGCATTGCCAATCCTCGGGATCACTATTGGGCGTATGGTCCAGAAT TTCGAGTTGCTACCACCACCAGGCGTTTCAAAGATCGACACGAGTGAGAAAGGTGGA CAGTTTAGCTTGCATATCTTGAAGCATTCCACCATTGTTGCTAAACCAAGATCCTTTTAA **Protein** MDLLLLEKTLLGLLAAILGAIFISKLRGKRFKLPPGPIPVPIFGNWLQVGDDLNHRNLTDLAK KFGEIFLLRMGQRNLVVVSSPDLAKDVLHTQGVEFGSRTRNVVFDIFTGKGQDMVFTVYG EHWRKMRRIMTVPFFTNKVVQQYRFGWEAEAAAVVEDVKKNPASATEGTVLRRRLQLM MYNNMFRIMFDRRFESEDDPLFLKLKALNGERSRLAQSFEYNYGDFIPILRPFLRGYLKLC KEVKEKRLQLFKDYFVDERRKLGSTKSLDNNQIKCAIDHILEAKDKGEINEDNVLYIVENINV AAIETTLWSIEWGIAELVNHPEIQAKLRHELDTKLGPGVQVTEPDIQNLPYLQAVVKETLRL RMAIPLLVPHMNLHDAKLGGFDIPAESKILVNAWWLANNPDQWKKPEEFRPERFLEEESK VDANGNDFRYLPFGVGRRSCPGIILALPILGITIGRMVQNFELLPPPGVSKIDTSEKGGQFSL HILKHSTIVAKPRSF. **RTqPCR** **F-**AAAGGAGGAAGTTGGGAAGC **R-**CCTTGTCCTTTGCTTCAAGG **NotI/PacI restriction primers** **F**-GCA**GCGGCCGC**ATGGATCTTCTCC **R-**TCG**TTAATTAA**AGAGTATGGATTAT

Ct23062 **(KC441537)**	**cDNA** ATGCAAACAGATTCCGTACAAGTATCACCATTTGATCTAGCATCATCACTTCTTAACGTAAAACTAA CCGAAACGTTAAACACATCAGAAGAGCTAACCATGTCACCAGCTATGAAGATGCTAGTTGAGAATC GTGACATGCTAACGTTGTTTACGACCACAATTGCTGTGTTGATTGGCTGTGTTGTTGTTTTGGTGT GGAGGAAATCGTTTACTAAGAAATCGGTCGTTAATAAGGAAGTTGAGACCATGAAGATTGTTTTGC CGAAAAAGGAGATAAAACATGAGGAGGTTGATGATGGGAAGAAAAAGGTTACGATTTTGTTTGGTA CGCAAACCGGAACTGCTGAAGGTTTCGCAAAGGCACTTTTAGAAGAAGCTAAAGTTCGATATGAAA AGGCTGTGTTCAAAGCAATTGATTTGGATGATTATGCTGCTGATGACGAAGAGTATGAGGAGAAAT TTAAGAAAGAATCTTTAGCATTTTTCTTTTTGGCTACATATGGAGATGGTGAGCCAACAGATAATGC GGCGAGGTTTTATAAATGGTTTACAGAGGGAGATGATAAAGGAGAGTGGCTTAAAAAACTTCAATA CGGGGTGTTTGGCCTCGGCAACAGGCAGTATGAGCATTACAACAAGATTGCAGTGGTGGTGGAT GATAAACTTGCAGAGCAGGGTGCAAAGCGTCTTGTTCCTGTTGGTATGGGAGATGATGATCAATG TATGGAAGATGATTTCTCAGCATGGAAAGAGCTTGTGTGGCCTCAGTTGGATCAATTGTTGCGTGA TGAGGATGACATGAGTGTTGCCACTCCATACACAGCTGCCGTTTTGGAATACCGTATCGTATATCA TGACAAACCAGACTCGTCTGCAGAAGATCAACATACGAATAGTCATGCTATTCCTGATGCTCAACA TCCAAGCAGATCCAACGTCGCAGTCAAAAAGGAACTCCACACCCCTGAATCTGACCGGTCTTGCA CTCATTTGGAATTTGATATATCTAATACTGGATTATCGTATGAAACCGGGGACCATGTTGGAGTCTA CTGTGAGAATCTAAGTGAAGTTGTGGACGAAGCTGAAAAATTAATAGGTTTACCGCCACACACTTA TTTCTCGGTACACACTGATAACGAAGACGGGACACCACTTGGTGGAGCCTCTTTGCCACCTCCTTT CCCTCCATGCACTTTAAGAAAAGCATTGGCTTCCTATGCTGATGTCTTGAGCTCTCCCAAAAAGTC AGCTTTGCTTGCTTTAGCTGCTCATGCTACTGATACCACTGAAGCTGATAGACTGAAATTTCTTGC GTCTCCTGCTGGAAAGGATGAATATGCTCAGTGGATAGTTGCAAGCCAGAGAAGTCTCCTTGAGG TCATGGAGGCATTCCCATCAGCTAAGCCTCCACTTGGTGTCTTTTTTGCATCTGTCGCCCCACGCT TGCAGCCGAGATACTATTCTATTTCTTCTTCCCCAAAGTTTGCGCCAAATAGGATTCATGTAACTTG TGCATTAGTGTATGAGCAAACACCATCAGGCCGTGTTCACAAGGGAGTCTGTTCAACATGGATGA AGAATGCCGTGCCTTTGACAGAAAGCCAGGATTGCAGTTGGGCCCCAATTTATGTTAGAACATCC AATTTCAGACTTCCTTCTGATCCTAAGGTCCCAGTTATCATGATTGGCCCAGGCACTGGATTGGCT CCATTTAGAGGTTTCCTTCAGGAAAGGTTAGCTCAGAAGGAAGCTGGGACTGAGCTCGGATCATC CATCTTATTCTTCGGATGCAGGAATCGCAAAGTGGATTTCATATATGAGGACGAGCTTAATAATTTC GTGGAGACTGGTGCTCTTTCCGAGCTTATTACAGCCTTCTCTCGTGAAAGTGCCACTAAGGAGTA CGTGCAACACAAGATGAGTCAGAAGGCTTCGGAAATCTGGAATTTACTTTCTGAGGGAGCATATTT GTATGTTTGTGGTGATGCCAAAGGCATGGCCAAAGATGTACATCGGACTCTGCACACTATTGTGC AAGAACAGGGATCTCTAGACTCGTCAAAGGCGGAGCTCTATGTGAAGAATCTACAAATGGCAGGA AGATATCTCCGTGATGTATGGTAA **Protein** MQTDSVQVSPFDLASSLLNVKLTETLNTSEELTMSPAMKMLVENRDMLTLFTTTIAVLIGCVVVLVWRK SFTKKSVVNKEVETMKIVLPKKEIKHEEVDDGKKKVTILFGTQTGTAEGFAKALLEEAKVRYEKAVFKAI DLDDYAADDEEYEEKFKKESLAFFFLATYGDGEPTDNAARFYKWFTEGDDKGEWLKKLQYGVFGLGN RQYEHYNKIAVVVDDKLAEQGAKRLVPVGMGDDDQCMEDDFSAWKELVWPQLDQLLRDEDDMSVA TPYTAAVLEYRIVYHDKPDSSAEDQHTNSHAIPDAQHPSRSNVAVKKELHTPESDRSCTHLEFDISNTG LSYETGDHVGVYCENLSEVVDEAEKLIGLPPHTYFSVHTDNEDGTPLGGASLPPPFPPCTLRKALASY ADVLSSPKKSALLALAAHATDTTEADRLKFLASPAGKDEYAQWIVASQRSLLEVMEAFPSAKPPLGVFF ASVAPRLQPRYYSISSSPKFAPNRIHVTCALVYEQTPSGRVHKGVCSTWMKNAVPLTESQDCSWAPIY VRTSNFRLPSDPKVPVIMIGPGTGLAPFRGFLQERLAQKEAGTELGSSILFFGCRNRKVDFIYEDELNN FVETGALSELITAFSRESATKEYVQHKMSQKASEIWNLLSEGAYLYVCGDAKGMAKDVHRTLHTIVQE QGSLDSSKAELYVKNLQMAGRYLRDVW. **RTqPCR** **F-**CTTCAATACGGGGTGTTTGG **R-**AGGAACAAGACGCTTTGCAC **NotI/PacI restriction primers** **F-**GCA**GCGGCCGC**ATGCAAACAGATTCC **R-**TCG**TTAATTAA**TTACCATACATCACG

Chapter 4

Cloning and Characterization of a Trichome-Specific 13-Lipoxygenase Expressed During Pyrethrin Biosynthesis in Pyrethrum (*Tanacetum cinerariifolium*)

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Abstract

The lipid precursor alcohols of pyrethrins - jasmolone, pyrethrolone and cinerolone - have been proposed to share parts of the oxylipin pathway with jasmonic acid. This implies that one of the first committed steps of pyrethrin biosynthesis is catalyzed by a lipoxygenase, catalyzing the hydroperoxidation of linolenic acid at position 13. Previously, we showed that the expression and activity of chrysanthemyl diphosphate synthase (TcCDS), the enzyme catalyzing the first committed step in the biosynthesis of the acid moiety of pyrethrins, is trichome-specific and developmentally regulated in flowers. In the present study we characterized the expression pattern of twenty-five lipoxygenase EST contigs, and subsequently carried out the molecular cloning of two pyrethrum lipoxygenases, *TcLOX1* and *TcLOX2*, that have a similar pattern as *TcCDS*. Only recombinant TcLOX1 catalyzed the peroxidation of the linolenic acid substrate at the C13 position. Just as *TcCDS*, *TcLOX1*, is exclusively expressed in trichomes. Phylogenetic analysis showed that the enzyme shared highest homology with chloroplast-localized 13-type-lipoxygenases that are involved in maintaining basal levels of jasmonate.

Introduction

Pyrethrins, the insecticidal constituents of pyrethrum flower extracts, are esters consisting of a combination of either one of two acids (chrysanthemic acid or pyrethric acid), and three alcohols (pyrethrolone, cinerolone, or jasmolone) (Figure 1). The three alcohols esterified to chrysanthemic acid are collectively known as type I pyrethrins, whereas the esters of pyrethric acid represent type II pyrethrins [1,2]. The alcohol moieties of pyrethrins resemble the plant hormone jasmonic acid (JA (**4**)), and have been suggested to be derived either from 7-OH-Jasmonic acid (**5**) or cis-jasmone (**7**) through the oxylipin pathway (Figure 2) [2]. Specific oxylipins have important regulatory roles in different aspects of plant development [3-5] as well as in plant defense towards pathogens and insects [6-8]. The generation of oxylipins is initiated by the action of lipoxygenases/LOXes (Linoleate:oxygen oxidoreductase, EC 1.13.11.12), which are non-heme, iron-containing di-oxygenases that catalyze the incorporation of molecular oxygen into poly-unsaturated fatty acids (PUFAs) containing a (1*Z*, 4*Z*)-pentadiene system, such as linoleic acid, linolenic acid (**1**), or arachidonic acid. Since linoleic and linolenic acid constitute the most common substrates found in plants, LOXes are classified with respect to the position (9 or 13) and stereo-specific oxygenation on the hydrocarbon backbone of these fatty acids. Based on the presence of a plastidic transit peptide on their primary structure, LOXes can be grouped into two subfamilies [9,10]. Those enzymes carrying no transit peptide are designated as type-1 LOXes, and those harboring putative chloroplast transit peptide sequences are classified as type-2 LOXes. All LOX-forms carrying a chloroplast signal peptide (type-2) catalyze the incorporation of oxygen at position 13 (13-LOXes) of the fatty acid substrate. Hence, it would be expected from the fact that subsequent steps of the JA biosynthetic pathway are in the plastids that for pyrethrin biosynthesis a type-2, 13-LOX gene is involved.

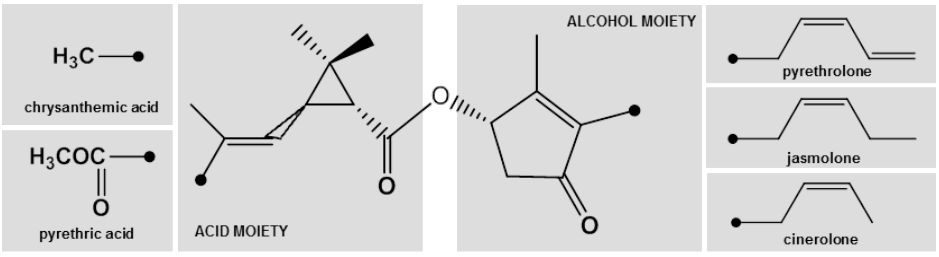

Figure 1: Chemical structures of natural pyrethrins from *Tanacetum cinerariifolium* The alcohol moieties, pyrethrolone, cinerolone, and jasmolone, form esters with the acid moieties, chrysanthemic acid and pyrethric acid, yielding the six pyrethrins esters.

Considerable progress has been made in the characterization of chrysanthemyl diphosphate synthase (TcCDS), the enzyme catalyzing the first committed step in the biosynthesis of the acid terpene moiety of pyrethrins [11], and GDSL-like lipase TcGLIP, an acyltransferase cloned and demonstrated to be responsible for the esterification of (1*R*,3*R*)-chrysanthemoyl-CoA and (S)-pyrethrolone [12]. We recently demonstrated that while the production of the acid moiety of pyrethrins (chrysanthemic acid) by TcCDS and other enzymes occurs in the trichomes, the esterification with the alcohol moiety (pyrethrolone) by TcGLIP happens in the pericarp [13]. However, neither the tissues in which the rethrolone precursors of pyrethrins are produced nor the enzymes catalyzing their first biosynthetic step are known yet.

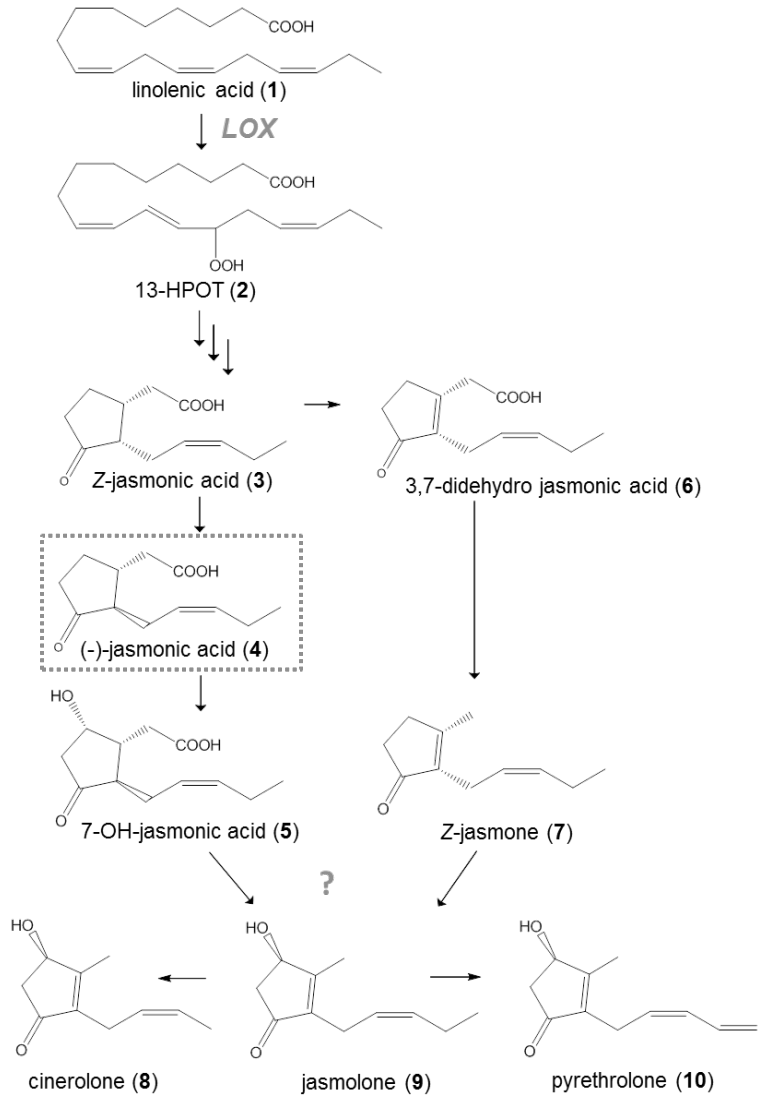

Figure 2: Proposed biosynthetic pathway of the alcohol moiety of pyrethrins. In grey the lipoxygenase (LOX) involved in the first dedicated step leading to cinerolone, jasmolone and pyrethrolone.

We also showed that the *TcCDS*, and *TcGLIP* genes were highly expressed in the early stages of flower development (stages 2 to 5), when there was an active accumulation of pyrethrins, and that their expression was down-regulated as the flower matured and the concentration of pyrethrins had reached its maximum (stages 6 to 7) [13]. Given these observations, we hypothesized that any gene involved in the formation of the alcohol counterpart of pyrethrins would be developmentally regulated in the same way as the *TcCDS* and *TcGLIP* genes. The aim of the present study was to clone pyrethrum LOX enzymes that were regulated in a *TcCDS*-like fashion, and to test the ability of the recombinant enzyme to catalyze the di-oxidation of the linolenic acid substrate.

Results

Isolation and selection of *LOX* EST candidates

To isolate LOX genes involved in pyrethrin biosynthesis, the pyrethrum annotated EST contig library was subjected to a keyword search using LOX or lipoxygenase as keywords. Among the 27,314 high-quality contigs, 25 EST sequences with homology to LOX genes were retrieved, but none were full length. Given our previous observations on the similar transcriptional profiles of *TcCDS* and *TcGLIP* during flower development and the respective high and low expression in trichomes [13], we hypothesized that EST contigs showing a similar expression pattern may be involved in pyrethrin biosynthesis. Among the 25 candidate contigs, six, named Ct20982, Ct4895, Ct20675, Ct2442, Ct938 and Ct425, showed a relative gene expression (RGE) profile similar to that of *TcCDS*, with high and constant or gradually decreasing expression during stages 2-5 followed by a pronounced decreased in stages 6 and 7, and with higher expression in trichomes than in ovaries. Two more ESTs, Ct5842 and Ct15096, followed the *TcCDS* and *TcGLIP* pattern of expression respectively. However, they were both discarded because of their very low expression levels (0.003% and 1.22%) relative to the expression level of *TcCDS* (Table 1, Column RLE). The six EST contigs with an expression similar to *TcCDS* were selected as genes possibly involved in pyrethrin biosynthesis. After RACE amplification of the 3' and 5' ends, assembly of the six selected ESTs resulted in two complete, full-length genes, *TcLOX1* (KC441523) and *TcLOX2* (KC441524).

Phylogenetic analysis of *TcLOX1* and *TcLOX2*

A phylogenetic analysis was performed in which the putative pyrethrum lipoxygenase sequences (*TcLOX1* and *TcLOX2*) were compared with those of 12 biochemically characterized plant lipoxygenases [14]. The phylogenetic tree (Figure 3) displayed 3 main clusters, designated as A, B, and C. All lipoxygenases in cluster A are 13-lipoxygenases

with a plastidic N-terminal localization peptide [14,15]. Two cytoplasmic localized lipoxygenases with 13 (*GmLOX1*) or with a dual 9/13 specificity (*GmLOX3*) [16,17], and the 9-lipoxygenase representatives with either a cytoplasmic (*AtLOX1* and *StLOX1.2*) or plastidic localization (*AtLOX5*) group in cluster B [14,15]. The remaining 13-lipoxygenases with plastidic localization (*OsLOX*, *StLOX2.1 and AtLOX2*) form Cluster C [14,15,18]. *TcLOX1*, shows a close sequence similarity with the genes of cluster A, which have been characterized as 13-lipoxygenases with chloroplast localization. Within this cluster pyrethrum *TcLOX1* shows a significant similarity (75%) to the *Solanum tuberosum LOX3.1* (X96406 (H3)), [15]. *TcLOX2*, on the other hand, falls into Cluster C, with a 60% similarity to the chloroplast localized *Solanum tuberosum LOX2.1 (StLOX2.1),* and sharing only 40% similarity with the *TcLOX1* (Figure 4).

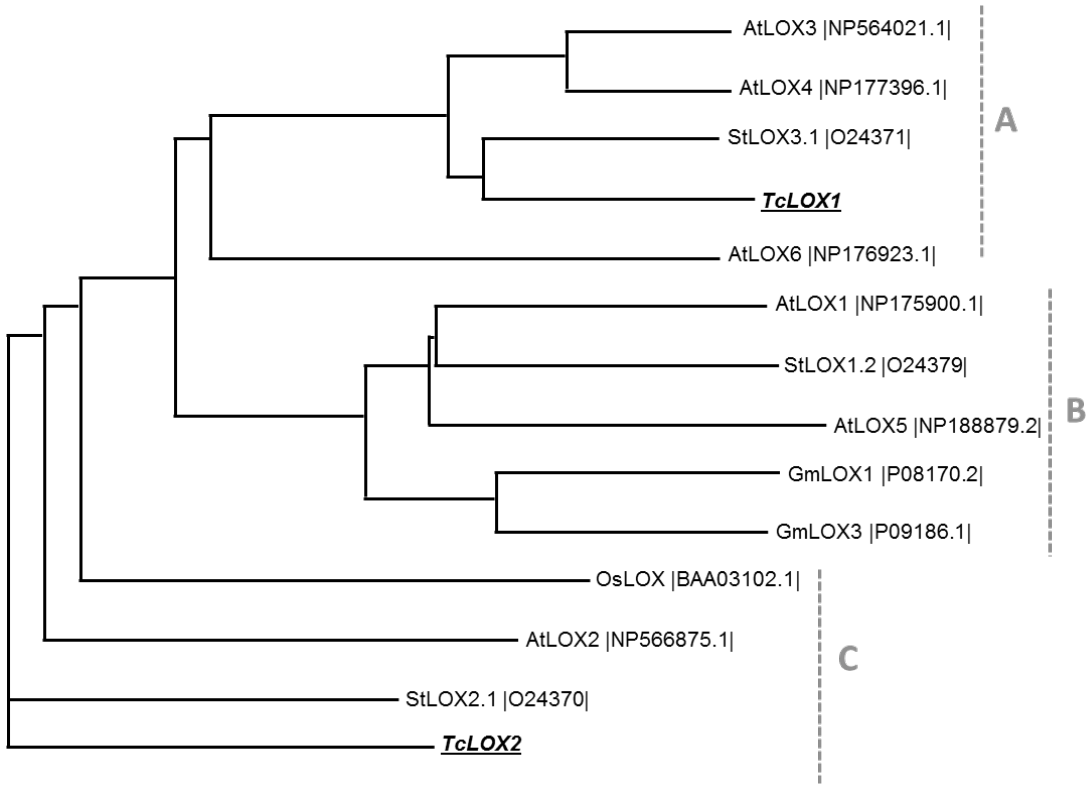

Figure 3: Phylogenetic tree constructed by pairwise comparison of the deduced amino acid sequences of the two putative pyrethrum *LOX* proteins (*TcLOX1* and *TcLOX2*) and other biochemically characterized plant LOXes. The tree was constructed by Neighbour-Joining method using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). The species abbreviations are At, *Arabidopsis thaliana*; Gm, *Glycine max; St, Solanum tuberosum; Os, Oryza sativa.*

Functional characterization of recombinant TcLOX1

The full-length *TcLOX1* cDNA consisted of 2724bp encoding a polypeptide of 907 amino acids , With a calculated molecular mass of 102.5 kDa and a predicted iso-electric point of 7.2, *TcLOX2* consisted of a 2700bp cDNA sequence encoding an 899 amino acids

polypeptide. In addition and as reported for other *13-LOX* genes from plants[14], they both contained a chloroplast targeting peptide, as predicted by the ChloroP1.1 prediction server (http://www.cbs.dtu.dk/services/ChloroP/) (Figure 4, grey highlighted area).

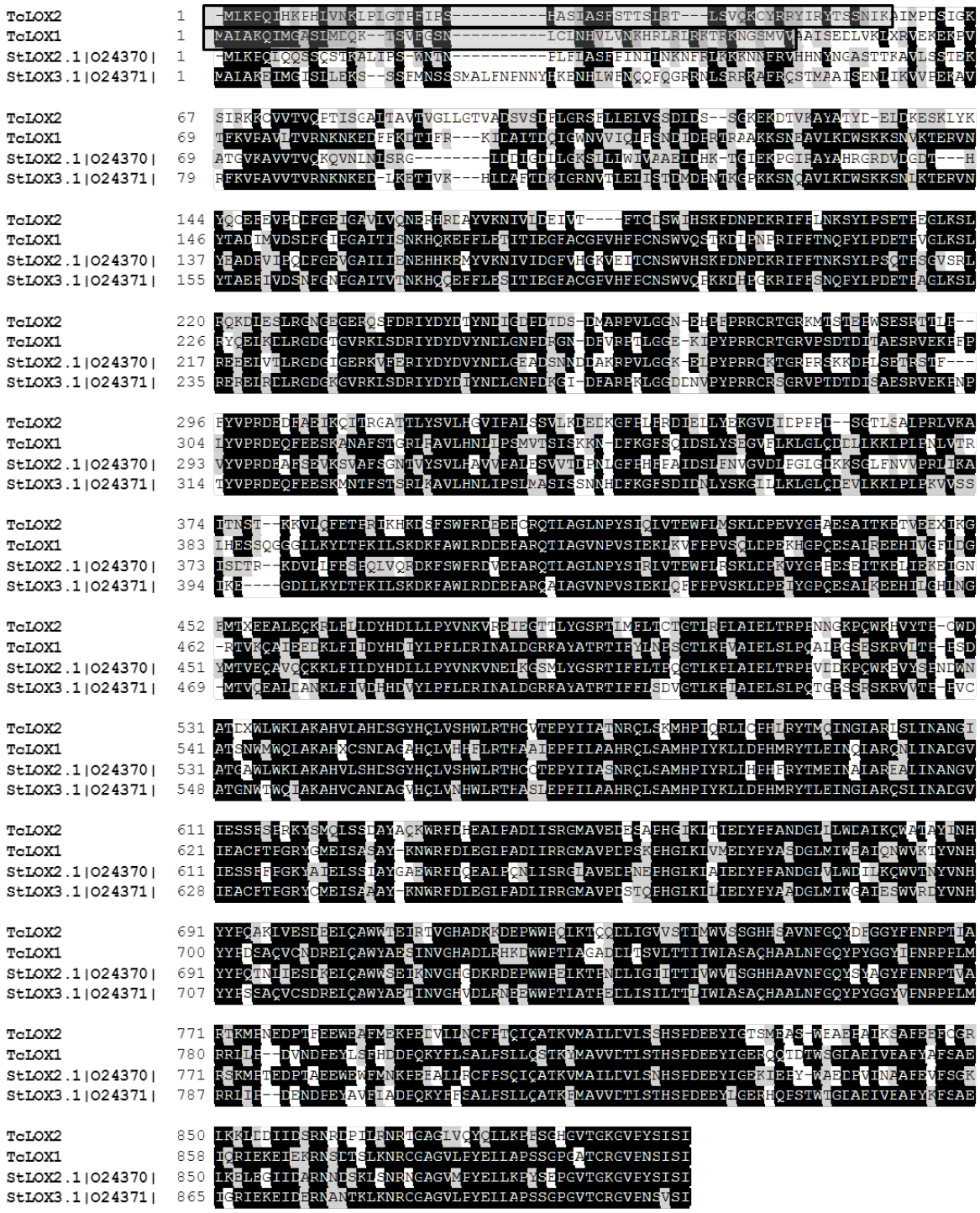

Figure 4: Sequence alignment based on the deduced amino acid sequence of the two pyrethrum LOX genes (*TcLOX1* and *TcLOX2*), and the two potato LOX genes (*StLOX2.1* and *StLOX3.1*) showing the chloroplast targeting peptide (in box) on the pyrethrum LOXes as predicted by the

ChloroP1.1 prediction server (http://www.cbs.dtu.dk/services/ChloroP/).The alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2).

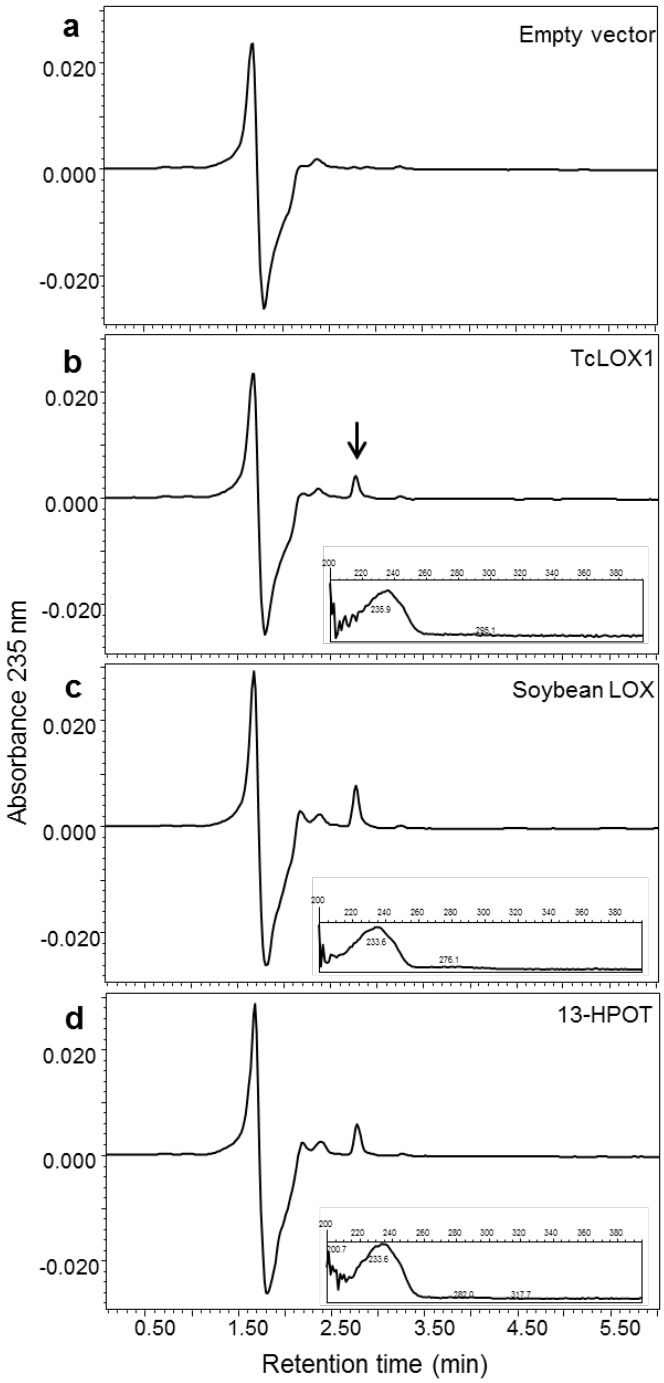

Figure 5: HPLC chromatograms and PDA spectra of hydroperoxylinolenic acid (arrow) of the enzyme assay performed with the empty vector control (A), purified recombinant *TcLOX1* protein (B), non-recombinant soybean LOX (C), and the 13-hydroperoxylinolenic acid standard (D).

To determine whether the cDNAs encoded functional enzymes, an empty pRSET-A vector and two recombinant ones containing the full-length *TcLOX* cDNAs were transformed into

E. coli and purified. The HPLC chromatogram of the products of the enzymatic assay performed with protein purified from cells transformed with *TcLOX1* showed a peak (Figure 5B, arrow), that was not detected in assays performed with protein derived from cells transformed with the control plasmid (Figure 5A) and *TcLOX2* (data not shown). *TcLOX1* activity was confirmed by running the same enzymatic assay with a non-recombinant purified soybean LOX protein sample. The assay showed a reaction product co-eluting with the reaction product of the recombinant pyrethrum LOX (Figure 5C). Comparison of the RT and UV spectrum with that of a 13-hydroxylinolenic acid authentic standard (14-1803-2, Loradan) demonstrated that pyrethrum TcLOX1 and non-recombinant soybean LOX are 13-lipoxygenases (Figure 5D).

Tissue specificity and developmental pattern of *TcLOX* gene expression.

Besides the initial RTqPCR carried out on cDNA templates isolated from flowers of different developmental stages and from stage 4 trichomes, the expression of *TcLOX1* was also assessed using cDNA samples from leaves, seedlings and trichomes isolated from stage 3 ovaries.

Comparison of the relative gene expression (RGE) of *TcLOX1* with *TcCDS*, [11], reveals that both genes consistently show a similar pattern of expression along flower development. That is a high expression in flowers of the early developmental stages (Figure 6A, stages 2 to stage 5), to considerably lower expression in later stages (Figure 6A, stages 6 and 7). Both genes are expressed considerably in isolated trichomes, higher than in intact ovaries (ovaries with trichomes) and leaves. The expression of both genes, however, was completely absent in seedlings, which are devoid of glandular trichomes (Figure 6B).

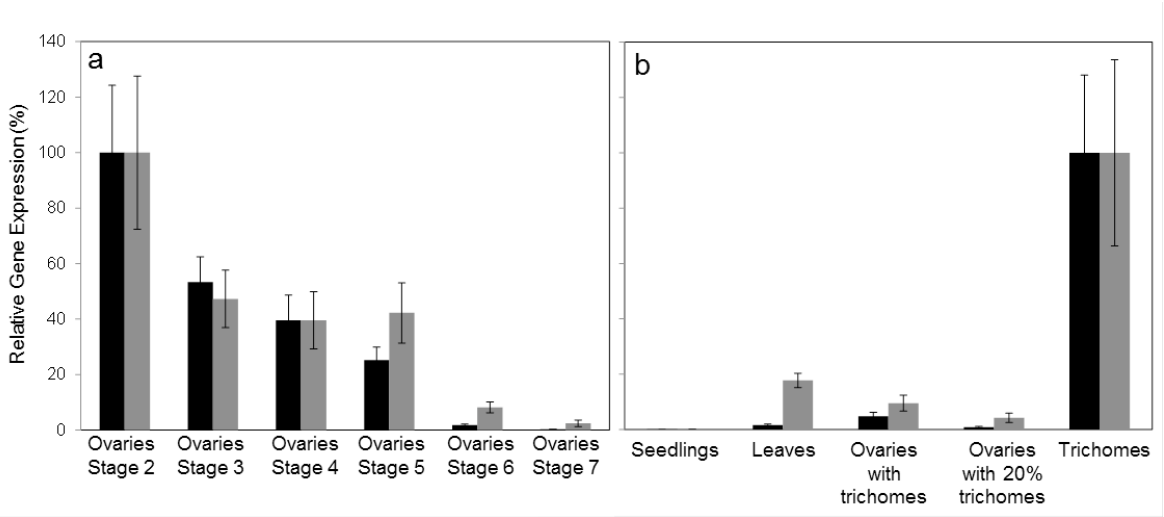

Figure 6: Relative gene expression of *TcCDS* (black bars) and *TcLOX1* (grey bars) in ovaries isolated from flowers in different developmental stages (A); and isolated glandular trichomes,

ovaries with trichomes, ovaries with 80% less trichomes, leaves and seedlings (B) after normalization against the *GAPDH* gene.

Table 1: Relative gene expression (%) of *LOX* ESTs and *TcCDS* in trichomes (stage 4) and along flower development (stages 2 through 7).

Identifier	Trichomes	Stage 2	Stage 3	Stage 4	Stage 5	Stage 6	Stage 7	RLE*
TcCDS	100.0±10.3	16.7±1.5	8.0±0.5	6.3±1.6	4.0±0.0	0.2±0.0	0.0±0.0	100
Ct20982	100.0±15.1	34.4±1.5	13.2±1.6	12.9±2.0	4.1±0.3	3.6±0.7	2.4±0.3	14.29
Ct5842	100.0±8.3	16.1±0.2	9.9±2.1	7.8±0.1	2.2±1.2	1.7±0.1	3.6±0.2	0.003
Ct4895	100.0±9.8	12.6±1.0	5.0±0.5	3.8±0.6	3.1±0.2	0.8±0.0	0.2±0.0	22.11
Ct20675	100.0±8.8	11.0±0.2	7.5±0.5	6.1±0.8	3.4±0.0	1.0±0.1	1.2±0.2	24.59
Ct2442	100.0±6.9	10.2±0.8	11.6±1.5	10.1±1.6	0.0±0.0	0.8±0.0	1.7±0.4	6.41
Ct938	100.0±9.8	9.8±0.6	7.2±0.7	4.2±0.7	3.1±0.1	0.6±0.0	0.6±0.0	48.51
Ct425	100.0±14.2	7.5±0.6	7.4±1.0	6.9±1.0	1.5±0.0	0.6±0.0	1.2±0.1	22.50
Ct4483	2.5±0.7	100.0±3.4	29.4±0.3	38.0±3.9	75.5±3.0	41.3±6.9	60.8±15.0	0.06
Ct1737	1.9±0.6	100.0±4.4	55.0±5.9	60.6±8.6	26.7±0.3	50.9±3.5	50.9±3.5	0.22
Ct15096	0.8±0.0	100.0±3.9	44.7±3.7	25.2±4.1	14.5±1.1	26.7±1.2	22.8±2.8	1.22
Ct22733	39.4±1.4	93.0±0.5	100.0±1.0	77.8±11.4	39.3±2.3	40.6±0.4	88.0±2.2	2.85
Ct26290	0.5±0.1	84.0±7.0	44.8±1.3	61.2±1.2	75.7±38.3	52.3±6.1	100.0±11.2	0.09
Ct20867	26.3±0.8	82.4±2.8	100.0±3.4	56.7±3.6	36.1±2.3	31.8±1.2	88.9±2.2	2.47
Ct366	2.2±0.3	71.6±4.9	24.4±3.0	26.5±3.4	46.4±1.1	67.7±0.7	100.0±9.8	4.64
Ct2382	1.5±0.0	64.3±3.2	74.5±5.1	92.8±14.5	82.0±1.6	74.9±8.4	100.0±7.3	62.38
Ct2729	1.2±0.4	63.8±11.5	15.8±1.8	22.6±0.0	31.9±3.3	42.5±0.8	100.0±1.5	1.44
Ct25085	2.3±0.1	56.1±1.6	34.0±1.0	56.2±9.1	74.9±7.0	51.5±14.5	100.0±3.4	0.06
Ct19896	27.7±3.0	54.8±5.6	100.0±3.4	56.4±0.6	16.1±1.1	16.9±3.5	50.9±3.5	1.93
Ct12107	0.5±0.0	47.4±1.4	46.8±3.7	77.6±9.5	36.6±1.3	40.3±1.8	100.0±7.3	16.65
Ct24032	1.4±0.1	43.2±1.5	18.7±1.4	26.8±1.4	31.9±0.9	24.8±0.4	100.0±7.3	0.60
Ct26547	1.6±0.3	38.4±16.9	18.6±1.8	22.9±1.9	36.0±4.6	28.2±2.5	100.0±5.9	0.84
Ct26038	1.2±0.3	32.7±2.9	19.5±1.1	22.5±0.3	19.8±2.0	27.3±1.6	100.0±1.5	0.38
Ct22571	59.8±9.3	20.8±0.7	14.5±0.0	25.5±2.6	46.3±4.8	45.6±1.1	100.0±11.2	8.09
Ct107	38.5±2.3	13.2±1.3	10.3±1.1	18.5±3.2	32.5±2.4	28.4±1.7	100.0±7.3	4.75
Ct22446	49.5±5.3	9.2±0.2	10.0±0.3	20.2±2.1	38.4±3.6	34.2±1.0	100.0±10.3	8.78

LOX ESTs with a TcCDS-likeexpression profile are underlined; double underlined ESTs represent fragments of *TcLOX2* cDNA and single underlined ESTs of *TcLOX1* cDNA.

*RLE stands for Relative Level of Expression and equals the average of the level of expression of a particular gene in all the tissues and stages relative to the level of expression of *TcCDS*. Errors represent the SD between two technical replicates.

Discussion

Matsuda *et al.* [2] demonstrated using ^{13}C-glucose as precursor in pyrethrum seedlings that the alcohol moiety of pyrethrins most likely derives from linolenic acid through the oxylipin pathway (Figures 1, 2). The oxylipin pathway is best known to lead to the formation of jasmonic acid and has been shown to start with the release of linolenic acid (1) from chloroplast cell membranes, which is subsequently oxidized at position C13 by a lipoxygenase. The resulting 13-hydroperoxylinolenic acid (13-HPOT (2)), is converted to JA (4) through reduction and a series of β-oxydations [8,19,20]. Given the high resemblance of jasmolone, pyrethrolone and cinerolone to jasmonic acid, Matsuda hypothesized that if they are indeed synthesized through the oxylipin pathway, a chloroplast located lipoxygenase capable of catalyzing a C13 peroxidation of the linolenic acid substrate would be required for the biosynthesis of the alcohol moiety of pyrethrins. In our previous study we showed that the monoterpene acid moiety of pyrethrin biosynthesis starts in the trichomes and is transcriptionally regulated along flower development [13]. In the early flower developmental stages (stages 1 to 5) when pyrethrins accumulate the expression of *TcCDS*, which is responsible for catalyzing the first step of the formation of the acid moiety of pyrethrins [11], is high. In the later stages (stages 6 and 7) when pyrethrin content is no longer increasing, the transcript level of *TcCDS* is very low (< 5 % of initial values). This is also true for *TcGLIP,* which is responsible for the final esterification step in pyrethrin biosynthesis although the decrease is slightly less pronounced (< 20% of initial values)[13].To identify and isolate a lipoxygenase gene potentially involved in the first step towards the biosynthesis of the alcohol moiety of pyrethrins, we retrieved 25 putative candidate EST contigs from a provisionally annotated EST library by using LOX or Lipoxygenase as keyword. Subsequently, expression profiling was done, resulting in the selection of six candidate EST contigs showing the pattern and level of expression most similar to *TcCDS*. A pattern of expression similar to *TcGLIP* was recognized in the EST Ct15096, but it was discarded because of a low expression in all flower stages (1.22%), (Table 1). After RACE amplification of these six selected ESTs, they assembled into only two full-length genes, named *TcLOX1* and *TcLOX2*. The cDNAs of both *TcLOX* genes were expressed in *E.coli* but only TcLOX1 was catalytically active as a 13-lipoxygenase. *TcLOX2* did not yield any product, which points to a possibly more labile nature of this enzyme, similar to what has been reported for *AtLOX2*, which failed to produce measurable amounts of products after the cells were sonicated [14], or a different optimal pH. Some lipoxygenases have been shown to have their optimal activity at pH values of 5.5 [18] rather than at neutral pH values (7.5) as used in this study.

Based on the primary structure of the enzyme, the positional specificity and subcellular targeting of plant lipoxygenases can be predicted with high confidence [21,22]. Our phylogenetic analysis clearly shows that both *TcLOXes* group together with other biochemically-characterized 13-lipoxygenases, which have also been reported to contain an N-terminal localization peptide that directs them to the chloroplast [14,15,18]. However, these 13-lipoxygenases only share a 40% similarity and therefore cluster into two distant groups (A and C, respectively). Interestingly, although according to the phylogenetic relationship both *TcLOXes* are classified as type-2 13-LOXes, *TcLOX1* clusters closer to the potato LOX3 (*StLOX3.1*) whereas *TcLOX2* shows closer sequence similarity to the potato LOX2 (*StLOX2.1*). Potato LOX2 is involved in de-novo wound induced JA accumulation, whereas potato LOX3 has been suggested to be implicated in maintaining basal JA levels hence, being present at constant level in both non-wounded and wounded tissues [15]. Although in pyrethrum seedlings wounding resulted in an enhanced content of pyrethrins [23], in both flowers and leaves biosynthesis is developmentally regulated [13]. Nevertheless, both genes may potentially work in parallel to supply the required precursors for pyrethrin biosynthesis in response to development and/or wounding.

One of the most fascinating features of the two lipoxygenases is that both *TcLOX*1 and *TcLOX2* display a very similar and dominant expression in trichomes. This would imply that also the rethrolone or an earlier precursor may be exported from glandular trichomes into the pericarp, where TcGLIP catalyzes the esterification of the acid and the rethrolone moieties. Our results show that potentially similar mechanisms of precursor export from trichomes to pericarp could be operating for the alcohol moiety, but an alternative production by for example the enzyme encoded by Ct15096 that followed a *TcGLIP* expression pattern, in the tissues that synthesize *TcGLIP* cannot be ruled out [13]. To prove either way, it will be necessary, for example, to generate transgenic pyrethrum with down-regulated expressing of these genes and observe the effect on accumulation of pyrethrins and free chrysanthemic acid [24]

Materials and Methods

Plant material and RNA isolation for EST library construction

T. cinerariifolium seeds were originally obtained from Honghe Senju Biology Co. Ltd. (Kunming, Yunnan, China) [25], and propagated without selection at Plant Research International. Plants were grown in the field in a sandy soil, supplied with a regular fertilizer regime, sampling flowers between the months of June and September.

Ovaries and ovary glandular trichomes were isolated from fresh stage-3 flowers of plant genotype 10. For the isolation of ovaries, ray florets were removed and the composite

flower heads were cut into half. Corollas of disc florets were excised at the calyx. The remaining ovaries were then isolated by cutting the achenes from the receptacle and immediate collection in a 2 ml Eppendorf tube filled with liquid nitrogen. Trichomes were removed from the excised ovaries with 4 vortexing pulses of 1 min, intermitted each time by 1 min cooling in liquid nitrogen. Frozen ovaries were spread on a pre-cooled 150 µm mesh mounted over a pre-cooled 50 ml screw-capped tube The trichomes that passed through the mesh were collected at the bottom of the tube and later used for RNA isolation. Excised ovaries, isolated trichomes and not yet fully developed young leaves were used to isolate RNA. To avoid interference of trichome content, the RNA of each sample was isolated with hexadecyltrimethylammonium bromide (CTAB).

Generation of three EST libraries.

Three micrograms of total RNA from each tissue was sequenced with 454 sequencing technology by Vertis Biotechnologie AG (Germany)[26]. cDNA libraries of the three different tissues were obtained with random hexamer primers which were labeled with different adapters. The three libraries were normalized, and then sequenced in a single 454 run. This run generated 281,264 reads for the ovary library (60% of total reads), 87,226 reads for the trichome library (19% of total reads) and 98,672 reads for the leaf library (21% of total reads). After adapter clipping, reads were discarded if the length of the reads was lower than 60 nt. For each library, less than 3% of reads were discarded. The remaining reads were clustered and assembled into contigs using CLC Main Workbench (CLCBio, Denmark). In every library, about 40-50 % reads that could not be assembled into contigs were left as singlets. Since all the plant material was harvested from the same plant, the reads from different libraries were pooled together to be assembled. In this way, 458,726 high quality reads from all three libraries were assembled into 27,314 contigs leaving 31.6% (144,825) reads as singlets. The average length of contigs was 411 bp. Using an in-house bioinformatics facility, potential gene functions of the resulting contigs were identified by blasting against the Nr database of annotated genes, saving the 50 best hits for each contig in a local database which could be interrogated with keywords.

Characterization of *LOX* candidates by RTqPCR

For RNA extraction, plant tissue was homogenized by adding one pre-cooled grinding ball to each 2 ml Eppendorf containing 50-100 mg of frozen plant tissue and using a pre-cooled Mikro-disembrator II (Braun; Germany) for 1 min at maximum speed. After careful removal of the beads, RNA was isolated using TriPure (Roche) and transcribed into cDNA

using TaqMan Reverse Transcription reagents (Applied Biosystems) according to the manufacturer's instructions.

RT-qPCR was used to study the expression of 25 candidate *LOX EST* contigs and the chrysanthemyl diphosphate synthase gene (*CDS*) in cDNA derived from ovaries at different flower developmental stages and trichomes isolated from ovaries at stage 4 of development. Gene specific primers were designed using Beacon Designer (Palo Alto, CA, USA) with expected product sizes of around 120 bp. *T. cinerariifolium* Glyceraldehyde 3-phosphate dehydrogenase gene (*GAPDH)* [13] was used for normalization. PCR reactions were prepared in duplicate by mixing in a 500 µL tube, 22.5 µL iQ SYBR green supermix 2x (Biorad), 4.5 µL sense primer (3 µM), 4.5 µL antisense primer (3 µM), 11.5 µL deionized water, and 2 µL cDNA template. After vortexing, each mix was distributed into two wells in 20 µL amounts. Quantification of the transcript level was performed in an MyiQ iCycler system (Bio-Rad Laboratories, USA) using a three-step program, which included (i) enzyme-activation at 95 °C for 3 min, (ii) 40 cycles of 95 °C for 10 sec, 60 °C for 30 sec, and (iii) 95 °C for 1 min, from 65 °C to 95 °C for 10 sec for dissociation curve analysis. At the end of each run, amplified products were sequenced to verify their identity. Relative gene expression (RGE) values were calculated using the efficiency δCt method [27].

Amplification and plasmid construction for expression in *E.coli*

SMART RACE cDNA amplification kit (Clontech, USA) was used to obtain the 5' and 3'-region of those six selected EST contigs showing a pattern of expression similar to the pattern of expression of *TcCDS*, according to the manufacturer's instructions. 5'-RACE and 3'-RACE products were cloned into pGEMT-easy vector (Promega, USA) and sequenced. Assembly of the six original fragments and newly sequenced products using SeqMan software resulted in two full-length cDNA sequences, named *TcLOX1* (genbank: KC441523) and TcLOX2 (genbank: KC441524). The full-length candidate LOXes were amplified from ovary cDNA using high fidelity Phusion polymerase (Finnzymes) and the primers TcLOX1-F, 5'- TTAT**CTGCAGC**ATGGCACTTGCAAAAC-3'; TcLOX2-F, 5'-TTAT**CTGCAGC**ATGTTGAAGCCCCAAATTC-3' (*Pst*I restriction site in bold); TcLOX1-R, 5'- AATA**GAATTC**GCAACCAAAATGTGCAC-3'; TcLOX2-R, 5'-AATA**GAATTC**CTGTAATTAGATGGAGATGC-3'(*EcoR*I restriction site in bold).. The amplified product was digested by *PstI/EcoRI* and cloned into the pRSET-A expression vector (Invitrogen) fused to an amino-terminal histidine tag, and expressed in *Escherichia coli* BL-21 under the control of an isopropylthio-β-galactoside inducible promoter.

Heterologous expression of *TcLOX1* and *TcLOX2* in *E. coli*

E. coli cells transformed with the recombinant and the empty constructs, were grown to an OD_{600} of 0.6 and then induced with 1mM IPTG at 18 °C for 20 h. After harvesting by centrifugation, the cells were resuspended in 1ml resuspension buffer (0.05 M Tris/HCl pH 8, 0.01 % β-mecaptoethanol) and 10 µL Lysozyme (100 mg/mL), disrupted by sonification (6 × for 10 s), and centrifuged at 13,000 *g* for 10 min at 4 °C. His-tagged proteins were purified with Ni2+-NTA column (Qiagen) according to the manufacturer's protocol. Enzyme induction and purification success were checked by SDS-PAGE and with a soybean LOX standard.

In vitro enzymatic activity assay

His-tag purified proteins were assayed for lipoxygenase activity [28,29]. The assay consisted of: 200 µL eluted protein (ca. 1 mg/mL) in 0.05 M Tris/HCl pH 8, 0.01 % β-mercaptoethanol, 200 mM imidazole, 700 µL 0.1 M Tris/HCl assay buffer (pH 7.5), 100 µL H_2O_2 (3 % in assay buffer), and 5 µL linolenic acid (50 mM in 100 % EtOH). Assays without enzyme and with 200 µg soybean lipoxygenase (Fluka 62340) were used as negative and positive controls respectively. The assays were incubated for 30 min at room temperature, and then filtered through 0.2 µm inorganic membrane filters (RC4, Sartorius, Germany) before HPLC analysis.

HPLC analysis of *in-vitro* assays.

HPLC analysis was performed using a Waters Alliance 2795 HPLC connected to a Waters 2996 PDA detector (Waters, MS technologies, UK). The column used was an analytical column (4.6 × 150 mm; Phenomenex, USA) attached with a C18 pre-column (2.0 × 4 mm; Phenomenex, USA). Degassed eluent A (MQ with 0.1% AcH) and eluent B (Methanol with 0.1% HAc) were pumped at 1 mL min^{-1} into the HPLC system. The gradient started at 10% B and increased linearly to 100% B in 23 min. Then the column was washed and equilibrated for 7 min before the next injection. The injection volume was 50 µl. An authentic standard of 13 (S)-hydroperoxy-9(Z),11-(*E*),15-(*Z*)-octadecatrienoic acid (13-(*S*)-HPOT) was purchased from Loradan (Malmo, Sweden).

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Supplemental data

Table S1: LOX EST sequences and RTqPCR primers

Identifier	RTqPCR primers	EST sequence
Ct107	AGGGAAATGCTTGCTGGAGTC GCTCAATATGGTGTCGTTCTATCG	AGACTACCAACCAAATCAGACCCCAATACTGAGAGCAGG CTTCCCCTTGTCATGAGCTTAAACATATATGTTCCAAGAG ACGAGCGGTTTGGACACCTGAAGTTGTCTGATTTTCTTGC TTATGGTCTAAAATCTATCGTTCAATTTCTTGTCCCCGAGT TTGAAGCCCTATTTGACAAAACTTTTGATGAATTCGACTCA TTTGAAGATGTCTTTGAGATTTACGAAGGAGGATTCAAGC TACCTCAAGGAGCTTTTGTTGAGCGCATTACAGAAAAGAT TCACTTGGAAATGTTGAAGATACTTCTTGAAACAGATAAC AATACTGGATTTGCTAGATATCCCAAACCACAAATCATCC AAGAGGAGAAGTATGCTTGGATGGAAGATGCAGAATTTG CAAGGGAAATGCTTGCTGGAGTCAACCCTGTTAATATTAG TCTTCTAAAAGAGTTCCCACCTACAAGCAAATTAGACGTC CAAGTCTATGGCAACCAAAACAGTTCGATAGAACGACAC CATATTGAGCAAAACCTAAACGGCTTAAAAATAGAGGAGG TATTGAAAGCAAATAGGTTGTTCATACTAGATCACCATGA TTCATTGATGCCATACTTGACGCGAATAAATGAAGGTGTG AACAAGATATATGCCTCAAGAACTTTGCTTCACCTACAAG AAGATGGAACTTTAAAACCATTAGTAATCGAGTTAAGCTT GCCTCATCCTGATGGAGATAATTTTGGTCCCGTTAGCAAT GTATACACCCCAGCTAAAGAGGGAGTAGAAGGCTCAGTT TGGCTGTTGGCAAAGGCATATGTGGCCGTTAATGATTCC GGGATTCATCAACTAATTAGCCACTGGTTGAATACGCATG CGGTTGTTGAGCCATTTGTGATTGCTGCAAATAGGCAGCT AAGCGTACTCCACCCCA
Ct366	CGTGTAAGGCATTTGAGCAGGTC CGGATAACGAGGCATTGGAAGC	AAAGAGGAATCAAACCCTTGAACCATAAACATTCAACCAG ATAACAAAAATGCATACAAGAATAGCATATTAAACAGAAC ACGATATGGGTCATGCAGAGCATCCAAAAACGCTTCATAT AGAGACACTGTTGGGAATACCACGACCCGTAAGACCTAT ATCACTGCTAGGATACAGCAACGTGTAAGGCATTTGAGC AGGTCCAGTCCTATTCCTCAACCTCTTATCCTGATTCATC TCGTCAATCTTCTTTTCAACCTCTCGCAAATTTGCACCAAA ATTTTGAAATGCTTCCAATGCCTCGTTATCCGTGGTCCAT TCAGGTGTATCTCTTTCCCCAAGATACACCTCATCAGCAG AATGCCTAGACAATATCTCAATCAATGACATCCCAAGAAT ACTTTGAAGCTGGGGAGTCACGGTTCTCAAAAATGCCTTT TCCGGGTTTGTCTCGAGTTCATCATAATCTGCAGTCCCTG GTGCAGGTAATAACCTACGGCTCTGTGGGGGTCGGTTAG GCATGTATCCTGCATAAGGGTACTGCCCGAAATTCACGG CTGCATGGAGTGCAGAAGCTACCCATATGATGGTGGTGC AAGACTGGATGAGTTCTTCAAGTGTGTGCATTTTAGGCCA CCATGGTTCATCCTTCTTGTCACCGTGTCCCTTTGTTCGG ACCTCTTCCCACCAGCGTTCGAGTTCACTGTCATTTTGTA TATCTTCATCGTTCTTGTAGTAGACAAAAACATAATCCTTC ACCCATGATTTTATGGCAGACCAT
Ct425	GGAGCACCGCCCTTAGTC AATTCCGTATCCAAGAAGATGTCG	GGTAGGTAAATGTCATGGTAGTCAATTATAAACAGCTTAT CTTCCTCAATTGCCTGCTTTACTGTCCTGCCATCGAGAAA TCCCACAATGTGTTCTTCTCGGAGAGCCGACTCTTGTGG GCCATGTTTTTCAGGATCAAGCTGACTCACTGGTGGGAA AACCTTAAGCTTCTCAATGCTAACAGGGTTCACCCCGGCA ATAGTTTGGCGAGCAAATTCATCGTCACGTAACCAAGCGA ATTTGTCCTTGGATAGAATCTTTGGGGTGTCATATTTGAG AAGGCCACCACCTTGGCTAGATTCATGTAGCCGAGTCAC CAAATTAGGCAAAGGAAGCTTCTTTAGAAGGTCATCTTGA AGGCCTAGTTTAAGAAAGACTCCTTCACTGTAAAGACTGT CGATCTGTGAGAACCCCTTAAAGTCGTTCTTCTTAGAAAT ACTTGTGACCATTGATGGCAATAGATTATGGAGCACCGC CCTTAGTCTTCCTGTGGAAAACGCATTTGCCTTTGACTCC TCAAATTGTTCGTCTCTTGGAACATACAATGGAAATGGCT TCTCCACTCGGCTCTCAGCCGTAATATCAGTATCACTAGG TACACGACCAGTCCGACATCTTCTTGGATACGGAATTTTT TCACCACCAAGTGTAGGCCTAACGAAGTCATTTCCCCTAT CTGGATTACCCAAATCATTATAAACATCA

Ct938	GACACATTATCCACTCACTCACC CTTCCACAATCTCAGCATCACC	AAACTGGGTTAAAACTTACGTGAACCATTACTACCCAGAT TCAGCCCAAGTATGCAACGACAGGGAGCTCCAAGCCTGG TACGCAGAGTCCATCAATGTGGGCCATGCTGATCTCCGC CACAAAGACTGGTGGCCAACTTTAGCCGGTGCAGATGAC CTTACTTCAGTCCTAACCACCATCATCTGGCTAGCATCAG CTCAACATGCAGCACTTAACTTCGGTCAATACCCATATGG TGGCTACATACCAAACCGTCCTCCACTAATGCGCCGGTT ACTCCCTGACGTAAACGACCCAGAATATCTAAGTTTCCAT GATGACCCACAAAAGTACTTCTTATCAGCTTTACCAAGCT TACTACAGTCAACAAAATACATGGCTGTAGTGGACACATT ATCCACTCACTCACCCGATGAGGAGTACATTGGTGAAAG ACAACAAACCGACACTTGGTCTGGTGATGCTGAGATTGT GGAAGCTTTTTACGCGTTTTCAGCTGAGATTCAACGGATA GAAAAGGAGATTGAGAAAAGGAACAGCGATACAAGTTTA AAGAACAGATGCGGTGCTGGTGTTTTACCATATGAACTTC TTGCACCAAGTTCCGGGCCTGGAGCAACATGTAGAGGTG TTCCAAATAGTATATCGATATGATTTATGATTAGCAACAAT TAACGGCTCTTTTTTTGTTAACCCGAATGAGGGATACAAT TATAGTATTATTAATGGTAAAGTAGAGTGCATATATGTAAT GTAAATCAAGCTTCTTAGGTGCACATTTTGGTTGCATATA CATGTAAAATCCTGATAAAGTACTTAAATAAAAGAGATGCT
Ct2382	GGCTTGGATTTGCGAAGGATATG GCGGTAAACTTTGGACAGTATGAC	GCCCCGTTATTCATAACTTTCTCGATTCAAGATACATGAAT CACCCCTTTATTTTGACCTTAATCATTAACATATTTAACCA ACCATTTAAATAGATATACTATTAGGAACCCCCATACTAGT GACACCAGCTTCCGAATAAGGCTTTAGAAGGCTGTATGG TACAACCCCGACTCCATTCCTGTTCTTCTTAGTTTCATCA GCATTCCTAGAATCAATGATCCCTTCCAGTTCCTTCAACT TTCCGGAAAAGATATTATAAGCTGCTTTTATTTGTGGAACA GCCTCAAAGGCTGGTTCCATCTTTTCCCCGATATACTCCT CATCCGGAGAATGATTTGATAACACATCCAAAACTGCCAT AACTTGTGAGGCTTGGATTTGCGAAGGATATGTAGATAAA AGTTCATCTTCGGGTCTACGTTTAAAAGCTTCCCATTCAT CATCAGTAGGGTCTTCACATGGCATTTTGACACGGGCTAT TGTGGCCCGGTTAGGAATGTACCCTGCATAGTCATACTG TCCAAAGTTTACCGCAGCATGGTGACCGGATGCTACCCA TATAATTGTTGTGAGTATACCGATAAGGTCTTGTGGTGTT TTGAGAACGGGCCACCATGGTTCGTCTTTCTTGTCTGCGT GGCCCTTTGTCCTGATCTCGGTCCACCATGCTTGAATCTC TTCATCAGATTCTACAAGATTTTCTTCTGGGTAGTAATGGT TTACGTAGTTTGTGACCCAATCTTTGATGATATCCCATAG GATTAAACCGTCATTTGCATACGGATAATCTTCCACAGTT AGCTTTAAACCATGTGGTGAATCTGGATCTTCCACAGCCA TGCCCCTTGCAATAAGGTCAGCTGGCAAAGCTTCCTGGT CAAAACGCCAGAATTGACCATATGCTATAGAACTCAACTC AATGCTATACTTTCCTGGTGAAAAGCACGATTCAATAATT CCCCCAGAGTTTATGAGGGCTTCTCGAGCCAAAGCATTG ATTTCCATTGTGTATCGGAAATGAGGGTGCAAGAGATTAT GTATAGGGTGCATTTTACTAAGCTGGCGATTCGTTGCAAT TATATATGGCTCAGTGCAACAATGTGTCCTCAACCAGTGA CTAACAAGTTGATGATAACCACTATCATGAGCAAGTGCAT GCATCTTAGCGAGCTTCCAGAGCCAAGCCCCGGTTGCAT CCCAACATGGAGTGTACGCTCGCTTCCATTGAGGTTTGC CATTACCGGGTGGTCGAACTAACTCAATAGCTAATGGCCT TAGTGTTCCAGTTGGAGTAAGGTACATGATTGTTCTTGAT CCGTATAAAGTTGTTCTCAAATTTGTTTGTTCTCTAACTTT GTTGACATATGGTAGGAAGATATCATGATAATCTAGCATG AAAAGCTTCTTGTTCTCTAATGCCTCCTCAAGCGTACAAT AGCCTTTGATTTGTCTCTCAATGACCTCTTTGGTTATTGCC GATTCAGGCGGACCATACACCTCAGGGTCCAGTTTACTT GCTATTGGCCATTCCGTCACCAAGGTAATACTCAAAGGGT TAAGACCAGCAAGGGTTTGTCGACAAAATTCTTCATCCCT AATCCATCCAAACTTATCTCTTTCAATCACTTCAGGAGTCT CGAAAAGCAAAACATTATTTTGGGCATCTGCTATAGACTT CAAAAGTCTAGGAAGAACATTTCCAAGGAAGCCATTGCTG TCCAACGGTGGCAAATTAACACCCTCGTTGAACAAAGAGT CGATGGCTGTGAAGTACGGGAACCCAAGATCTTTGTCAA TCAGAGTTGTCTCCAACGAAGGCAGGACTGCATGCAGAA CTGAGTATACAGTCTTGAACGAAAATGTTAGATTCTTCAC TTCAGAAAAGGCTTCATCCCTT

Ct2442	TTCAACACTGCTTCATTGCTCTTC TGACCGTTAGAAACAAGAACAAGG	ATGGCGTCTCGTCTGGCAAATACGGCTGATTAGTAAAGA AAATCCTGGGATTAGGAAGATCTTTAGTAGATTGAACCCA AGAATTACAAGGGAAATGAACAGGGCCACAAGCAAATCC TTCAATAGTAATAGTTTCCAAAAAGAACTCTTTCTGATGCT TATTACTAATAGTAATAGCTCCGGGTATTCCGAAATCAGA ATCAACCATTATATCAGCAGTATAATTCACCCTTTCCGTTT TAACATTCGACTTCTTCGACCAATCCTTCAACACTGCTTC ATTGCTCTTCTTTGCAGCTCTTGTTCTTGGATCAATATCAT TACTAAAAAGCTGAATAACAACATTCCATCCAATCTGATCA GTAATAGCATCAATCTTTCTAAAAATAGTATCCTTAAAAAA ATCCTCCTTGTTCTTGTTTCTAACGGTCAAAACAGCTCGA ACCTTAAACGTAACCGGTTTTTCTTTTTCGACACGTACAA GTTTAACCAAATCTTCACTGATTGCTGCCACAACCATTGA GCCATTTTTTCGGGTTTTTCGCAACCTTAACCTATGTTTAT TTACCAAAACATGGTTCAAACATAAATTTGAACCAAAAACT GATGTTTTTTGGTCCATTAAGGAAGCACCCATGATTTGTT TTGCAAGTGCCATTACAAAGAAGAAATGAAATGAAGTTAT GTTTTATC
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Ct4483	TTGACTGGATCTTCTGGTGGATAG TGATGGTCTACTTCTTTGGGATGC	AGGCATTTTGATTCTAGCAATGGTTGGTCTGTTGGGGAAA TAGCCTGCGAAATCATATTGACCAAAGTTAACAGCTGCAT GGTGGCCAGAAGCCACCCAAATCATGATTGACACAATTTT AATCAAGTCATCTTTGGTTTCGAGTTGTGGCCATCCTTCC GCGTTTTTCTTGTCTTCATGTCCAACTGTTCGAATTTCACT CCACCATGCTCGAAGCTCTGCATCAGAGTTGACTGGATC TTCTGGTGGATAGTAGTGCTCGACATAAGAAGTTACCCAT TCTTTGATGGCATCCCAAAGAAGTAGACCATCATTTGCAA ATGGGTAGTCCTGTATTGTTAGTTTTAGCCCATGTCGTTC ATTTTGATCTTTAACAGCCATGCCCCTGCT
Ct4895	CGGCTATAAAGTCTGCGTTTGAAG GATGGAGATGCTATAAGGAACACC	GCAGACAAAAAAGACGAACCATGGTGGCCACAACTTAAA ACACAACAAGATTTAATTGGAGTTGTGTCAACGATAATGT GGGTATCATCTGGCCATCATTCAGCTGTGAACTTTGGTCA ATACGATTTTGGTGGTTATTTTCCAAACAGACCAACTATA GCACGTACTAAAATGCCAAATGAAGATCCAACTTTTGAAG AGTGGGAAGCCTTTATGGAGAAGCCCGAGGACGTGTTAT TAAATTGTTTCCCGACTCAGATTCAAGCTACCAAGGTAAT GGCAATCTTGGATGTTTTATCAAGTCATTCACCAGATGAG GAGTACATTGGTACCTCCATGGAGGCTTCGTGGGAAGCA GAACCGGCTATAAAGTCTGCGTTTGAAGAGTTTTGTGGAA GGTTAAAGAAACTTGATGATATCATAGACTCGAGAAACCG TGATCCTATTCTAAGGAACCGAACTGGAGCAGGGTTGGT TCAGTATCAGCTTCTTAAGCCATTTTCTGGACACGGGGTG ACTGGTAAAGGTGTTCCTTATAGCATCTCCATCTAATTAC AGTTTTACATCGAAAAA
Ct5842	ACCGTGGCTGATAGTGTTTCG CTTCACCGTATCCTTCTCTTCTCC	GTTGAAGCCCCAAATTCACAAACCACACTTGGTTAACAAA CTACCACTCGGTACGCCATTCATCCCTAGTCATGCATCAA TCGCCTCGTTCTCCACTACCTCTCTTAGGACGTTGTCCGT ACAGAAATGCTACCGCAGATATATTCGCTACACCTCTAGT AACATAAAAGCCATTATGCCGGATTCTATTGGCAAGTCCA TAAGGAAGAAATGTGTCGTCACTGTACAGCCAACTATTAG CGGTGCGCTTACTGCTGTAACCGTGGGTCTCCTAGGAAC CGTGGCTGATAGTGTTTCGGACTTTTTAGGACGATCGTTT TTGTTAGAGCTTGTGTCTAGTGACCTTGATTCAGGAGAAG AGAAGGATACGGTGAAGGCGTATGCAACCTATGACGAGC TCGACAAAGAAAGTAAGCTGTACAAATATCAGTGTGAATT

		TGAGGTTCCTGATGATTTTGGAGAAATAGGTGCCGTATTA GTACAGAATGAACGTCATAGAGACGCCTACGTCAAGAAT ATTGTTCTTGATGAGATTGTCACCTTCACTTGTGATTCTTG GATTCACTCCAAATTTGATAATCCTGATAAAAGAATCTTCT TCCTTAACAAGTCATATCTACCATCCGAAACACCAGAAGG GCTCAAGTCGTTAAGGCAAAAA
Ct12107	TCTATTACCCCACCAACAGTCATC ATCCCATAACACTCTAGCATTCCC	GTTAAACCCGTCTTAGGGTCAGTCTCGGCTGCAACAAGC TCCAAAAGAAGCGATTTTCCAAGCAAATCGGTGATATCAT CAAGTGGTCTCGTAAAACTAAGGTTCTCTATTACCCCACC AACAGTCATCTGGACCGTAATCACAGCTTTCACACTAACA GTTTTTACCACATCAGCCGTGCTAAATTCACCCGCCACAG CTTTGATTTGGCCTACTGAGTTTTTGCTATTTCGCTTAGAA CAAAAAGGTATGTGACGTTTAACAGGGAATGCTAGAGTGT TATGGGATAGAAATGAAGTAGGGTTTACACTAGCATCACT ACCACTACCATCTCCGGTGATAAACGCCTTCTTGGCACGT GCGATTATTGGCGAACCGGTTGTTTGGTGGTGGTGGTGA TTGAGGTGTGGTTTCAACATGTTTTGGGTGGAAAGTGGTA TACATC
Ct15096	CCAGTCATGGTGGACAGAGTTG TGGTGGATGAATCAATAAGTTCGG	AGAAGATTATCCATACGCAGTTGATGGACTTGAAATTTGG TCAGCAATCCATCTTTGGGTTAAAGACTATTGTTGTGTATA TTACCAATCAGACGAAATGGTTCAAACTGATTCAGAACTC CAGTCATGGTGGACAGAGTTGAGAACCGAGGGCCACGG TGACAAGAAAGACGAGCCATGGTGGCCTAAATTGCAAAC AGTAACCGAACTTATTGATTCATCCACCATCATCATATGG GTTGCTTCAGCTCTCCATGCAGCGGTC
Ct17307	GATTTTCGCAGCCTTTCC ATTCTTGATCTTAATGATTTCGG	CCGCAGCCGATGTCATCTTCGTCGCCCAATCTTCGATTTT CGCAGCCTTTCCAATCACTCCACTCGACTCACTCGATGG ATCACAATGAGTCGAACTAATAAGTTGGATCGAGACATGT TTGCCAAAGATTTCATAAATCCGATCAAGAAGCGATGCAC CGAAATCATTAAGATCAAGAATATTTTTCTTCATTAAAACG ACTGTTCCTTTAATT
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Ct20675	CACCAGCATCATTTGAGCACAC TAGCCTACCACAAGCACTGC	CCGCCAATTCTTATAAGCAGAAGCACTGATCTCCATACCA TAGCGTCCAGGAGTGAAGCACGCCTCAATGACACCATCA GCGTTGATCAAGTTCTGGCGAGCCAACTGATTGATCTCTA ATGTATATCTCATATGTGGGTCCAGAAGCTTATATATAGG GTGCATCGCACTCAATTGCCTATGTGCAGCCAATATGAAT GGTTCAATAGCCGCGTGTGTCCGTAAAAAGTGGTGAACC AGTTGGTGAGCACCAGCATCATTTGAGCACACGTGGGCT TTAGCTAGTTGCCACATCCAGTTACTAGTGGCATCAGATG GTGGTGTGAGTACACGTTTTGACTCTGAACCAGGCAGTG CTTGTGGTAGGCTAAGCTCGATTGCGACGGGCTTGAGAG TGCCAGATGGATTCAAGTAAAATAT
Ct20867	TTCTTGACCCCATCAGGCACTC GGCGTCTACTGGAGGTGTGAG	GATATCTTAACGGCATGAATGTACAACAGGCTATCGAGGA AAATAAGCTATTCATAATAGACTACCACGACATATACCTAC CATTTCTTGACCGGATAAATGCACTCGATGAGCGTAAAGC ATATGCAACCCGAACTATCTTTTTCTTGACCCCATCAGGC ACTCTTAAGCCCATTGCAATTGAGCTCAGCTTACCACGGG CTTTACCAGGTTCACAACCGAAGCGTGTTCTCACACCTCC AGTAGACGCCACTAGCAACTGGACATGGCAGTTGGCTAA AGCGCATGTCTGCTCTAACGATGCTGGGGTCCACCAGCT TTCACACCACTGGTTACGAACACATGCAGCCATGGAACC
Ct20982	GACCAGCAGAATCAGCAATTACC TCCTTGAACCGTATAGAGTTGTCC	AAACATAAGGATTCATTTTCATGGTTTCGTGATGAGGAGT TTTGTCGACAAACACTTGCTGGTCTCAATCCATATAGCAT ACAGTTGGTCACGGAGTGGCCGTTGATGAGCAAACTAGA

		CCCTGAAGTTTATGGACCAGCAGAATCAGCAATTACCAAA GAGACTGTTGAGGAACAGATAAAAGGTTTTATGACTCTTG AAGAGGCATTGGAACAAAAGAGACTATTTTTGTTGGATTA TCATGATTTGCTATTACCTTATGTGAATAAAGTACGAGAAA TTGAAGGGACAACTCTATACGGTTCAAGGACATTAATGTT CCTCACATGTACGGGAACGTTGAGACCATTAGCCATTGA GTTGACTCGCCCACCAAACAATGGGAAACCACAGTGGAA GCATGTTTACACACCTTGTTGGGATGCTACTGACGGCTG GCTCTGGAAGCTAGCCAAGGCTCATGTCCTTGCGCATGA TTCCGGTTATCACCAGCTTGTCAGCCACTGGCTACGGAC TCATTGTGTTACTGAGCCTTATATAATTGCTACGAACCGC CAACTTAGCAAAATGCACCCTATACAGAGACTATTGTGTC CTCATCTACGATATACAATGCAGATTAATGGCTTAGCTCG ACTTT
Ct22446	TGGTGGAAGGAACTACGAGATGG ACGACTTAGAGTTGGGCGATTAGG	AATCATGGTGGAAGGAACTACGAGATGAGGGACATGGTG ACAAGAAACATGAGTTGTGGTGGCCCATTATGGATTCCTG TCAGGCGCTAATAGACATTTGCACAACTTTCATATGGGTG GCTTCTGCTCTTCATGCAGCTGTAAACTATGGGCAATACC CTTATGCTGGTTTCCTACCTAATCGCCCAACTCTAAGTCG TAGGTTTATGCCTGAACCAAATACTCCCGAGTATGATGAA CTTGAACAGAATCCTGAAAAGGTTTTCTTAAAGACCATTA CTCCGCAACTACAAACACTTCTTGGGATTGCACTAATAGA ATTATTATCTAGACATCCATCAGATGAGGTGTACCTTGGA CAAAGAGAGTGTCCTGAGTGGACACTGGACGCGGAACCT CTCAAGGCATTTGAGAAATTTGGGAATAAGCTGAAAGAGA TTGAGGAGAGGATTAAAAAGATGAACACTGACAAAAGGAT GAAGAATAGGGCTGGACCAGTGAATGTACCTTATACGCT ACTCTACCCGACCAGCGAAGAGGGACTTACTGGAAAGGG AATACCTAACAGCACCGCAATGTAAAACAAATCCCCATCT GCAAAAAACA
Ct22571	TGGTGGAAGGAACTACGAGATGAG ACGACTTAGAGTTGGGCGATTAGG	AAGGATTGGGTCTTCCCTGAACAAGCATTGCCGGCGGAT CTTGTCAAGAGGGGAATGGCAGTTGAGGACTCAAATTCT CATCATGGAATTCGCCTACTTATTGAGGATTACCCATATG CAGTTGACGGGCTTGAAATTTGGTCAGCAATCAAATCATG GGTAGAGGATTACTGCAAATTCTACTACACAAATGATGAT ATTGTCCAGAACGACGTAGAACTGCAATCATGGTGGAAG GAACTACGAGATGAGGGACATGGTGACAAGAAACATGAG TTGTGGTGGCCCATTATGGATTCCTGTCAGGCGCTAATA GACATTTGCACAACTTTCATATGGGTGGCTTCTGCTCTTC ATGCAGCTGTAAACTATGGGCAATACCCTTATGCTGGTTT CCTACCTAATCGCCCAACTCTAAGTCGTAGGTTTATGCCT GAACCAAATACTCCCGAGTATGATGAACTTGAACAGAATC CTGAAAAGGTTTTCTTAAAGACCATTACTCCGCAACTACA AACACTTCTTGGGATTGCACTAATAGAATTATTATCTAGAC ATCCATCAGATGAGGTGTACCTTGGACAAAGAGAGTGTC CTGAGTGGACACTGGACGCGGAACCT
Ct22733	GCCCGACAGAACCTCCTTAATG TTATAGGCAGCAGCACTTATTTCC	AAAATTTTGGACCCACACATGAGATATACTCTAGAGATTA ACGCTATTGCCCGACAGAACCTCCTTAATGCTGATGGTGT CATAGAGCAAGGCTTTACCCCTGGACGCTACTGCATGGA AATAAGTGCTGCTGCCTATAAGCATTGGCGCTTCGATTTA GAAGGCCTCCCAGCCGATCTCATTAGAAGGGGTATGGCA GTTCCAGACCCGAGTCAAAGACACGGATTAAAGCTTCTAA TCGAAGACTACCCATATGCTTCCGACGGACTTTTGATTTG GGAAGCAATACAAAACTGGGTCCAAACTTACGTAACCCG AT
Ct24032	GATCACTGTGCTCGTCCAACTC CTGAACCTACTGCGAAGGGATTG	ACCCCAATATCTTCTTCGTAATCAAAGTCTAATTCGAAGG TTGAATCGCCTTTTGTTGACTTACTGATCACTGTGCTCGT CCAACTCTTCAAGTTTGTCTCTTTTCCATGCTTACCTTTCA TCAATCCCTTCGCAGTAGGTTCAGAATGAAGAGCACTAAT AAGCTGCAAAGAAACTTTACGGCCCAAAAGCTCATCTTTG CTACTAGCAACAGAATGATTATTCATCAAGATCAATGTCC CTTTCATCTTCTTTTCTTCAAGTTTCATCCTCTTGCTCCGG TCAACTTCTGCCATAGCTAAGATAAATAATTTGAAACTTTT TTTATTATATTTTTAAAAACAAATGTGAAGTGAGATTTGAT AAGATTGTATC

Ct25085	ATCTGCTTCTGCCTTATGTTGC CATCCGTGTTGGTTGGTGTG	TAAATTGGATCATGAAACTTTTGGACCACTTGAATCAGCA ATCACCAAGGAAATTGTAGAGGAAGAGATTCAAGGTTTCA TGACTTTCAATAAGGCTTTGGAACAAAAGAAATTGTTCATT TTGGATTACCATGATCTGCTTCTGCCTTATGTTGCTAAAA CGAGAGATCTGAAAGGGACAACTCTCTATGGTTCAAGAA CTTTAATGTTCCTTTCTCCTGCTGGTACATTGATGCCTATA GCCATTGAGTTGACTCGCCCAAAATCACCATCAAAGCCA GATCAGTGGAAGCACGTGTACACACCAACCAACACGGAT GCTACAAATGCCTGGCTCTGGAAGCTAGCCAAGGCTCAT GTCCTTGCCCATGATTCTGGCTTTCACCAGCTTGTTAGCC ACTGGCTAAGACTCATTGTGCAAC
Ct26038	CTACTGCGAAGGGATTGATGAAAG GATCACTGTGCTCGTCCAACTC	GACATTAAACTTATTTAATTACAAACCACTCTTTATAAATA CCCATTAAATTCCCTTTCTCTATTATACAATCTTATCAAAT CTCACTTCACATTTGTTTTTAAAAATATAATAAAAAAAAGTT TCAAATTATTTATCTTAGCTATGGCAGAAGTTGACCGGAG CAAGAGGATGAAACTTGAAGAAAAGAAGATGAAAGGGAC ATTGATCTTGATGAATAATCATTCTGTTGCTAGTAGCAAAG ATGAGCTTTTGGGCCGTAAAGTTTCTTTGCAGCTTATTAG TGCTCTTCATTCTGAACCTACTGCGAAGGGATTGATGAAA GGTAAGCATGGAAAAAGAGACAAACTTGAAGAGTTGGAC GAGCACAGTGATCAGTAAGTCAACAAAAGGCGATTCAAC CTTCGAATTAGACTTTGATTACGAAGAAGATATTGGGGTA CCAGGGGCATTCTTGATCATTAACAGACACCAC
Ct26290	CATCCGTGTTGGTTGGTGTG ATCTGCTTCTGCCTTATGTTGC	GCATTTGGCTTAGATTACGGTGCGTAGCAATTATGTAAGG CTCTGTTGCACAATGAGTTCTTAGCCAGTGGCTAACAAGC TGGTGAAAGCCAGAATCATGGGCAAGGACATGAGCCTTG GCTAGCTTCCAGAGCCAGGCATTTGTAGCATCCGTGTTG GTTGGTGTGTACACGTGCTTCCACTGATCTGGCTTTGATG GTGATTTTGGGCGAGTCAACTCAATGGCTATAGGCATCAA TGTACCAGCAGGAGAAAGGAACATTAAAGTTCTTGAACCA TAGAGAGTTGTCCCTTTCAGATCTCTCGTTTTAGCAACAT AAGGCAGAAGCAGATCATGGTAATCCAAAATGAACAATTT CTTTTGTTCCAAAGCCTTATTGAAAGTCATGAAACCTTGAA TCTCTTCCTCTACAATTTCCTTGGTGATTGCTGATTCAAGT GGTCCAAAAGTTTCATGATCCAATTTA
Ct26547	CTGAACCTACTGCGAAGGGATTG GATCACTGTGCTCGTCCAACTC	GACATTAAACTTATTTAATTACAAACCACTCTTTATAAATA CCCATTAAATTCCCTTTCTCTATTATACAATCTTATCAAAT CTCACTTCACATTTTTTTTTAAAATATAATAAGAAAAGTTTA AAGTTTTTTATCTTAGCTATGGCAGAAGTTGACCGGAGCA AGAGGATGAAACTTGAAGAAAAGAAGATGAAAGGGACAT TGATCTTGATGAATAATCATTCTGTTGCTAGTAGCAAAGAT GAGCTTTTGGGTCGTAAAGTTTCTTTGCAGCTTATTAGTG CTCTTCATTCTGAACCTACTGCGAAGGGATTGATGAAAGG TAAGCATGGAAAAGAGACAAACTTGAAGAGTTGGACGAG CACAGTGATCAGTAAGTCAACAAAAGGCGATTCAACCTTC GAATTAGACTTTGATTACGAAGAAGATATTGGGGTACCAG GGGCATTCTTGATCATTAACAGACACCAC

Chapter 5

Biosynthesis of Sesquiterpene Lactones in Pyrethrum (*Tanacetum cinerariifolium*)

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Abstract

The daisy-like flowers of pyrethrum (*Tanacetum cinerariifolium*) are used to extract pyrethrins, a botanical insecticide with a long history of safe and effective use. Pyrethrum also produces other potential defense compounds, particularly sesquiterpene lactones (STLs). The STLs are stored in glandular trichomes present on the pyrethrum achenes, and have been shown to be active against herbivores, micro-organisms and in the competition with other plants. Despite these reported bioactivities, the biosynthetic origin of pyrethrum sesquiterpene lactones remains unknown. In the present study we show that germacratrien-12-oic acid is most likely the central precursor for the sesquiterpene lactones present in pyrethrum. The formation of the lactone ring depends on the regio- (C6 or C8) and stereo-selective (α or β) hydroxylation of germacratrien-12-oic acid. Candidate genes implicated in the first three committed steps leading from farnesyl diphosphate to 6-hydroxy-germacratrien-12-oic acid were retrieved from a pyrethrum trichome EST library, cloned, and characterized in yeast and *in planta*. The diversity and distribution of sesquiterpene lactones in different tissues and the correlation with the expression of these genes are shown and discussed.

Introduction

Pyrethrum has a long history of cultivation to produce insecticides based on pyrethrins that are extracted from the flower heads. The flower heads consist of a collection of small flowers: ray and disc florets that are set on a receptacle. Microscopic examination of the disc florets shows that the surfaces of the achenes is densely covered by glandular trichomes (Figure 1). In our previous work, we showed that glandular trichomes covering pyrethrum achenes and leaves are filled with a mixture of compounds dominated by sesquiterpene lactones (STLs) [1]. In earlier publications these STLs were characterized in more detail [2-6]. Pyrethrosin, the first recognized STL isolated from the flower heads of *Tanacetum cinerariifolium,* exhibited several biological properties including cytotoxic [7], phytotoxic [8], antibacterial [9], antifungal [10] and root growth inhibitory [4] activities. Before, we also showed that the trichome content - with STLs as the major constituent - acts as antifeedant against herbivores and is fungistatic against seedling-specific pyrethrum pathogens, suggesting a maternal protection mechanism promoting survival of the next generation [1].

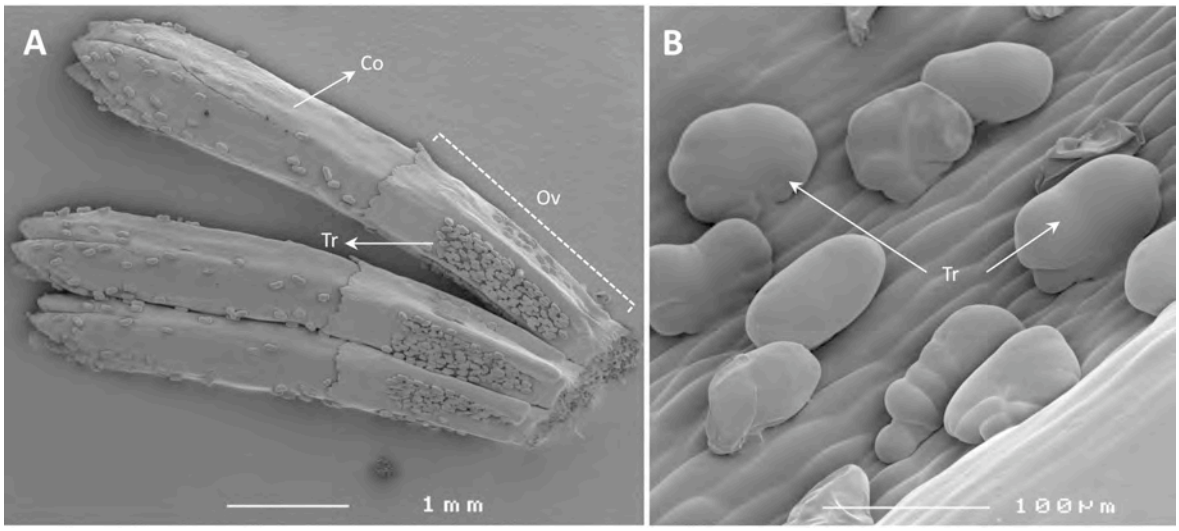

Figure 1: SEM of complete, unopened disk florets showing the highest density of glandular trichomes in the indentations between the ribs of the ovaries (A), closer view of trichomes (B). Ov, ovary; Co, corolla; Tr, trichome.

The insight that, besides pyrethrins, pyrethrum plants are also a source of other bioactive compounds, which by the industry are now mainly regarded as a contaminating residue being removed as waste, raised our interest to elucidate the mechanism by which these compounds are produced. Even though the detailed structures of STLs vary across the Asteraceae family, their basic structure consists of a C15 sesquiterpene backbone and a

lactone moiety. The sesquiterpene backbones are mainly the germacranolide, eudesmanolide, and guaianolide skeletons [11,12]., which have all been suggested to be derived from germacrene A (Figure 2) [13]. In addition to their sesquiterpene backbone, the regio- (C_6 or C_8) and stereo-selective (α or β) formation of the lactone ring also contributes to the diversity in STLs reported in nature. The proposed pathway to 6-hydoxy- germacratrien-12-oic acid (6-OH-GAA) starts with the cyclization of farnesyl diphosphate (FPP) to germacrene A by germacrene A synthase (GAS). In the next step, germacrene A is oxidized at its isopropenyl side chain by a single cytochrome P450 enzyme, germacrene A oxidase (GAO), to form germacra-1(10),4,11(13)-trien-12-ol (GOL), which is then further oxidized to germacra-1(10),4,11(13)-trien-12-al (GAL) and germacra-1(10),4,11(13)-trien-12-oic acid (GAA) [14] (Figure 3). Hydroxylation of GAA at the C6 position by costunolide synthase (COS) results in an unstable intermediate, 6-OH-GAA, which after spontaneous cyclization of the C6 hydroxyl and C12 carboxylic group leads to formation of costunolide, the precursor of the C6-C7 costunolide-types of sesquiterpene lactones (C6-C7 STLs) [15,16]. Hydroxylation of GAA at the C8 position, in a reaction that also depends on a cytochrome P450 enzyme [17], gives a C8-OH-GAA. Computational modeling showed that the atomic distance between the hydroxyl oxygen and the carbonyl carbon that forms the C-O bond in the lactonization is 4 times longer in C8-OH-GAA than in C6-OH-GAA. This longer atomic distance does not favor spontaneous lactonization, suggesting that an enzyme is involved in the formation of the precursor of the C7-C8 type of sesquiterpene lactones (C7-C8 STLs) [17] (Figure 3).

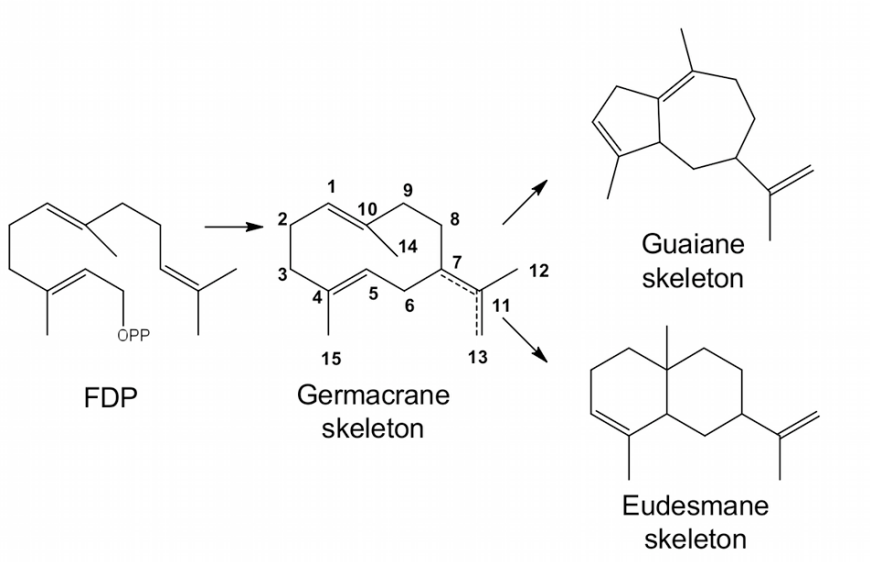

Figure 2: Simplified scheme for the formation of germacrene, eudesmane and guaiane-type sesquiterpene lactones [18].

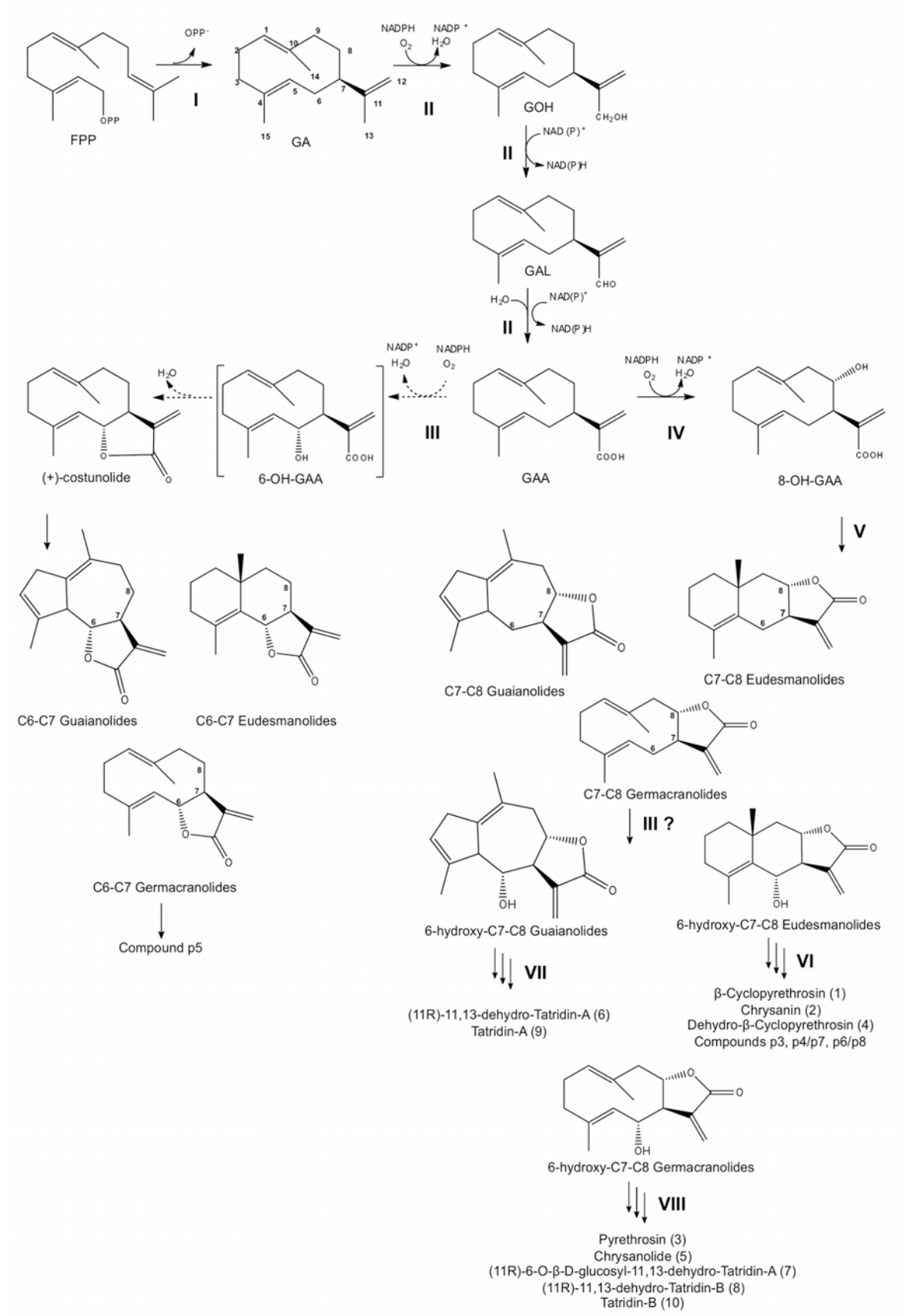

Figure 3: Biosynthetic route to 6-hydroxy-germacrene A acid (6-OH-GAA) and to 8-hydroxy-germacrene A acid (8-OH-GAA). I, Cyclization of farnesyl diphosphate (FPP) to (+)- germacrene A (GA) by (+)- germacrene A synthase (*TcGAS*). II, Oxidation of GA via germacra-1(10),4,11(13)-

trien-12-ol (GOH) and germacra-1(10),4,11(13)-trien-12-al (GAL) into germacra-1(10),4,11(13)-trien-12-oic acid (GAA) catalyzed by an NADPH dependent single P450 enzyme (*TcGAO*). III, Hydroxylation at the C6 position of GAA acid or of various C7-C8 lactones by a second P450 enzyme (*TcCOS*). IV, Hydroxylation at the C8 position of GAA, and V, subsequent enzymatic lactonization. VI, VII, and VII involve extra oxidative steps and esterification with glycosyl, tigloyl and acyl groups, and cyclization of the sesquiterpene backbone to get to the structures in Figure 4 and 5.

Although most of the pyrethrum STLs for which the structure has been elucidated have a C7-C8, rather than a C6-C7 lactone [3-5], they all share the same basic sesquiterpene backbone and, interestingly, all of the former also are hydroxylated at the C6 position (Figure 4). We hypothesized that the three enzymes catalyzing the formation of 6-OH-GAA from FPP in Asteraceae relatives of pyrethrum, are present in pyrethrum and catalyze the formation of the pyrethrum C7-C8 STLs, and potentially also the non-characterized, putatively identified C6-C7 type STLs found in pyrethrum trichomes. To identify the corresponding genes, trichomes of pyrethrum were isolated and used to generate an EST contig library from which homologous genes were retrieved, cloned and characterized in yeast and *in planta*. Transcriptional profiles in specific tissues and along flower development were correlated with chemical profiles of the reaction products to confirm the involvement of these genes in the production of pyrethrum sesquiterpene lactones.

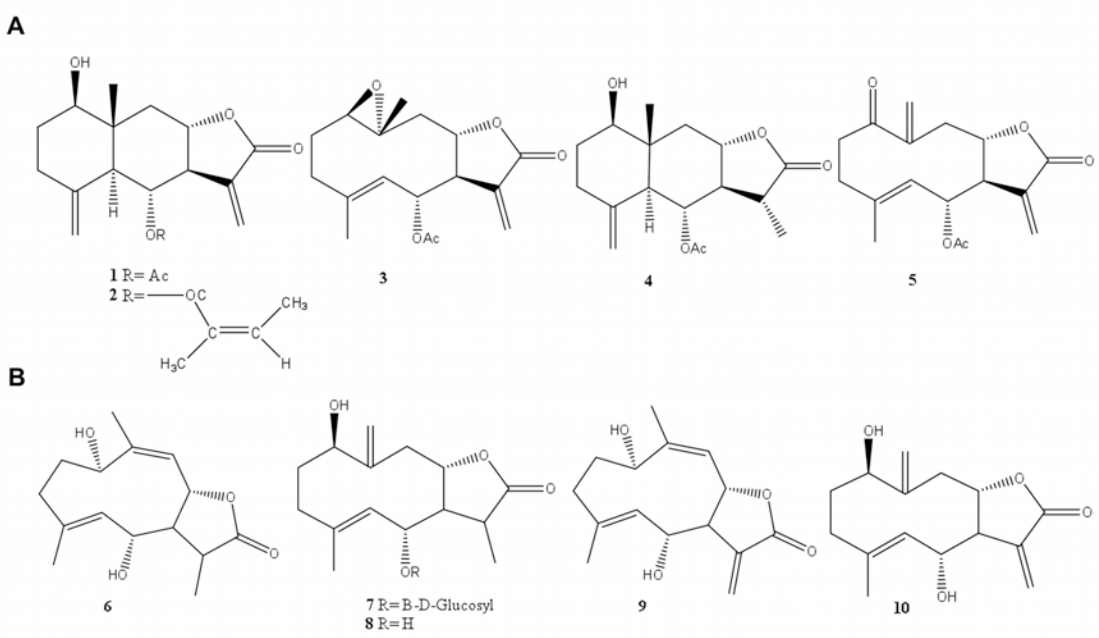

Figure 4: Major sesquiterpene lactones reported for pyrethrum. A, β-cyclopyrethrosin (1), chrysanin (2), pyrethrosin (3), dehydro-β-cyclopyrethrosin (4), chrysanolide (5) [3]. B, (11R)-11,13-dehydro-tatridin-A (6), (11-R)-6-O-β-D-glucosyl-11-13 dehydro-tatridin-B (7), (11R)-11,13-dehydro-tatridin-B (8) tatridin-A (9), and tatridin-B (10)[4].

Results

Localization of sesquiterpene lactones in pyrethrum.

In our previous study we showed that sesquiterpene lactones (STLs) accumulate in glandular trichomes of achenes and leaves [1]. To establish the localization of sesquiterpene lactones in other tissues as well, we analyzed by GC-MS, dichloromethane (DCM) extracts of (i) leaves, (ii) stems, (iii) disk florets and (iv) ray florets. Comparison of the relative peak areas of one C7-C8 STL (Figure 5, peak 4/7) and one C6-C7 STL (Figure 5, peak 5) shows that the highest concentrations were found in the disk and ray florets, while leaves and stems displayed lower concentrations (Figure 6). Both types of lactones were present in all studied tissues except for leaves, in which the C6-C7 STL was not detected.

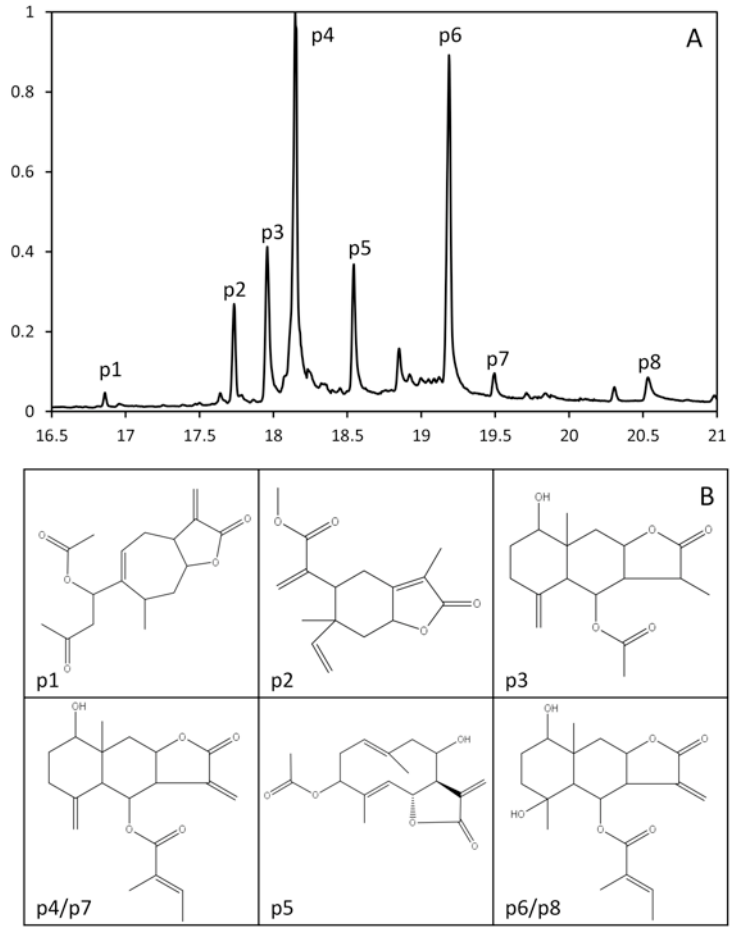

Figure 5: GC-MS analysis of chloroform dip seed extracts, representing the content of trichomes (A). Putative chemical structures of the STLs found in pyrethrum trichomes according to the NIST library search. Xanthinin (p1), deoxysericealactone (p2), 1.8-hydroxy-3, 8a-dimethyl-5-methylene-2-oxododecahydronaphthol(2,3-b)furan-4yl acetate (p3), 1.8-hydroxy-8a-methyl-3,5-dimethylene-2-oxododecahydronaphthol(2,3-b)furan-4yl(2E)-2-methyl-2-butenoate (p4/p7), 1.4-hydroxy-6,10-dimethyl-3-methylene-2-oxo-2,3,3a,4,5,8,9,11a-octahydrociclodeca(b)furan-9-yl acetate (p5), 2-

methylbut-2enoic acid (5,8-dihydroxy-5,8a-dimethyl-3-methylene-2-oxo-dodecahydronaphtho(2,3-b)furan-4-yl) ester (p6/p8). The prefix “p” stands for putative. Table S1 provides further details.

To determine how STLs accumulate during flower development, extracts of flower heads in different developmental stages (Figure 7B) were analyzed for STL content. For both STLs analyzed (Figure 5 peak 4/7 and peak 5), the concentration per flower increased gradually from stage 1 to stage 5 (Figure 7A).

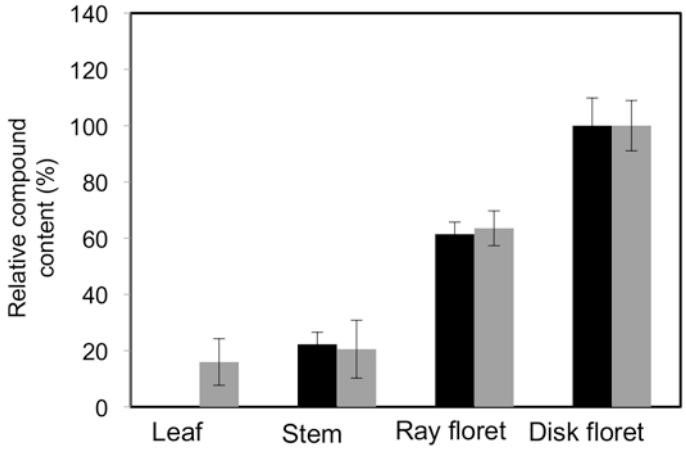

Figure 6: Peak areas of a C7-C8 type sesquiterpene lactone (Grey bars: compound p4/p7 of Figure 4), and a C6-C7 type (Black bars: compound p5 of Figure 4) in pyrethrum leaves, stems, ray florets and disk florets relative to the peak areas in disk florets (100%). Error bars represent SEM (N=3)

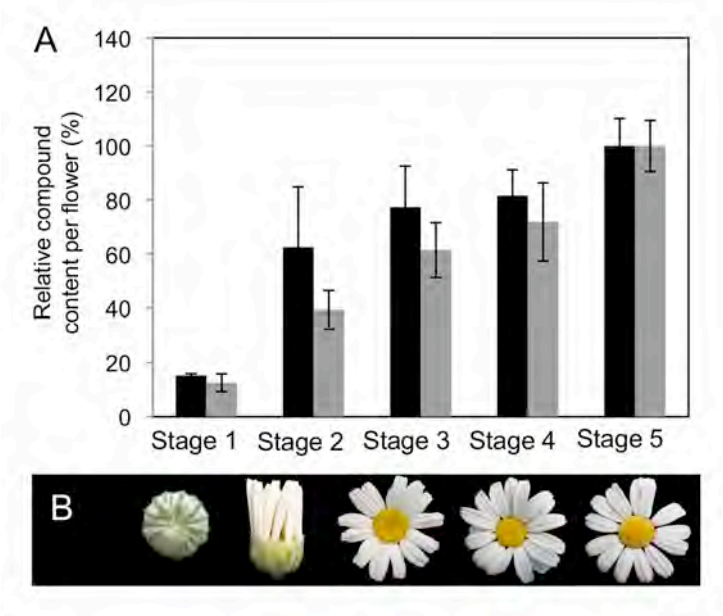

Figure 7: Peak areas of a C7-C8 type sesquiterpene lactone (Grey bars: compound p4/p7 of Figure 4), and a C6-C7 type (Black bars: compound p5 of Figure 4) in pyrethrum flowers in different

developmental stages relative to the peak areas in stage 5 flowers (A). Images of the corresponding pyrethrum flower heads (B) Error bars represent SEM (N=3)

Isolation of Putative STL Biosynthesis Related Genes.

Young leaves, ovaries isolated from stage 3 flowers, and trichomes isolated from ovaries of stage 4 and 5 flowers were used to generate cDNA libraries. 454 Sequencing gave 484.392 reads of an average size of 400bp, which after assembly resulted in 27.317 contigs and 144.825 reads that remained as singletons. Although 85% of the contigs had an average size of 400bp, a small percentage of them (1.2%) had a size bigger than 1200bp, which represents the average size of a full-length gene. Sequences were annotated by blasting against the GenBank (http://blast.ncbi.nlm.nih.gov) database.

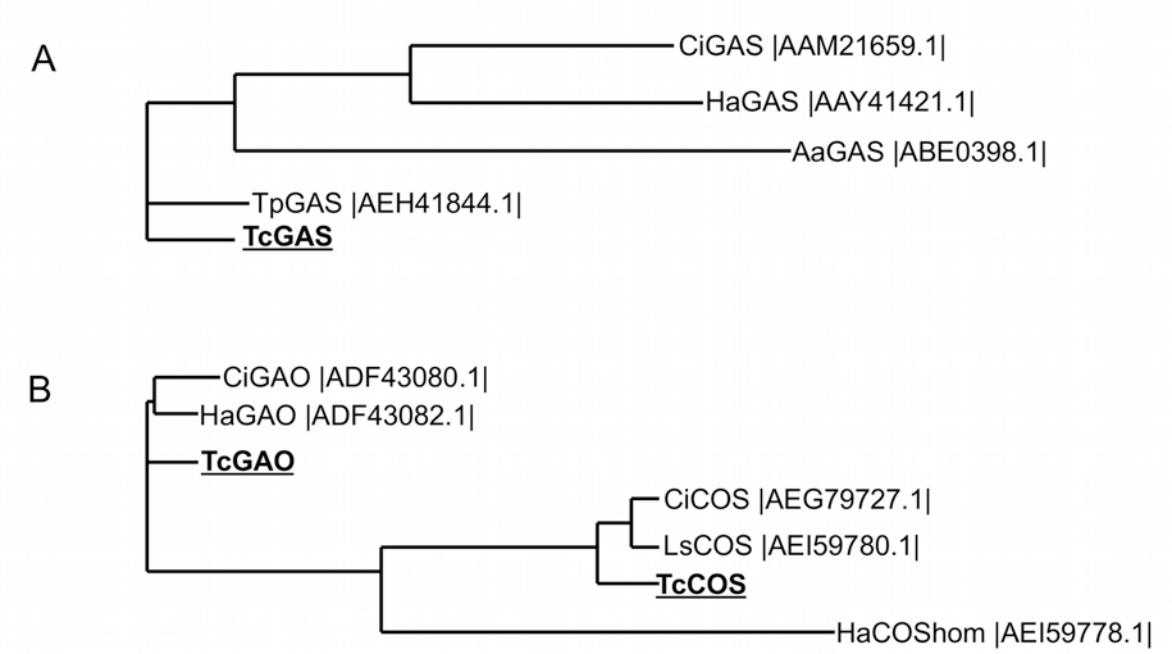

Figure 8: Phylogenetic analyses of the germacrene A synthases and oxidases. Tree based on the deduced amino acid sequences of the putative pyrethrum germacrene A synthase (*TcGAS*) and other characterized plant GASs (A). Tree based on the putative pyrethrum germacrene A oxidase (*TcGAO*) and costunolide synthase (*TcCOS*) and other characterized plant GAO and COS genes. The tree was constructed by Neighbour-Joining method using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). The species abbreviations are Ci, *Cichorium intybus*; Ha, *Helianthus annuus; Ls, Lactuca sativa; Tp, Tanacetum parthenium.*

The genes responsible for C6-C7 costunolide-type of STL biosynthesis in *Tanacetum parthenium* (feverfew) (germacrene A synthase, *TpGAS*) (AEH41844.1) [19]; in *Cichorium intybus* (germacrene A oxidase, *CiGAO*) (ADF43080.1) [20] and costunolide synthase (*CiCOS*) (AEG79727.1) [16]; and the *Helianthus annuus* costunolide synthase (*HaG8H*)

(AEI59778.1) responsible for C7-C8 STL biosynthesis in sunflower [17] were blasted against the pyrethrum trichome specific EST contig library. The blast of the three first sequences resulted in the identification of three highly homologous pyrethrum full-length ESTs. However, the EST trichome database did not have ESTs highly homologous to *HaG8H*, and the most homologous EST found was short and displayed an identity of less than 45%. The first highly homologous sequence (*TcGAS*) (KC441526) encoded a protein with 95% similarity to the feverfew germacrene A synthase (AEH41844.1) and 83 to 86% similarity to the germacrene A synthases from *Artemisia annua* (ABE0398.1), *H. annuus* (AAY41421.1) and *C. intybus* (AAM21659.1) (Figure 8A). The second sequence (*TcGAO*) (KC441527) encoded a protein with 88% similarity to the *C. intybus* (ADF43080.1) and the *H. annuus* (ADF43082.1) germacrene A oxidases, and the third one (*TcCOS*) (KC441528) showed 89% similarity to the *C. intybus* (AEG79727.1) and *Lactuca sativa* (AEI59780.1) costunolide synthases, sharing only 32% identity with the *H. annuus* costunolide synthase (AEI59778.1) (Figure 8B and supplemental data Figures S1 S2, and S3).

Functional Characterization of pyrethrum GAS/GAO/COS in yeast and *in planta*

The full length sequence of the putative germacrene A synthase identified in pyrethrum, *TcGAS*, was cloned into a binary expression vector under the control of the Rubisco small subunit promoter and introduced into *A. tumefaciens* for *in planta* expression. *Nicotiana benthamiana* leaves were agro-infiltrated and analyzed after 5 days according to van Herpen et al [21]. In the headspace of *TcGAS* agro-infiltrated *N. benthamiana* leaves we detected a peak, which was not present in the headspace of empty vector infiltrated leaves (Figure 9A), and for which the mass spectrum matched the mass spectrum of β-elemene (Figure 9B), the on-column, heat-induced Cope rearrangement product of germacrene A (Figure 9C) [11] . Expression in yeast gave similar results confirming the product specificity of TcGAS (data not shown).

To assess the enzymatic activity of TcGAO, its open reading frame (ORF) and the ORF of *TcGAS* were expressed using the yeast dual expression vector pESC-TRP under the control of GAL10 and GAL1 promoters. This plasmid (*TcGAS*+*TcGAO*::pESC-TRP) and the previously characterized feverfew *GAS* and chicory *GAO*, *TpGAS*+*CiGAO*::pESC-TRP [16], were transformed into the yeast strain WAT11. After induction of expression of the two genes, metabolites were extracted and analyzed using GC-MS. Two compounds that were not present in yeast transformed with the empty vector were detected in yeast expressing pyrethrum *TcGAS*+*TcGAO* as well as *TpGAS*+*CiGAO* (Figure 10A). The mass fragmentation (Figure 10B) patterns of the two peaks matched the fragmentation patterns of the cyclization products of GAA under acidic conditions, γ-costic acid and β-costic acid

(Figure 10C) [14,20], therefore the encoded enzyme was designated as germacrene A oxidase (TcGAO) (CYP71AV2).

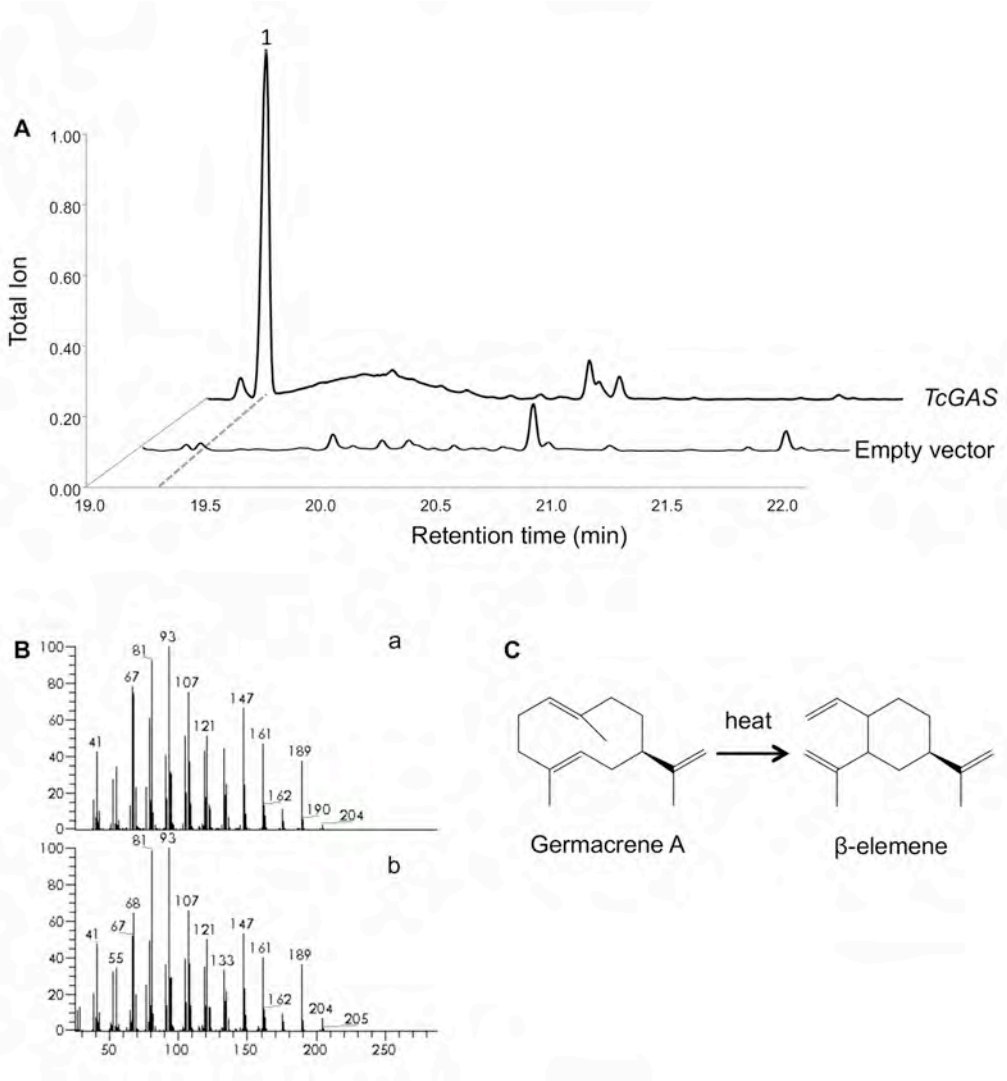

Figure 9: Headspace analysis of volatiles emitted from *Nicotiana benthamiana* leaves agro-infiltrated with *TcGAS* (A). Mass fragmentation pattern of compound 1 (Ba) and β-elemene from the Wiley library (Bb). Cope rearrangement of germacrene A to β-elemene by heat (C).

The full-length cDNA sequence of the costunolide synthase candidate, *TcCOS*, was cloned into a yeast expression vector and co-transformed with *TcGAS* and *CiGAO* into yeast. The transformed yeast culture was able to produce costunolide, however, only a very small amount was detected by GC-MS, which was confirmed by a parallel injection of a commercially available standard (data not shown) indicating that *TcCOS* encodes a costunolide synthase (TcCOS) (CYP71BL4). Low levels of costunolide production by COS have been explained to be due to the increasing acidic conditions during yeast culturing, which can be improved by buffering [20]. During our *in-vitro* yeast assay we took care of this aspect, and, therefore, we presume that the relatively poor reactions may be due to

the fact that *TcCOS* prefers C7-8 lactonized substrates rather than GAA alone, as those would inevitably spontaneously lactonize to the C6-C7 types of STLs.

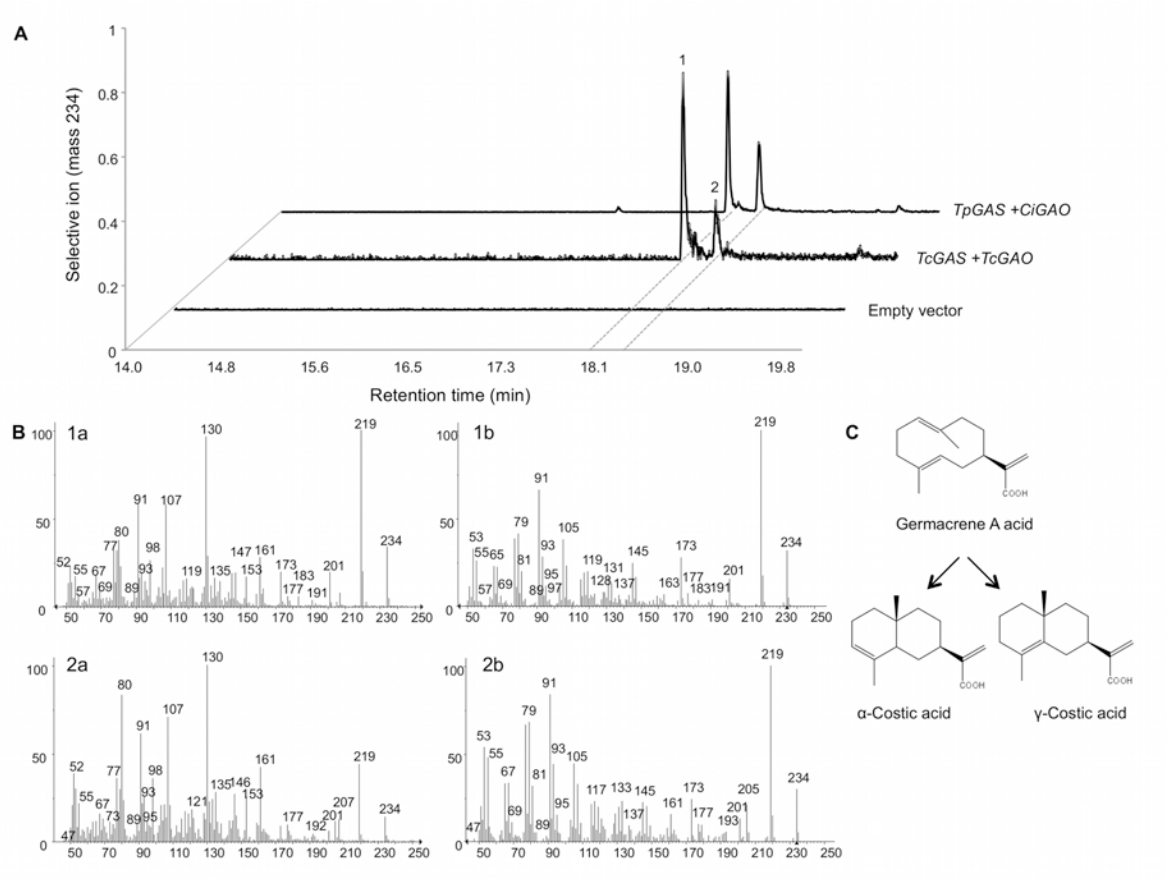

Figure 10: (A) GC-MS chromatograms for the terpenoids with a parent mass of m/s 234 isolated from yeast transformed with the indicated genes. Front line showing the metabolites for the empty vector control, middle line showing the metabolites of yeast transformed with *TcGAS* and *TcGAO*, and last line showing the metabolites of yeast transformed with *Tanacetum parthenium TpGAS* and *Cichorium intybus CiGAO*. (B) Mass fragmentation pattern of peak 1 (1a), α-costic acid (1b), peak 2 (2a), and γ-costic acid (2b). (C) Acid induced rearrangement of germacrene A acid to α-costic acid and γ-costic acid.

To confirm the activity of this putative pyrethrum TcCOS, cDNAs of *TcGAS*, *TcGAO* and *TcCOS* were cloned into a binary vector under control of the Rubisco promoter, and used to transform *A. tumefaciens*. *A. tumefaciens* cultures with RBC::*TcGAS*, RBC::*TcGAO* and RBC::*TcCOS* were co-infiltrated in *N. benthamiana* leaves. After 5 days, methanol extracts were prepared and analysed using LC-QTOF-MS. Comparison of chromatograms showed two new compounds, eluting at 22.21 and 22.66 min, in leaves infiltrated with *TcGAS*+*TcGAO*+*TcCOS*, which were not present in leaves infiltrated with *TcGAS*+*TcGAO*, with *TcGAS*, or with the empty vector control (Figure 11). The parent masses of these two new peaks, 352.1609 (at 22.21) and 538.2184 (at 22.66) deviated only 6 and 22 ppm from

the elemental formulas of costunolide-cysteine ($C_3H_7NO_2S$) and costunolide-glutathione ($C_{10}H_{17}N_3O_6S$) conjugates. Such costunolide conjugates were also previously identified in a similar study where *N. benthamiana* leaves were agro-infiltrated with the chicory counterparts [16].

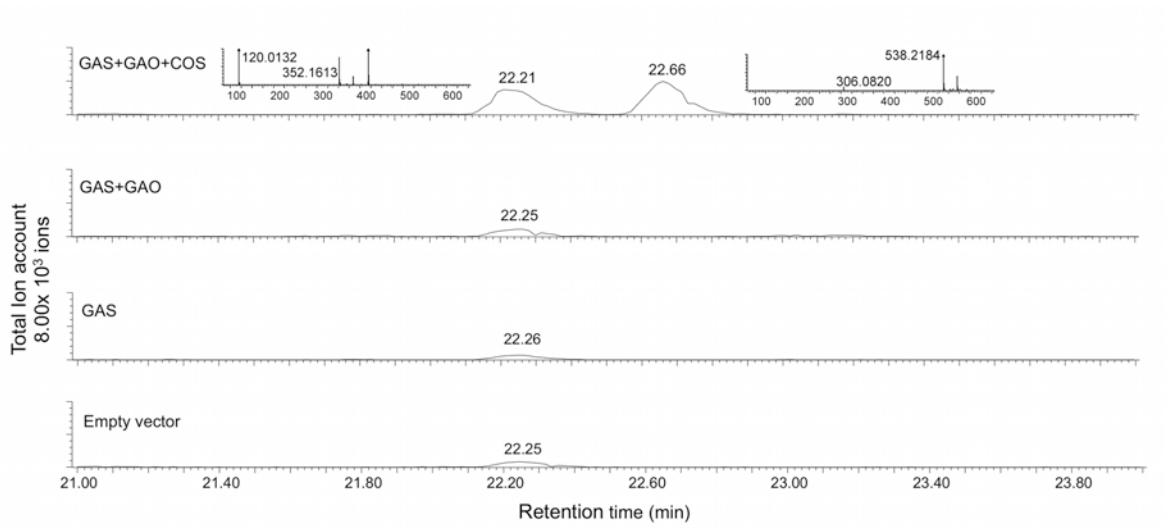

Figure 11: LC-MS/MS analysis of non-volatiles metabolites from *Nicotiana benthamiana* leaves agro-infiltrated with empty vector, *TcGAS*, *TcGAS*+*TcGAO*, and *TcGAS*+*TcGAO*+*TcCOS*. The MS/MS spectrum of the two new peaks (peak 22.21 and peak 22.66) in leaves agro-infiltrated with pyrethrum GAS+GAO+COS and their respective parent ions ($[m/z]^-$ 352.1613 and 538.2184, respectively) are shown.

Expression analysis of pyrethrum STL-biosynthesis related genes

In order to assess the expression of *TcGAS*, *TcGAO* and *TcCOS*, and to confirm that the expression is exclusively in trichomes, RT-qPCR experiments were carried out using cDNA samples from seedlings (without trichomes), young leaves, and trichomes alone (isolated from achenes of stage 3 flowers).

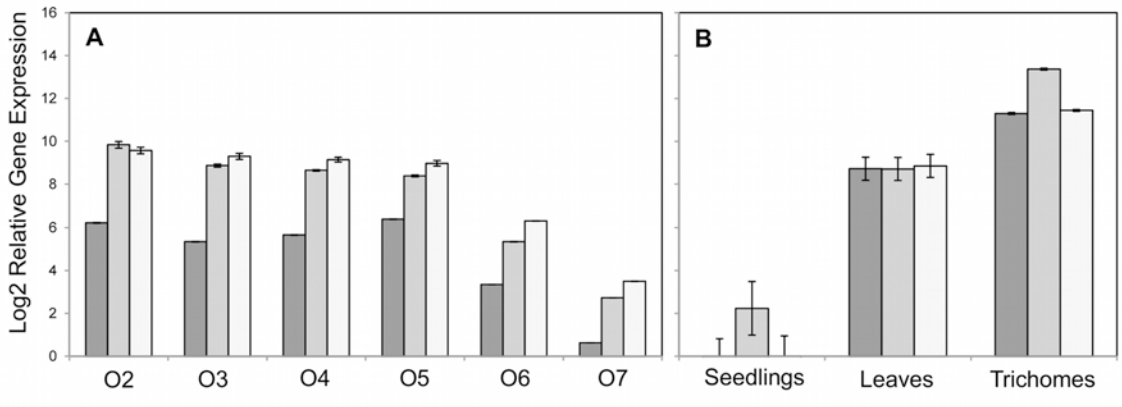

Figure 12: Log_2 of the Relative Gene Expression of pyrethrum *TcGAS* (black bars), *TcGAO* (grey bars) and *TcCOS* (white bars) in different tissues (A) and in ovaries isolated from flowers at

different developmental stages (O2 to O7) (B). The Ct value for each sample was normalized using *GAPDH* as housekeeping gene. Error bars represent SEM (N=3)

Comparison of the relative gene expression of the three genes revealed that expression was highest in the trichomes, lower in the leaves, and absent in seedlings (Figure 12A). RT-qPCR analysis of ovaries derived from flowers in different stages of development revealed a similar pattern of expression for all three genes (Figure 12B). Expression was high and almost constant in stages 2, 3, 4, and 5 and gradually decreased in stages 6 and 7.

Discussion

In the present study we demonstrate in yeast and *in planta* that the three pyrethrum genes, *TcGAS*, *TcGAO* and *TcCOS* encode enzymes, that catalyze the formation of C6-OH-GAA from FDP. C6-OH-GAA is a crucial intermediate in the biosynthesis of many C6-C7 costunolide-like sesquiterpene lactones with a range of biological activities [22-25], and a putative intermediate in the biosynthesis of most pyrethrum STLs, which are oxidized at both the C6 and C8 positions.

Accumulation of different types of STLs in trichomes

Previous studies in pyrethrum and other species of the Asteraceae family have shown that sesquiterpene lactones (STLs) are exclusively stored in trichomes and probably also produced there [1,19,26-28]. Here, we investigated this for pyrethrum and observed that the concentrations of different STLs in various tissues, indeed correlate with the presence of trichomes (Figure 6). Quantitative expression analysis in isolated trichomes in comparison to other tissues confirmed that all three genes putatively involved in pyrethrum STL biosynthesis are highly expressed in trichomes, and share a highly similar expression pattern (Figure 12B). Consequently, based on the results presented here and on what has been reported for feverfew, a close relative of pyrethrum [19], glandular trichomes are the sites of sesquiterpene lactone accumulation and production also in pyrethrum. STL accumulation per flower increases gradually in flower development stages 1 to 5 and is accompanied by a high and constant expression of the three genes involved in their production, as long as disc florets are still opening (Figure 6, 12A). After all florets have opened gene expression is down regulated. Consistent with other reports on terpene biosynthesis [29-31], and with our previous report on pyrethrin accumulation, the results reported here indicate that also STL accumulation is developmentally regulated in pyrethrum. Even though both types of secondary metabolites accumulate in a similar

pattern during flower development, and are both at least partially produced in the glandular trichomes, the final concentrations of STLs are 5-10 fold lower compared to pyrethrins. These differences might represent ecologically optimal adaptations to specific herbivores and pathogens or even surrounding competing flora [1,4] at the lowest fitness cost [1].

Role of C6 hydroxylation of GAA backbones in the biosynthesis of pyrethrum STLs

Most of the pyrethrum STLs reported in the literature seem to belong to the C7-C8 type (Figure 4), rather than to the C6-C7 costunolide types of STL [3-5]. Yet, their basic sesquiterpene backbone, before lactone formation, is likely to be derived from the same germacratrien-12-oic acid (GAA) precursor and hence the same enzymes are expected to be involved. The formation of the reported pyrethrum STLs [3-5], seems to depend on C8 hydroxylation of GAA. However, the pyrethrum trichome ESTs do not contain a *HaG8H* homolog. Interestingly however, all pyrethrum STLs display oxidation at the C6 position (Figure 4). A possible explanation would be that pyrethrum has a C8 hydroxylase gene that is not homologous to HaG8H or that costunolide or its derivatives is a pathway intermediate for the C7-C8 STL biosynthesis in pyrethrum. However, considering the spontaneous and irreversible lactonization of C6-OH-GAA and the enzymatically driven lactonization of C8-OH-GAA [17], it seems more likely that C6 hydroxylation by TcCOS *in planta* occurs after the formation of the C7-C8 lactone ring by an as yet unknown pyrethrum enzyme not highly homologous to HaG8H. Figure 3 shows how we envisage the precursors of the reported STLs are formed. We presume that the relatively poor efficiency of TcCOS may be due to the fact that it prefers a C7-8 lactonized substrate rather than GAA. Preferred C6-hydroxylation of GAA would inevitably lead to the formation of C6-C7 types of STLs, which are not common in pyrethrum.

Additional enzymatic reactions would require other P450 enzymes, for example catalyzing hydroxylation of C3 and C8 (compound p5, Figure 5) and of C1 (Figure 4, all compounds; Figure 5, compounds p3, p4/p7, p6/p8) in some cases followed by subsequent oxidation (Figure 4, compounds 3 and 5) catalyzed by the same or a second P450 enzyme or a dehydrogenase. In some compounds the C3 and C6 hydroxyl groups are further esterified to acetyl (Figure 4, compounds 1,3,4,5; Figure 5, compound p3 and p5), glycosyl (Figure 4, compound7), or tigloyl conjugates (Figure 4 compounds 2; Figure 5 compounds p4/p7 and p6/p8), by presumably acetyl glycosyl and acyl transferases, respectively. And finally germacrene cyclases would be necessary to catalyze the cyclization of the sesquiterpene backbone (Figure 4, compounds 1, 2 and 4; Figure 5 compounds p3, p4/p7 and p6/p8). Out of the 27.317 ESTs, the pyrethrum EST database contains, 155 ESTs with homology to acyl transferases, between 100 and 250 ESTs that match to acetyl and glycosyl

transferases respectively, 30 ESTs matching cyclases and more than 150 matching cytochrome P450s. In consequence, in the absence of close homologues that would facilitate the selection of the enzymes required to catalyze the missing steps in the pyrethrum STLs biosynthesis, other strategies, like similarity in the pattern and level of expression with the genes already characterized in the present study, would have to be applied to narrow down these candidate numbers.

The lack of proper standards, the more polar nature of some of the reported pyrethrum STLs (glycosylated), which probably resulted in the inability to detect them by GC-MS, and their almost complete absence in the NIST database, resulted in poor or even the same identifications for most pyrethrum STLs. Only compounds p6, p7 and p8 were satisfactorily identified as crysanin (Figure 4, compound 2, Supplemental Table S1) and compound p3 although not with high reliability shows good resemblance to dehydro-β-cyclopyrethrosin (Figure 4, compound 4).

Besides the C7-C8 types of STLs, pyrethrum trichomes also seem to produce some C6-C7 STLs, that have not been reported before, and that were only putatively identified in this study (Figure 5, compound p5). It has been proposed [14] and shown later on in chicory and feverfew that after the cyclization of farnesyl diphosphate (FPP) to germacrene A, its C12 methyl group can be oxidized in three steps to yield GAA (Figure 3), which is catalyzed by a single cytochrome P450, germacrene A oxidase (GAO) [16,20]. Hydroxylation at the C6 position of GAA, in a reaction catalyzed by costunolide synthase (COS), followed by spontaneous lactonization, yields costunolide, the precursor of C6-C7 STL in chicory and feverfew [15,16]. Similarly, the pyrethrum COS was demonstrated to produce costunolide in yeast and *in planta*, which, in addition to the genomic data suggests that the costunolide biosynthetic pathway is conserved in pyrethrum and that probably costunolide plays a role as precursor of C6-C7 STLs also in pyrethrum.

In summary, we have isolated and characterized two genes (*TcGAS* and *TcGAO*) involved in the biosynthesis of the pyrethrum sesquiterpene lactone backbone, GAA, which is the central precursor of all known STLs found in pyrethrum. Furthermore, a gene encoding an enzyme capable of catalyzing the C6 hydroxylation of GAA was characterized. In heterologous expression, this hydroxylation yields costunolide, the precursor of putatively identified C6-C7 STLs. However, the enzyme possibly also (or preferably) catalyzes hydroxylation at the C6 position of the – likely already lactonized - precursor of the reported C7-C8-type STLs.

Materials and Methods

Plant material.

Seeds were obtained from Honghe Senju Biology Co. Ltd. (Kunming, Yunnan, China).
T. cinerariifolium plants were grown in open field conditions at PRI with irrigation and fertilization when needed.

Extraction and analysis of sesquiterpene lactone contents

Plant materials, including leaves, stems, seedlings, ray florets, disk florets, receptacle and flowers of five developmental stages (1 to 5) as defined by Head (1966) [32] were dissected from flowers picked during summer, flash frozen in liquid nitrogen and ground to a fine powder. Apolar metabolites were extracted from these tissues (50 mg) by 30 sec of vortexing and 5 min sonication in 1 ml chloroform. The extracts were centrifuged for 5 min at 3500 rpm, dehydrated using anhydrous Na_2SO_4 and analyzed by gas chromatography-mass spectrometry (GC-MS). Ovary secretory trichomes, ovaries without trichomes and intact ovaries were isolated from stage 3 flowers and extracted as previously described[1]

GC-MS analysis of plant extracts

The GC-MS measurements were conducted on an Agilent 7890A gas chromatograph consisting of a 7683 series autosampler, 7683B series injector, and 5975C inert MSD with triple-axis detector. Control of the equipment, data acquisition, processing, and management of chromatographic information were performed using the Agilent Enhanced ChemStation E.02.00.493 software. A Zebron ZB-5MS GC13 capillary column (30 m x 0.25 mm i.d. x 0.25 µm film thickness; Phenomenex, USA) with 5m guard column was employed for the chromatographic analyses, which were based on an established pyrethrin protocol. The injector temperature of the GC was set at 250 °C and helium was the carrier gas with a column flow rate of 1.0 ml/min. The injection volume was 1µl and samples were injected in splitless mode. The oven temperature was held at 45 °C for 2 min and programmed to 300 °C at 15 °C/min, the final temperature was held for 4 min. Total run time per sample was 23 min. The mass spectrometer was operated in the electron ionisation mode (70 eV) with an ion source temperature of 230 °C. The detector was switched on after 4.5 min solvent delay and the full mass-range mode was used for the analyses of the samples with a mass to charge ratio range (m/z) from 45–250 atomic mass units (amu), and a scan time of 0.2 sec and an inter-scan delay of 0.1 sec. If not described otherwise, samples were prepared in $CHCl_3$ and diluted 5x before injection. Constituents of the essential oil were identified by comparing their mass spectra with those of the reference library, the NIST 08 mass spectral database.

Isolation and amplification of putative STL biosynthesis related genes

An expressed sequence tag (EST) database of three cDNA libraries derived from pyrethrum leaves, ovaries and trichomes was produced using the GS FLX Titanium platform. Reads were clustered and assembled into contigs. Using an in-house bioinformatics facility, potential gene functions of the resulting contigs were identified by blasting against the Nr database of annotated genes and storing the first 10 hits in a local database. The three candidate STL biosynthesis related contigs were identified by sequence homology to known sesquiterpene synthases and P450s. The candidate genes were amplified from trichome cDNA using high fidelity Phusion polymerase (Finnzymes), cloned into pGEMT-easy vector (Promega), and sequenced. The cDNA sequences for the pyrethrum germacrene A synthase (*TcGAS*), the germacrene A oxidase (*TcGAO*) and the costunolide synthase (*TcCOS*) have been deposited in GenBank under the accession numbers KC441526, KC441527, and KC441528, respectively. The sequences for *TcGAO* and *TcCOS* were also submitted to David Nelson's cytochrome P450 homepage (http://drnelson.uthsc.edu/cytochromeP450.html) and were assigned the names CYP71AV2 and CYP71BL4, respectively [33].

Co-expression of *TcGAO* and *TcCOS* with *TcGAS* in yeast

For the production of GAA in yeast, *TcGAO* and *TcGAS* were both cloned into the pESC-Trp yeast expression vector (Agilent technologies) with the TRP1 auxotrophic selection marker. *TcGAS* was amplified from trichomes cDNA using high fidelity Phusion polymerase (Finnzymes) with the addition of *Not*I/*Bgl*I restriction sites. The amplified product was digested by *Not*I/*Bgl*I and ligated into the pESC-Trp plasmid. Subsequently, *TcGAO* was amplified and cloned into *TcGAS* pESC-Trp using *Sal*I/*Kpn*I restriction sites, yielding the final plasmid *TcGAS+CiGAO* pESC-Trp. Finally, *TcCOS* was clone into modified PYEDP60K [34] using *Not*I/*Pac*I restriction sites.

The *TcGAS+CiGAO* pESC-Trp plasmid was transformed into the WAT11 [35] yeast strain and the clones were selected on synthetic dextrose (SD) minimal medium (0.67% Difco yeast nitrogen base medium without amino acids, 2% D-glucose, 2% agar) supplemented with amino acids, but omitting L-tryptophane for auxotrophic selection of transformants.

TcGAS+CiGAO pESC-Trp and pYEDP60k plasmids containing *TcCOS* were co-transformed into the WAT11 yeast strain. After transformation yeast clones containing both plasmids were selected on SD minimal medium supplemented with amino acids, but omitting uracil, adenine sulphate and L-tryptophane for auxotrophic selection of transformants.

For the induction of gene expression in yeast, the transformed WAT11 yeast strain with *TcGAS+CiGAO* pESC-Trp or co-transformed with *TcGAS+CiGAO* pESC-Trp and *TcCOS*

PYEDP60k-Ura-Ade were inoculated in 3 mL SD minimal medium (0.67% Difco yeast nitrogen base medium without amino acids, 2% D-dextrose) but omitting Trp or Trp-Ura-Ade amino acids, respectively. The yeast was cultured overnight at 30 °C and 300 rpm. The start culture was diluted to OD 0.05 in SG (0.67% Difco yeast nitrogen base medium without amino acids, 2% D-galactose) minimal medium omitting Trp or Trp-Ura-Ade amino acids, respectively. All yeast induction experiments were performed in triplicates in 50 mL of culture volume. Cultures were buffered at pH 7.5 using 75 mM HEPES. After fermentation for 48h at 30 °C and 300 rpm, the medium was extracted with 20 mL ethyl acetate. From this, a 10 mL sample was taken and the ethyl acetate evaporated with a stream of N2 to a final volume of 1 mL, which was analyzed by GC-MS.

Plasmid construction for expression in *Nicotiana benthamiana*

For expression in *N. benthamiana*, *TcGAS*, *TcGAO* and *TcCOS* were cloned into ImpactVector1.1 (http://www.impactvector.com/) to express them under the control of the Rubisco (RBC) promoter [36]. An LR reaction (Gateway-LR Clonase TM II) was carried out to clone each gene into pBinPlus binary [37] vector between the right and left borders of the T-DNA for plant transformation.

Transient expression in *N. benthamiana*

A. tumefaciens infiltration (agro-infiltration) was performed according to the description of van Herpen et al. [21]. *A. tumefaciens* batches were grown at 28ºC at 220 rpm for 24 hours in YEP media with kanamycin (50 mg L-1) and rifampicillin (34 mg L-1). Cells were harvested by centrifugation for 20 min at 4000xg and 20ºC and then resuspended in 10 mM MES buffer containing 10 mM $MgCl_2$ and 100 µM acetosyringone (4′-hydroxy-3′,5′-dimethoxyacetophenone, Sigma) to a final OD600 of *c.* 0.5, followed by incubation at room temperature under gentle shaking at 50 rpm for 150 min. For co-infiltration, equal volumes of the *A. tumefaciens* batches were mixed. Batch mixtures were infiltrated into leaves of three-week-old *N. benthamiana* plants by pressing a 1 mL syringe without metal needle against the abaxial side of the leaf and slowly injecting the bacterium suspension into the leaf. *N. benthamiana* plants were grown from seeds on soil in the greenhouse with a minimum of 16 h light. Day temperatures were approximately 28 ºC, night temperatures 25 ºC. After agro-infiltration the plants were grown under the same greenhouse conditions for another 3 days and then harvested for analysis.

GC-MS analysis of yeast and agro infiltrated plant extracts

Seven mL yeast culture was extracted three times with 2 mL ethyl acetate, which was concentrated, dried using anhydrous Na_2SO_4 and used for GC-MS analysis. Agro-

infiltrated leaves (100 mg) were ground in liquid nitrogen and extracted by brief vortexing and sonication for 10 min in 800 µl dichloromethane. The extracts were centrifuged for 15 min at 3000 rpm, dehydrated using Na_2SO_4 and then used for GC-MS analysis as described above. Compounds were identified by comparison of mass spectra and retention times (RT) with those of the authentic standards of germacrene A (GA), germacra-1(10),4,11(13)-trien-12-ol (GOH), germacra-1(10),4,11(13)-trien-12-al (GAL) [34] and costunolide (COS) (TOCRIS bioscience). Quantification of these sesquiterpenoids was conducted by determination of the total ion current (TIC) peak areas of the sesquiterpenoid peaks from three independent fermentation experiments. An absolute concentration of sesquiterpenoids was calculated from the peak area by comparison with calibration curves of the authentic standards. At the routine injection port temperature of 250 °C germacrene A, germacra-1(10),4,11(13)-trien-12-oic acid (GAA) and costunolide are thermally converted into β-elemene, elematrien-12-oic acid, and saussurea lactone, respectively as discussed by de Kraker et al. [13,15,38]. We also regularly injected samples with an injection port temperature of 150°C to confirm the presence of non-rearranged germacrene A, germacra-1(10),4,11(13)-trien-12-oic acid and costunolide.

LC-QTOF-MS and MS/MS analysis

Non-volatile metabolites were analysed by LC–QTOF-MS (liquid chromatography, coupled to quadrupole time-of-flight mass spectrometry) according to a protocol for untargeted metabolomics of plant tissues [39]. A Waters Alliance 2795 HPLC connected to a Waters 2996 PDA detector and subsequently a QTOF Ultima V4.00.00 mass spectrometer (Waters, MS technologies, UK) operating in negative ionization mode was used. An analytical column (Luna 3 µ C18/2 100A; 2.0 × 150 mm) attached to a C18 pre-column (2.0 × 4 mm) (both from Phenomenex, USA) was used. Degassed eluent A [ultra-pure water: formic acid (1000:1, v/v)] and eluent B [acetonitril:formic acid (1000:1, v/v)] were used at a flow rate of 0.19 mL min^{-1}. Masses were recorded between m/z X and m/z Y; leucine enkaphalin ([M-H]− =554.2620) was used as a lock mass for on-line accurate mass correction. For agro-infiltrated *N. benthamiana*, 100 mg infiltrated leaf from each treatment was ground in liquid nitrogen and extracted with 300 µl methanol:formic acid (1000:1, 495 v/v). After brief vortexing and sonication for 15 min, the extracts were centrifuged for 5 min at 13,000 rpm and filtered through a 0.2 µm inorganic membrane filter (RC4, Sartorius, Germany). The gradient of the HPLC started at 5 % eluent 497 B and increased linearly to 75 % eluent B in 45 min, after which the column was washed and equilibrated for 15 min before the next injection. The injection volume was 5 µl. Data-directed MS-MS measurements were done at collision energies of 10, 15, 25, 35 and 50 eV.

Headspace analysis and GC-MS thermodesorption

Volatile collection from agro-infiltrated *N. benthamiana* leaves and GC-MS analysis were performed according to van Herpen et al. [21]. Steel sorbent cartridges (89 mm 429 × 6.4 mm O.D.; Markes) containing Tenax were used for volatile collection. Cartridges were conditioned at 280 °C for 40 min under a nitrogen flow of 20 psi in a TC-20 multi-tube conditioner and were capped airtight until use. *N. benthamiana* leaves were detached and placed on water in a small vial and were enclosed in a glass container. To trap the leaf-produced volatiles, air was sucked through one Tenax cartridge (to purify the incoming air) and then through the containers and a second cartridge to adsorb volatiles at a flow rate of 90 mL min^{-1} for 24 h. Sample cartridges were dried for 15 min at room temperature with a nitrogen flow of 20 psi before GC-MS analysis on a Thermo Trace GC Ultra connected to a Thermo Trace DSQ quadruple mass spectrometer (Thermo Fisher Scientific, USA). Cartridges were placed in an automated thermodesorption unit (Ultra; Markes, Llantrisant) in which they were flushed with helium at 50 mL min^{-1} for 2 min to remove moisture and oxygen just before thermodesorption. The volatiles were desorbed by heating of the cartridges at 220°C for 5 min with a helium flow of 50 mL min^{-1}. The compounds released were trapped on an electrically cooled sorbent trap (Unity; Markes, Llantrisant) at a temperature of 5°C. Subsequently, the trapped volatiles were injected on the analytical column (ZB-5MSI, 30 m × 0.25 mm ID, 1.0 µm film thickness, Zebron, Phenomenex) in splitless mode by ballistic heating of the cold trap to 250°C for 3 min. The temperature program of the GC started at 40°C (3 min hold) and rose 10°C min^{-1} to 280 °C (2 min hold). The column effluent was ionised by electron impact (EI) ionisation at 70 eV. Mass scanning was done from 33 to 280 *m/z* with a scan time of 4.2 scans s^{-1}. Xcalibur software (Thermo, USA) was used to identify the eluted compounds by comparing the mass spectra with those of authentic reference standards.

Gene expression analysis

For RNA extraction plant tissue was homogenized by adding one pre-cooled grinding bead to each 2 ml Eppendorf vial containing 50-100 mg of liquid nitrogen frozen plant tissue and using a pre-cooled Mikro-disembrator II (Braun; Germany) for 1 min at maximum speed. After careful removal of the beads, RNA was isolated using TriPure (Roche) and transcribed into cDNA using TaqMan Reverse Transcription reagents (Applied Biosystems) according to the manufacturer's instructions.

RT-qPCR was used to study the expression of *TcGAS*, *TcGAO*, *TcCOS* in cDNA derived from different tissues. Gene specific primers were design using Beacon Designer Software. *T. cinerariifolium* glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (TcGAPDH-F: 5'- AGACGAGTTTCACAAAGTTG-3' and TcGAPDH-R '5-

AGGAATCTGAAGGCAAGC-3') was used for normalization. PCR reactions were prepared in duplicate by mixing 22.5 µL iQ SYBR green supermix 2x (Biorad), 4.5 µL sense primer (3 µM), 4.5 µL antisense primer (3 µM), 11.5 µL deionized water, and 2 µL cDNA template in a 500 µL Eppendorf vial. After vortexing, 2x20 µL of each sample was distributed into two wells in 20µL amounts. Quantification of the transcript level was performed in an MyiQ iCycler system (Bio-Rad Laboratories, USA) using a three-step programme, which included (i) enzyme-activation at 95 °C for 3 min, (ii) 40 cycles of 95 °C for 10 sec, 60 °C for 30 sec, and (iii) 95 °C for 1 min, from 65 °C to 95 °C for 10 sec for dissociation curve analysis. At the end of each run, amplified products were sequenced to verify their identity. Relative expression values were calculated using the efficiency δCt method.

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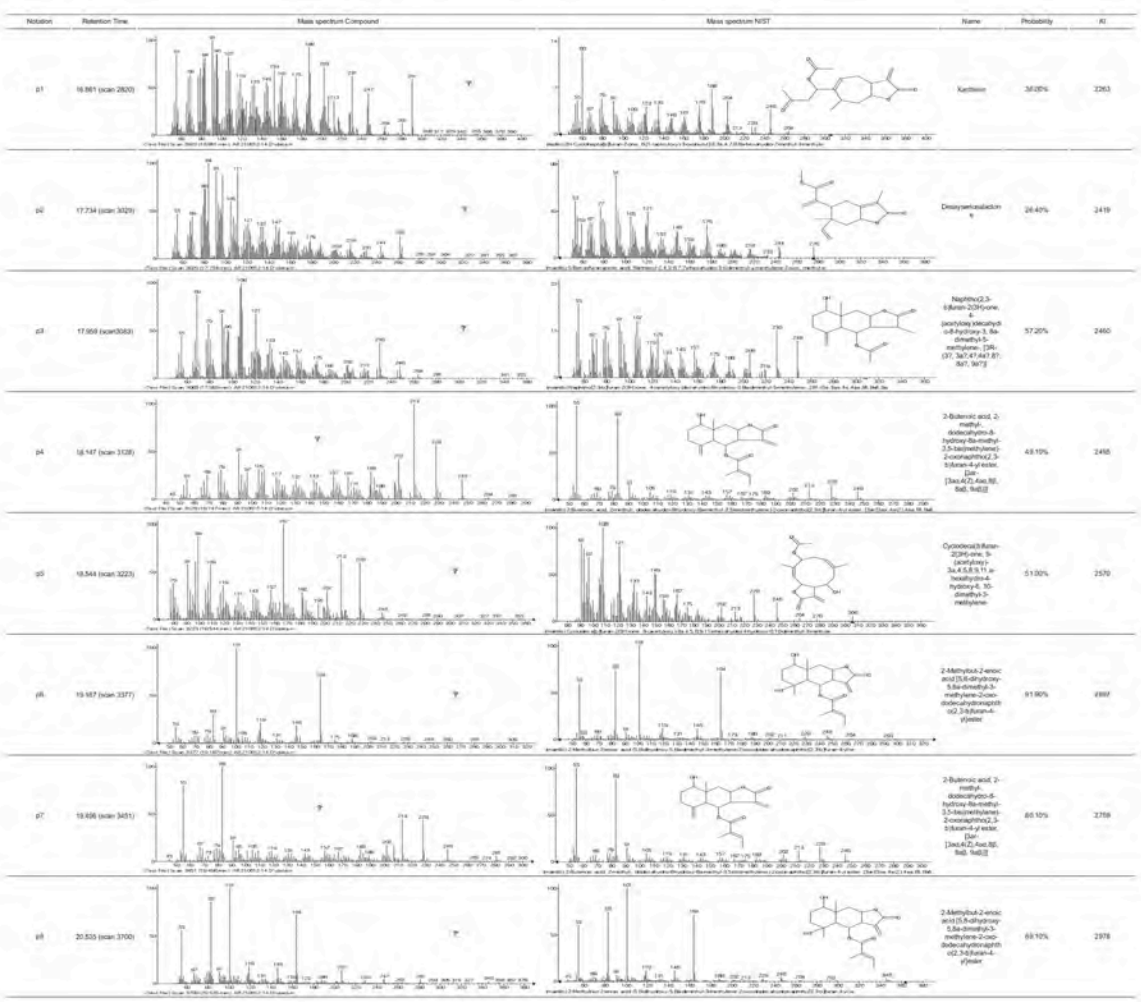
Supplemental Data

Table S1 : List of compounds detected with GC-MS on pyrethrum chloroform dip extracted seeds.



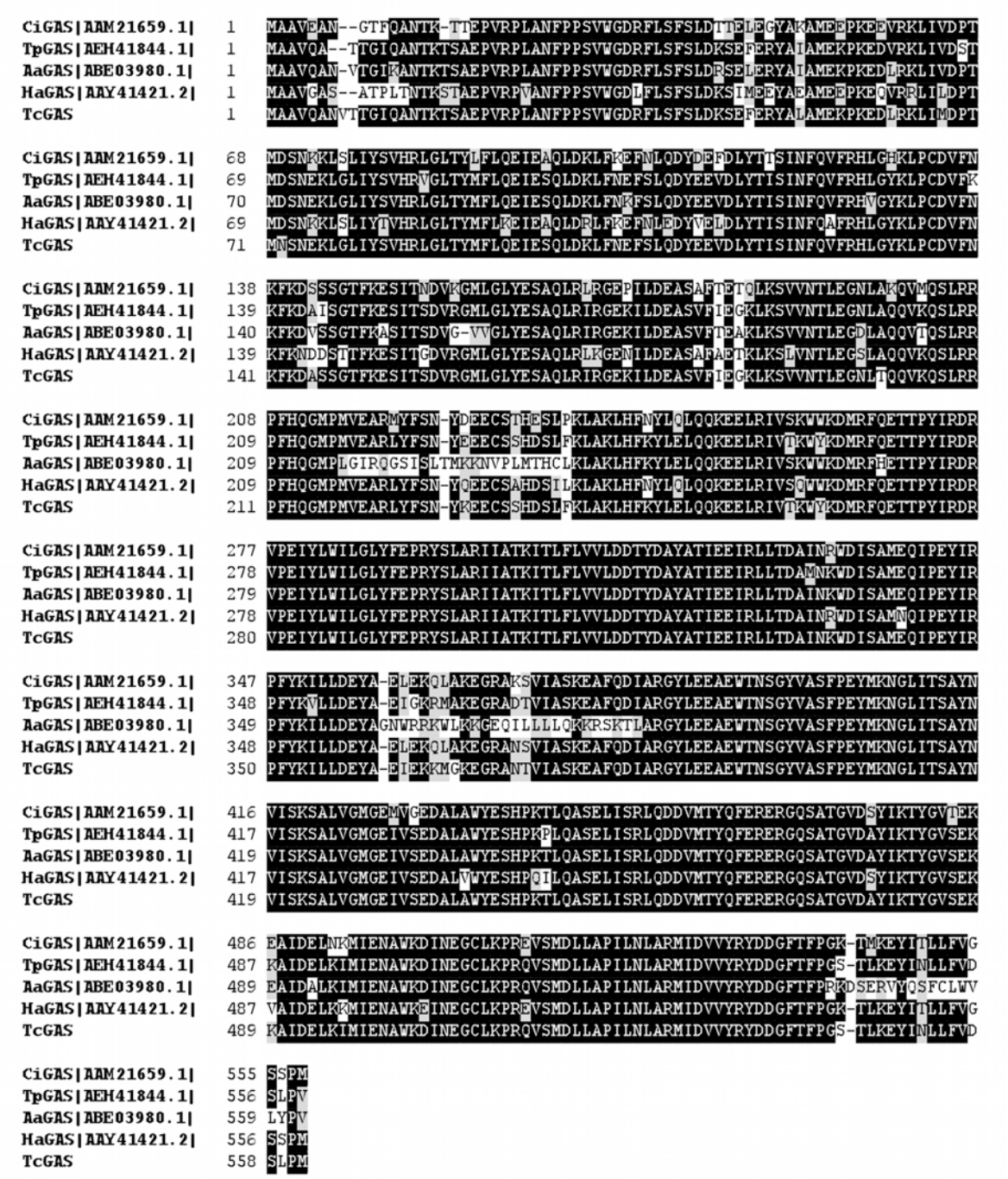


Figure S1: Multiple protein sequence alignment based on the deduced amino acid sequence of pyrethrum germacrene A synthase (TcGAS) (KC441526) and other characterized plant GASs. The alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). The species abbreviations are Ci, *Cichorium intybus*; Ha, *Helianthus annuus*; Aa, *Artemisia annua*; Tp, *Tanacetum parthenium*.

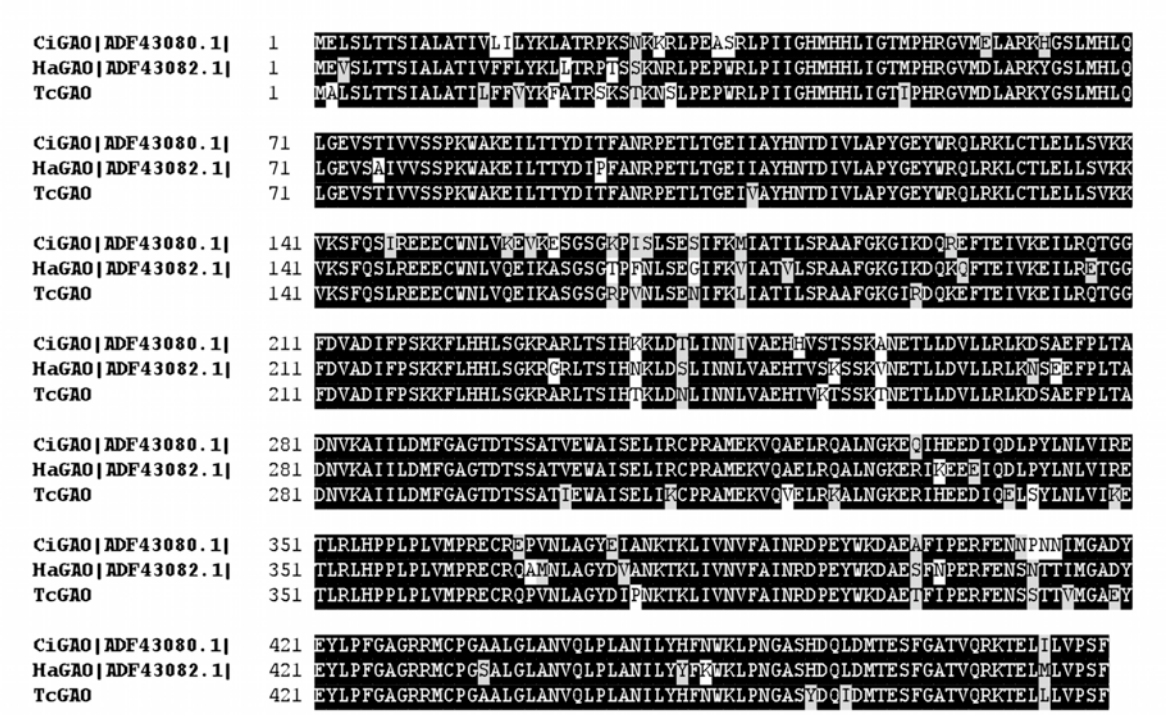


Figure S2: Multiple protein sequence alignment based on the deduced amino acid sequences of pyrethrum germacrene A oxidase (TcGAO) (KC441527) and other characterized plant GAOs. The alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). The species abbreviations are Ci, *Cichorium intybus*; Ha, *Helianthus annuus*.

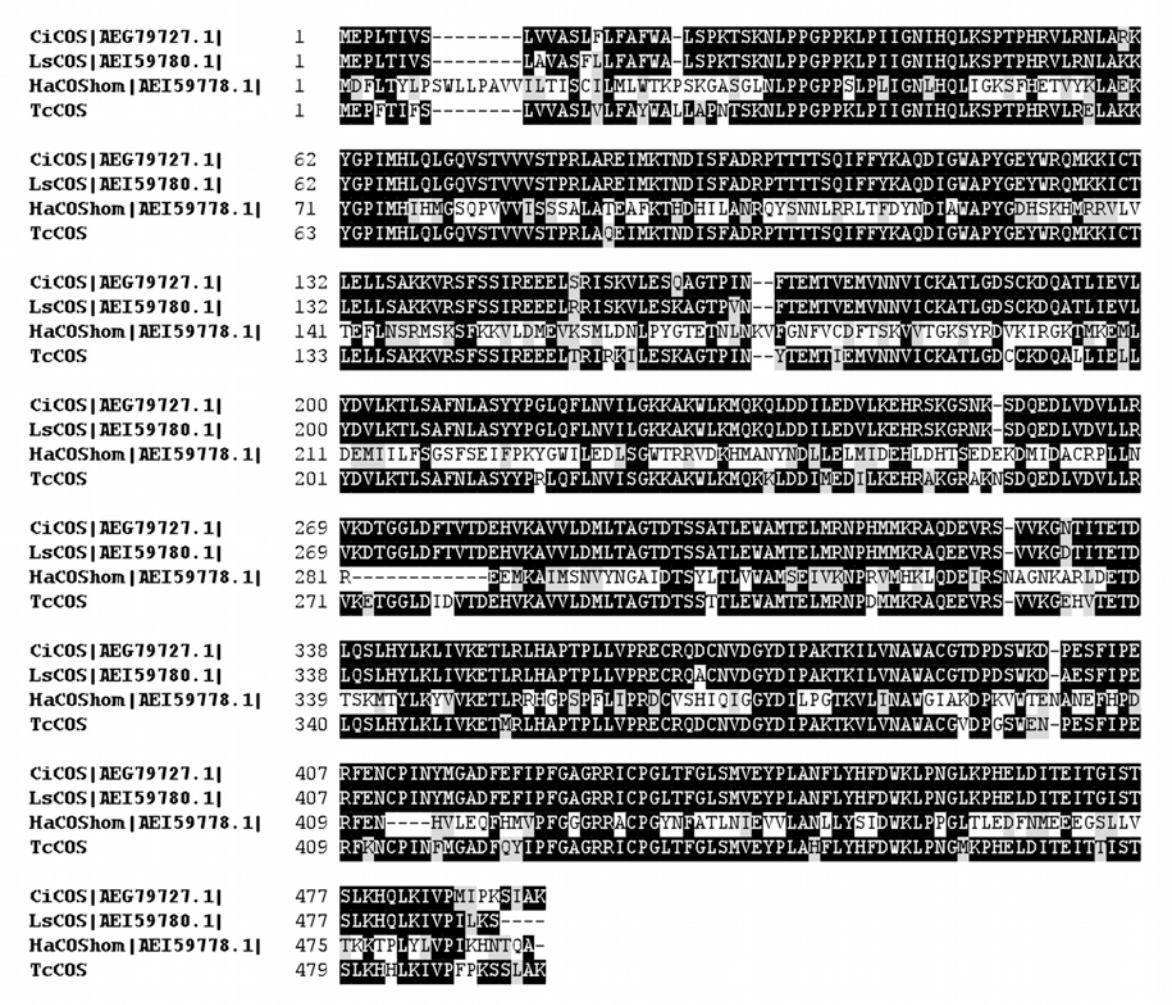


Figure S3: Multiple protein sequence alignment based on the deduced amino acid sequences of pyrethrum costunolide synthase (TcCOS) (KC441528) and other characterized plant COSs. The alignment was performed using ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2). The species abbreviations are Ci, *Cichorium intybus*; Ha, *Helianthus annuus*; *Ls, Lactuca sativa.*

Chapter 6

General Discussion

The perennial herbaceous plant *Tanacetum cinerariifolium*, also known as pyrethrum, is a daisy-like flower with an inherent ability to produce considerable amounts of biologically active metabolites, especially pyrethrins, probably intended for self-defence. The discovery of pyrethrin toxicity towards insect pests triggered the exploitation of pyrethrum for commercial purposes in the late 19th century. Despite having a long history of safe and effective use as a source of a versatile botanical insecticide, pyrethrum lost its popularity when in the mid-20th century more cost-effective, active and persistent synthetic variants became available. In recent years, a shift in the general consumers' preferences towards more selective, safer, non-persistent and more environmentally friendly pesticides has renewed the interest in the use of pyrethrum, renewing pyrethrum's economic significance. Despite the fact that the plant has been under commercial cultivation in many parts of the world for the last 160 years, surprisingly little breeding, ecological and genetic work has been performed to achieve important economic targets of the industry. Increasing the yield of pyrethrins in its natural host, or the mass production of pyrethrins in cultured cells or even a microbial host, would offer new possibilities to the pyrethrin industry that could potentially contribute to placing pyrethrins in a more favourable competitive position in today's insecticide market. Similarly, insights into the biological role of secondary metabolites found in pyrethrum, could potentially greatly benefit the economics of the pyrethrum industry. However, in an era in which advanced breeding and genetic modification techniques are not the limiting factor, the luck of basic biochemical information, like the identification and isolation of key enzymes involved in the formation of pyrethrins and sesquiterpene lactones constitutes the major hurdle in the genetic engineering of these secondary metabolites, either in the natural host or other species. Genes encoding enzymes involved in the biosynthesis of certain metabolites are expected to be actively transcribed at specific moments and/or specific tissues, hence the determination of the exact site of accumulation and synthesis of secondary metabolites constitute a necessary tool to help pick out the genes of interest. Developing knowledge around different aspects of pyrethrum secondary metabolism will, therefore, contribute to generating the necessary tools for breeding and/or engineering of varieties with enhanced pyrethrin contents and decreased contents of unwanted metabolites. Potentially, in the longer run it will also be possible to engineer the biosynthesis of pyrethrins into other crop species. Ideally such crops would then no longer require the external application of pesticides to protect them against microbial diseases and pests.

In this section I will discuss the most important findings obtained in the course of this thesis, ranging from localization and biochemical aspects of the synthesis of pyrethrum defence compounds to their possible biological role in the young emerging seedling as well as in the adult plant.

Pyrethrum selectively stores defence compounds in different compartments of the achenes

The main insecticidally active constituents of pyrethrum, the pyrethrins, occur throughout the whole plant, but are concentrated in the dry fruits (achenes) [1]. A distinctive feature of pyrethrum achenes is the presence of a high density of glandular trichomes covering their surfaces. Both glandular trichomes on the outside and secretory ducts inside achenes, have been implicated in the production and storage of pyrethrins [2,3]. Cryo-scanning electron microscopy images of the surface and cross sections of the achenes of florets (Figure 1A) showed the presence of similar oil-like substances in the subcuticular cavity of trichomes (Figure 1B), in the intracellular spaces of the pericarp (also referred to as secretory ducts) (Figure 1C), as well as in the space immediately surrounding the embryo (Figure 1D).

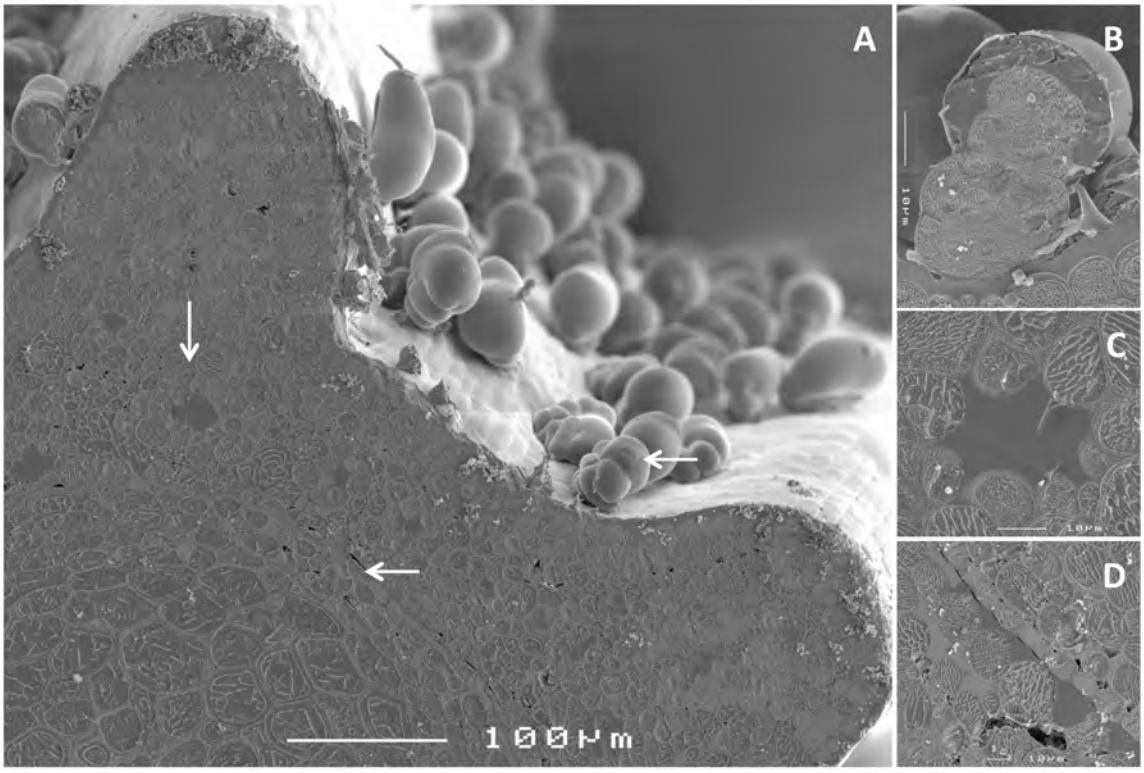


Figure 1: Cryo-scanning electron microscopy image of a perpendicular section of a disc floret showing the trichomes on the outside of the ovary, and the oil in the pericarp (arrows) (A). Closer view of a trichome (B), and of the oil in the intercellular space of the pericarp (C) and in the space between the embryo and the pericarp (D).

GC-MS analysis revealed that trichomes and pericarp of achenes are composed of very distinct sets of metabolites (Chapter 2, Figure 3). The pericarp composition consists mainly of the six pyrethrin esters, cinerin I, jasmolin I, pyrethrins I, cinerin II, jasmolin II

and pyrethrins II [4], whereas the trichomes mainly contain sesquiterpene lactones (STLs). STLs were reported to form part of pyrethrum flower head extracts [5,6,7], but there were no reports of their exact localization. Pyrethrum glandular trichomes were shown, however, to be not only the sites of storage of STLs, but also responsible for their biosynthesis, involving the same basic steps and enzymes that are responsible for the biosynthesis of STLs in close relatives of pyrethrum like parthenium, sunflower and artemisia [8,9,10] (Chapter 5). Similar to the *Artemisia annua* glandular trichomes [11], the two apical cells of pyrethrum glandular trichomes do not have chloroplasts, and if, like for *A. annua,* morphological differences translate into cellular specialization [12], it is likely that the two chloroplast-free apical cells of pyrethrum trichomes are mainly specialized in the production of cytoplasmic mevalonate-derived sesquiterpene lactones [13] that are selectively transported in the apical direction (Figure 2).

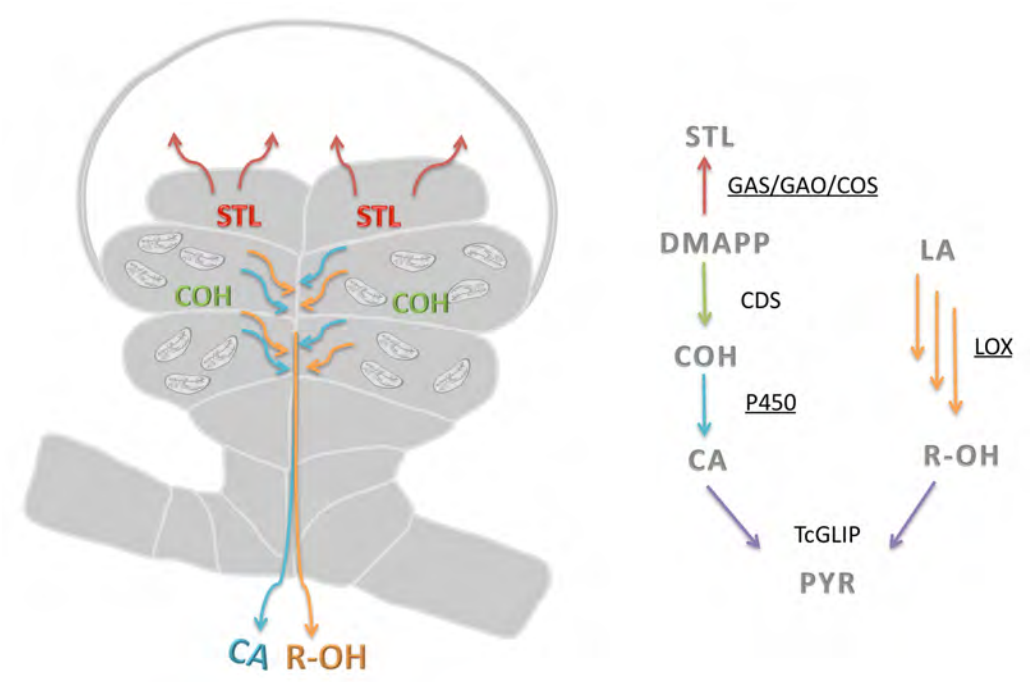


Figure 2: Schematic representation of a pyrethrum trichome and the proposed bidirectional transport of pyrethrum sesquiterpene lactones (STL), the acid portion, chrysanthemic acid (CA) and the rethrolone portion (R-OH) of pyrethrins (PRY). Chrysanthemol (COH), the precursor of CA, is not secreted out of the trichome. On the right, a simplified schematic representation of the steps and enzymes involved in biosynthesis of STL and CA from dimethylallyl pyrophosphate (DMAPP), R-OH from linolenic acid (LA), and the final esterification of CA and R-OH into pyrethrins. Underlined are the enzymes characterized in this thesis

Glandular trichomes are not only implicated in the production of mono- and sesquiterpene compounds that are stored in the subcuticular cavity right outside the apical cells [14,15],

but also in the production and secretion of monoterpenoids in basipetal direction (Chapter 2). In pyrethrum glandular trichomes, the irregular monoterpenoid moiety of pyrethrins, chrysanthemic acid (CA), is produced from chrysanthemol (Figure 2, COH) through the chloroplast-dependent methylerythritol phosphate (MEP) pathway, localized in the chloroplast containing subapical trichome cells. After synthesis, CA is transported in basipetal direction towards the pericarp where its CoA-activated form is joined to the alcohol moiety (Figure 2, R-OH) by the a ester-forming GDSL-like lipase enzyme (TcGLIP) [16]. The tissue in which the activation of CA takes place, is not yet known, but the enzyme catalysing this step is expressed exclusively in the trichomes [17]. The tissue producing R-OH is also not yet proven directly. However, in Chapter 4 transcriptional characterization of all LOX contigs revealed that the only gene with the same level of expression and developmental regulation as other pyrethrin genes, *TcLOX1,* was dominantly expressed in trichomes. This supports the possibility that for the alcohol moiety of pyrethrins, a similar mechanism of precursor biosynthesis in the trichomes and export to the pericarp, may be operating (Figure 2, R-OH) (Chapter 4).

Elucidating the pyrethrin biosynthetic pathway

The botanical insecticides obtained by solvent extraction of pyrethrum flowers, the pyrethrins, are 6 esters consisting of a combination of two acids (chrysanthemic acid and pyrethric acid) derived from a monoterpene pathway, and three alcohols (pyrethrolone, cinerolone and jasmolone) proposed to be derived either from 7-OH- jasmonic acid or Z-jasmone, and in consequence sharing parts of the oxylipin pathway with jasmonic acid [18].

The acid moieties are irregular monoterpenes that have their origin in the MEP pathway (Figure 3) [18]. The formation of the cyclopropane ring is catalyzed by chrysanthemyl diphosphate synthase (CDS) to give chrysanthemyl diphosphate using two molecules of dimethylallyl pyrophosphate (DMAPP) [19]. Recently, it was found that under the low substrate conditions prevalent in plants, chrysanthemyl diphosphate is immediately dephosphorylated by CDS as well in a two-step reaction [17]. The resulting product, COH, undergoes two consecutive oxidations in a reaction catalysed by a single cytochrome P450 enzyme to give CA. The gene encoding this cytochrome P450 enzyme expresses exclusively in the trichomes and shows a transcriptional regulation during flower development completely parallel to the CDS gene (Chapter 3). In the next step, CA is activated by conjugation to co-enzyme A. Recently, the enzyme for this reaction was discovered [17]. Also in 2012, a GDSL-like lipase (GLIP) responsible for the esterification of the CoA-activated form of CA to the rethrolone counterpart was cloned and characterized [16]. Thus, after CDS in 2001 [19], all four remaining enzymatic steps in the

monoterpene branch of the pyrethrin biosynthetic pathway from DMAPP to pyrethrin I have been elucidated in 2012 (Figure 3).

If at the start of this research program little was known about the biochemistry of the acid portion of pyrethrins, even less was known about the biochemistry of the rethrolone moiety. Matsuda and co-workers demonstrated in their research, that regardless of the immediate precursor (7-OH-jasmonic acid or *Z*-jasmone), the rethrolone portion of pyrethrins derives from linolenic acid through the jasmonic acid (JA) or oxylipin pathway (Figure 3). As a consequence, a series of enzymes involved in the biosynthesis of JA, like lipoxygenases (LOXes), are expected to be involved in pyrethrin biosynthesis as well. By assessing the similarities in expression levels and developmental regulation of all pyrethrum *LOX* contigs to *TcCDS* and *TcGLIP*, two potential *LOX* genes were selected. Both candidates turned out to be specifically expressed in trichomes, but only one could be shown to catalyse the hydro-peroxidation of the linolenic acid substrate during pyrethrin biosynthesis (Chapter 4). The trichome localization implies that, like the monoterpene acids, also production and export of rethrolone precursors could be operating from trichomes. However, given the esterification of the two portions by TcGLIP which localizes in the pericarp (Chapter 2), we cannot rule out the possibility of a lipoxygenase with a pericarp localization, as an alternative candidate for the biosynthesis of the rethrolone portion of pyrethrins. Constructs to induce silencing of these genes by RNAi have been made within the project to prove this point, but so far in pyrethrum no stable transgenic events have been generated with them, as only recently the protocol was developed [20]. Effects of gene silencing of specific LOX candidates in trichomes (with a trichome specific promoter) could provide definite evidence to mark the role of trichomes in the generation of lipid precursors for pyrethrin biosynthesis.

If the rethrolone portion of pyrethrins follows the biosynthesis of phytohormone JA, the initial steps of its biosynthesis will have a chloroplast localization. This would imply that large quantities of linolenic acid (LA) would be required to be either synthesized *de novo*, or released from chloroplast membranes by the action of phospholipases [21,22,23]. In both cases, the synthesis of large quantities of JA analogues for pyrethrin biosynthesis, could potentially compromise a normal wound response in sites where the production of pyrethrins is higher, hence, it is conceivable that pyrethrum flowers developed insensitivity to jasmonates and started utilizing alternative activation systems to allow reliable responses to tissue damage. Indeed, it was shown that pyrethrin concentrations in adult plants and flowers were unaffected by mechanical damage which would normally trigger the release of jasmonates and possibly induce such defence systems [24]. Furthermore, more recently in seedlings, when mechanical wounding actually did enhance the levels of pyrethrins, the authors show that only a combination of green leaf volatiles (GLVs) and

(*E*)-β-farnesene could induce the biosynthesis of pyrethrins type I [25]. Apparently, pyrethrin biosynthesis, which involves the jasmonate pathway, only responds to these specific GLV volatile combinations and less to the generic jasmonates which are always formed upon plant damage. Unfortunately the direct experiment by applying JA and measuring the response to prove that important point was lacking in the paper [23].

In plants, fatty acid biosynthesis and desaturation is localized in photosynthetically active chloroplasts [26,27], and one of the most conspicuous features of cells secreting lipophilic compounds is the presence of osmophilic (=lipid) materials in the plastids, which in most cases are entirely or partially surrounded by endoplasmic reticulum (ER) [14]. In glandular trichomes of *A. annua*, which are highly similar to pyrethrum glandular trichomes, the choroplasts of the four subapical secretory cells were the only organelles containing large amounts of osmophilic material (Chapter 2) [11]. The nature of this osmophilic material was not determined, but was suggested to be either the substrate or the final terpenoid products characteristic of this species. Although more detailed utrastructural and microdissection studies of pyrethrum subapical trichome cells would be required to determine the presence and nature of osmophilic materials, it is possible that also in pyrethrum these chloroplast containing cells, do not only specialize in the biosynthesis of the monoterpene portion of pyrethrins (Chapter 2) but also in the partial or total biosynthesis of the chloroplast derived rethrolone portion of pyrethrins.

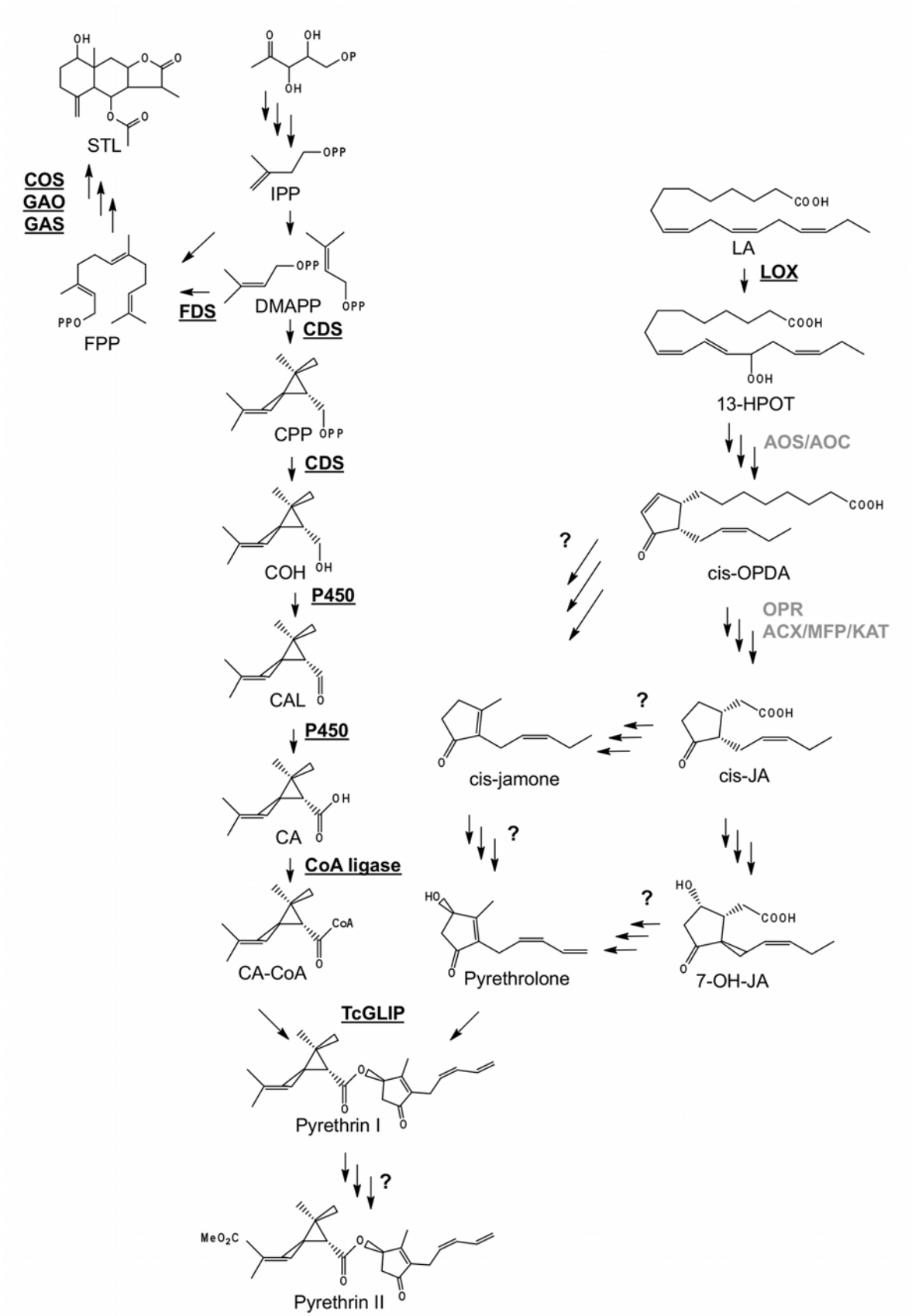


Figure 3: Simplified biosynthetic pathways to pyrethrins and sesquiterpene lactones. The identified enzymes involved in the biosynthetic pathways are underlined and the enzymes involved in the known parts of the JA pathway are shown in grey. Steps that have not been elucidated are shown

with question marks. Pyrethric acid (methyl ester of CA) is not found in free form and most likely formed in a conversion of pyrethrin I to pyrethrin II, Jasmolone is likely the primary product of the oxylipin pathway. Conversion to pyrethrolone and cinerolone may happen either before or after ester formation.

Whether or not trichomes are involved in the biosynthesis of the alchohol moiety of pyrethrins, whether and how pyrethrolone derives form JA or Z-jasmone, and whether and how the type II pyrethrins are formed from type I pyrethrins remains to be elucidated. In this regard, it is interesting to speculate on the formation of type II pyrethrins (Figure 3). The observation that the ratio PI/PII decreases during flower development, and the fact that free pyrethric acid has never been observed, neither in previous reports [28], nor in this research, suggest that the methylesters of type II pyrethrins could arise from modifications of type I pyrethrins by, for example, the action of a single or multiple cytochrome P450 enzymes catalysing the hydroxylation and subsequent oxidation at C8 of the chrysanthemic acid moiety, followed by a methylation of the carboxyl group in a reaction catalysed by possibly a carboxyl methyltransferase enzyme. *O*-methyltransferases (OTMs) constitute a large family of enzymes that methylate the oxygen atom of a variety of secondary metabolites, such as flavonoids, phenylpropanoids and some alkaloids [29,30]. The reaction involves the transfer of the methyl group of S-adenosyl-L-methionine to the hydroxyl group of an acceptor molecule with the formation of its methyl ether and S-adenosyl-L-homocysteine as products [29]. In plants, *O*-methylation play important roles in the biosynthesis of structural macromolecules (lignin), in the synthesis of molecules involved in disease resistance (phytoalexins), and in reducing the reactivity of the hydroxyl group of secondary metabolites, changing their degree of antimicrobial activity, mutagenicity, and carcinogenicity [31,32]. The methylation of a variety of acids, including benzoic, salicylic, indoleacetic and jasmonic acid by the action of carboxyl OMTs also plays important roles in different aspects of plant biology [33]. The methylated product of indoleacetic acid plays an important role in the regulation of plant growth and development and methylated jasmonic acid acts as a signal molecule mediating intra and interplant communication for defence responses [34,35,36]. In order to gain more insight in whether the carboxylation followed by methylation of the side chain of the cyclopropane ring of pyrethrins occurs before or after esterification to the rethrolone counterpart, and in addition to more detailed/targeted biochemical studies to confirm the complete absence of pyrethric acid in pyrethrum extracts, the already cloned cytochrome P450s (Chapter 3) and the 17 pyrethrum ESTs contigs with homology to carboxyl methyltransferases could be tested for their ability to specifically carboxylate and

subsequently methylate either chrysanthemic acid or any of the type I pyrethrins [37,38,39].

Elucidating pyrethrum sequiterpene lactone biosynthesis

Apart from being the source of the most potent natural insecticide known today, pyrethrum also produces a range of other putative defence compounds, most prominently the germacranoid sesquiterpene lactones. These compounds may be useful to the plant, but they are a nuisance to the industry. Notably pyrethrosins are residues contaminating pyrethrin extracts and have been implicated to cause the allergenic properties of pyrethrum products [40,41]. A better knowledge of how these compounds are synthesized is, therefore, also relevant from an industrial perspective. Most pyrethrum STLs for which the structure has been elucidated share the same basic C15 sesquiterpene backbone with a C7-C8 [5,6,7] rather than C6-C7 lactone ring. However, remarkably nearly all pyrethrum STLs are oxidised at both the C6 and C8 positions. In parthenium and chicory hydroxylation of the C6 position in germacratrien-12-oic acid leads to the spontaneous formation of a C6-C7 lactone ring in a compound called costunolide. In Chapter 5 I showed that the enzymes required for the production of costunolide are also present in pyrethrum. In pyrethrum, trichome-specific enzymes (germacrene A synthase, TpGAS and germacrene A oxidase, TcGAO) catalyse the biosynthesis of the sesquiterpene backbone, GAA, and a third enzyme (costunolide synthase, TcCOS) catalyses hydroxylation at the C6 position of GAA, which upon spontaneous lactonization results in costunolide (Chapter 5). We only obtained weak evidence that costunolide STLs occur in pyrethrum, however (Chapter 2). The formation of the more common C7-C8 STL, requires a cytochrome P450 capable of hydroxylating the GAA backbone at the C8 position, and presumably a second enzyme to perform the subsequent lactonization to form the C7-C8 STLs [42]. Presently, only one C8-hydroxylase enzyme has been isolated and characterized from sunflower [42], and this gene does not seem to have a highly expressed orthologue in pyrethrum trichomes. Considering, however, that all pyrethrum C7-C8 STLs show a hydroxylation at position 6 of the GAA backbone, we propose that this hydroxylation is catalysed by TcCOS after the C7-C8 lactone ring has been formed. The STL biosynthesis in pyrethrum requires further study to understand all steps, but this work is currently hindered by the lack of proper standards and the limited availability of mass spectra and chromatographic information of known structures in the NIST database.

Roles of pyrethrins and STLs in pyrethrum defence

During flower development and seed maturation pyrethrum trichomes are involved in the production of (i) sesquiterpenoid compounds that are transported in apical direction and

stored in the subcuticular cavity surrounding the two apical cells, and of (ii) the monoterpenoid, chrysanthemic acid, that is transported in basipetal direction towards the pericarp where esterification with the rethrolones results in the formation of pyrethrins (Figure 2).

In the adult plant, trichomes of seeds have a more complex sesquiterpenoid composition than leaves (Chapter 2, Figure 3C/F). Mixtures of STLs derived from seed trichomes showed deterrent effects towards caterpillars and slowed down the mycelial growth of the soil-born pathogen *R. solani* (Chapter 2, Figure 8). Based on these observations, we propose that the differences in STL composition may reflect functional adaptations to specific herbivores or microbial pathogens of the leaves and seeds.

In the adult plant, STLs, may play a more important role in protecting the leaves against herbivores, whereas in the early stages of germination, the STLs stored in trichomes outside the seeds, diffuse into the soil and may play roles as antibiotics of soil-borne pathogens and/or inhibitors of the root growth of competing plant species. Pyrethrosin was shown to inhibit the growth of the roots of Chinese cabbage seedlings at concentrations as low as 10 ppm [7], which is equivalent to the concentration of STLs found in 1 seed (6-10 μg) when dissolved in 1mL of water.. Additional assays on seed germination, plant pathogenic micro-organisms and insect herbivores with STLs from leaves and seeds separately would help to understand whether the differential composition indeed represents an adaptation to particular enemies, pathogens and/or nutrient competitors. In pyrethrum cultivation, the agronomic performance mainly depends on the effective management of weeds and fungal diseases with herbicides and fungicides [43,44,45]. The major diseases affecting pyrethrum production in Tasmania are ray blight disease caused by *Phoma ligulicola*, sclerotinia crown rot and sclerotinia flower blight caused by *Sclerotinia minor* and *S. sclerotiorum*, respectively, and botrytis flower blight caused by *Botrytis cinerea* [46]. In Kenya, the diseases affecting pyrethrum that pose the most serious threat to production are wilt diseases (caused normally by *Fusarium* spp.) These diseases can cause damping-off of seedlings and chlorosis, or necrosis followed by death in larger plants [47]. Considering the fungistatic and allelopathic properties of pyrethrum STLs, the selection of varieties with enhanced contents of STLs, or external application of STLs that during refining are discarded as waste products, could potentially present an alternative means to synthetic herbicides and fungicides to manage weeds and diseases.

In later stages during flower development, pyrethrins stored in the intracellular spaces of the pericarp are being absorbed by the desiccating embryo (Chapter 2, Figure 4) and end up in very high concentrations in the germinating seedling (Chapter 2,Figure 6A). The seedling stage is a highly vulnerable stage with a high risk of mortality, due to both abiotic and biotic stress [48,49,50]. Yet, investing resources in defence rather than in growth

during this early stage compromises the seedling's current and future fitness. Therefore, receiving a maternal immunization at a stage of vulnerability and inability to generate its own defences could be a highly successful strategy. A similar mechanism of maternal immunization has been observed in the Brassicales, where glucosinolates, synthetized in both the vegetative parts and siliques, are transported to the embryo to finally end up in the seedling, where they function as defence compounds [51]. Similar to STLs, the pyrethrins present in the seedling occur at such high concentrations that they may play a more important role against microbial pathogens (Chapter 2, Figure 8), than against insects [52]. Thus, both pyrethrins and sesquiterpene lactones actually combine to promote the survival of the seedling through protection against insects, pathogens and competitors.

Concluding remarks

The results described in this thesis present an overview of the progress that has been made towards understanding the biochemical and molecular basis of a number of steps in the biosynthesis of two defence-related secondary metabolite classes in pyrethrum, the pyrethrins and sesquiterpene lactones. In addition, important progress in our understanding of the tissue distribution, transport as well as bio-activity of these compounds was achieved, which as a whole offered new insights in their so far un-explored biological functions in the seedling as well as the adult plant. As a whole, the work presented here will raise the interest of and greatly help future researchers to unravel the still missing aspects of biosynthesis and trafficking of these economically important botanical metabolites. Moreover, it will contribute to the exploration of new production and engineering possibilities for the pyrethrum industry, which should in the future help to fulfil the worldwide growing public demand for safer and environmentally-friendly methods of pest control.

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Summary

Summary

The use of botanical insecticides is in today's world an attractive alternative to the less safe and environmentally malign synthetic chemicals, whose overall longer persistence in the environment does not only make them more contaminant, but also increases their chances of causing a rapid development of resistance in the target pest and having negative-side effects on beneficial organisms. Yet, at present only a handful of botanical products are in commercial use for insect control on crops. Among the most important botanical insecticides (pyrethrins, rotenone, neem and essential oils), pyrethrins have the longest history of effective use against a wide range of insects and best record of low toxicity to mammals. Although pyrethrins were relegated from their once prominent position in the mid-1930's, the recent market trends towards "reduced risk" pesticides and host plant resistance have brought pyrethrins back to the attention and initiated the generation of knowledge around them. Pyrethrins refer to an oleoresin extracted from the dried daisy-like flowers of pyrethrum (*Tanacetum cinerariifolium*). The active constituents are six esters formed by a combination of two acids (chrysanthemic acid and pyrethric acid) and three alcohols (pyrethrolone, cinerolone and jasmolone, collectively called rethrolones). The esters of chrysanthemic acid with the rethrolones constitute type I pyrethrins, whereas the esters of pyrethric acid are collectively known as type II pyrethrins. Apart from being the source of pyrethrins, pyrethrum also produces a range of other defense compounds collectively known as sesquiterpene lactones, which have also been implicated in plant defense against herbivores, pathogens, and competing plant species. Although pyrethrins and sesquiterpene lactones are found throughout the whole plant, the highest concentrations of both types of compounds are found in the achenes of the flowers, which are densely covered with glandular trichomes. GC-MS analysis revealed that trichomes of mature achenes contain sesquiterpene lactones and other secondary metabolites, but no pyrethrins. Although glandular trichomes were known to participate in the production of mono- and sesquiterpene compounds that were stored in or emitted from the subcuticular cavity just outside the apical cells, here we demonstrate that basipetal secretion can also occur. In pyrethrum, the monoterpene-derived portion of pyrethrins, chrysanthemic acid (CA), is translocated from the trichomes to the pericarp, where it is esterified into pyrethrins that accumulate in the intercellular space. We also show that during seed maturation, pyrethrins stored in the pericarp are absorbed by the developing embryo, and that during seed germination these embryo-stored pyrethrins are recruited by the germinating seedling, which, due to the lack of trichomes, cannot produce defense compounds themselves. At early stages, not only sesquiterpene lactones that

diffuse to the soil from the seed coat, but also the pyrethrins found in the seedlings, seem to play a more important role as antimicrobials than as insecticides.

Although there has been considerable progress on the chemistry of pyrethrins, the molecular/biochemical basis of their biosynthesis was largely unknown. The acid and alcohol moieties of pyrethrins derive from distinct pathways. Whereas the alcohol portion is believed to be derived from linolenic acid and share a large part of its biosynthetic pathway with jasmonic acid, the acid moieties are monoterpenes with a cyclopropane ring that are supposed to be derived from an irregular monoterpene pathway. Before the start of this project, only one enzyme, chrysanthemyl diphosphate synthase (TcCDS), had been isolated and demonstrated to catalyze the first step in the biosynthesis of the acid portion of pyrethrins, which consist of the condensation of two molecules of DMAPP to produce chrysanthemol (COH) via chrysanthemyl diphosphate. During this project an additional acyltransferase enzyme (TcGLIP) was isolated by another group and demonstrated to be responsible for the esterification of (1*R*,3*R*)-chrysanthemoyl-CoA and (*S*)-pyrethrolone, one of the last steps in the biosynthesis of pyrethrins, which was demonstrated in this thesis to likely take place in the pericarp.

To identify additional genes of the pyrethrin biosynthetic pathway, we generated three EST libraries derived from ovaries, trichomes and leaves. Gene candidates were obtained either by keyword interrogation of the annotated contigs or by blasting the libraries with known genes catalyzing similar reactions in other plants. Given the likelihood of cytochrome P450s as potential candidates to catalyze the missing steps in the biosynthesis of the acid moiety of pyrethrins, the pyrethrum EST libraries, were first interrogated for genes encoding cytochrome P450 (CYP) enzymes with a developmental expression pattern similar to *TcCDS*, and a specific expression in CA-producing glandular trichomes. Experiments with yeast microsomes allowed the selection of two enzymes capable of converting COH into chrysanthemal. Although after agro-infiltration none of these enzymes affected the background level of CA, one of them (*Ct21854)* resulted in a strong reduction of COH emission, which correlates with a significantly higher amounts of a CA conjugate, confirming that *Ct21854* qualifies as a chrysanthemic acid synthase efficiently converting COH into CA.

Rethrolones have been proposed to originate from linolenic acid and share part of the oxylipin pathway with jasmonic acid, which in turns implies that one of the first committed steps should involve a lipoxygenase enzyme, catalyzing the hydroperoxidation of linolenic acid at position 13 of the hydrocarbon chain. Based on this assumption the pyrethrum EST libraries were interrogated for genes encoding LOX enzymes. The expression patterns of twenty-five lipoxygenase EST contigs were characterized, and the ones with a developmental regulation similar to *TcCDS* and *TcGLIP* were selected. Subsequently, the

molecular cloning of a lipoxygenase, *TcLOX1,* was carried out. Recombinant TcLOX1 was demonstrated to catalyze the peroxidation of the linolenic acid substrate at the C13 position. The gene shares the developmental and trichome-specific expression pattern with *TcCDS*, suggesting that a trichome production and translocation could be operating for the alcohol moiety of pyrethrins as well.

Finally, besides pyrethrins, pyrethrum plants are also a source of sesquiterpene lactones. Even though considerable information on bioactivity and industrial significance of pyrethrum sesquiterpene lactones is available, the localization and biosynthetic origins were largely unknown. Like in other species of the Asteraceae family, pyrethrum sesquiterpene lactones are exclusively stored in trichomes and it is shown that germacratrien-12-oic acid (GAA) is most likely the central precursor of all known sesquiterpene lactones found in pyrethrum. Candidate genes implicated in the first two committed steps leading from farnesyl diphosphate to GAA were retrieved from the pyrethrum trichome EST library, cloned, and characterized in yeast and *in planta*. Furthermore, a gene encoding an enzyme capable of catalyzing the C6 hydroxylation of GAA was characterized. This hydroxylation results in spontaneous lactonization likely resulting in the putatively identified C6-C7-costunolide-derived STLs in pyrethrum. However, the enzyme may also catalyze the hydroxylation at the C6 position of the lactonized precursor of all reported C7-C8-type STLs.

In conclusion, the work compiled in this thesis presents new insights into the participation of pyrethrum trichomes in the biosynthesis and selective trafficking of pyrethrins precursors and STLs in opposite directions, and contributes to understanding the role of these flower-stored secondary metabolites in the immunization of the next generation against insect and fungal pathogens. Moreover, this study makes an important contribution towards understanding the biochemical and molecular bases of pyrethrins and STLs, the two most relevant bioactive compounds found in pyrethrum.

Samenvatting

Samenvatting

Het gebruik van botanische insecticiden is tegenwoordig een aantrekkelijk alternatief voor minder veilige en milieuonvriendelijke synthetisch-chemische middelen. De slechte afbreekbaarheid in het milieu, maakt die verbindingen niet alleen vervuilender, maar verhoogt ook de kans op de snelle ontwikkeling van resistentie in het plaaginsect en negatieve gevolgen voor nuttige insecten. Niettemin zijn er op dit moment slechts een gering aantal gewasbestrijdingsmiddelen van botanische oorsprong in de handel. Tussen die middelen (pyrethrines, rotenon, neem en essentiële oliën) hebben pyrethrines de langste en beste staat van dienst ten aanzien van effectieve inzetbaarheid tegen een groot aantal insecten zonder noemenswaardige toxiciteit voor zoogdieren. Alhoewel pyrethrines midden jaren 30 van de vorige eeuw verdrongen zijn van hun vooraanstaande marktpositie, hebben recente ontwikkelingen in de richting van pesticiden met een verminderd risicoprofiel en de introductie van gewasresistenties, natuurlijke pyrethrines weer onder de aandacht van ondernemers en onderzoekers gebracht. Pyrethrines vormen het hoofdbestanddeel van een niet-vluchtige olie die uit gedroogde margriet-achtige bloemen van pyrethrum (*Tanacetum cinerariifolium*) geëxtraheerd wordt. De actieve bestanddelen zijn 6 esterverbindingen die gevormd worden uit de combinatie van twee zuren (chrysanthemyl en pyrethryl zuur) en drie alcoholen (pyrethrolone, cinerolone en jasmolone, gezamenlijk rethrolonen genoemd). De esters van chrysanthemyl zuur met rethrolonen heten type I pyrethrines, en de esters van pyrethryl zuur met rethrolonen, type II pyrethrines. Pyrethrum produceert naast pyrethrines ook een scala aan andere afweerstoffen die sesquiterpeen lactonen genoemd worden, en die eveneens een rol toegedicht wordt in de afweer van planten tegen herbivoren, pathogenen en ondergrondse wortelcompetitie met andere planten. Alhoewel pyrethrines en sesquiterpenen in alle bovengrondse delen aangetroffen worden, worden de hoogste concentraties van beide verbindingen gevonden in de vruchten of nootjes (zaden in de volksmond) van de bloemen. De zaden zijn dicht bezet met klierhaartjes. GC-MS analyse daarvan toonde aan dat klierharen van rijpe zaadjes sesquiterpeen lactonen en andere secundair metabolieten bevatten, maar geen pyrethrines. Van klierharen is bekend dat ze actief zijn in de productie van mono- en sesquiterpenen die opgeslagen worden in, of afgegeven worden door, de sub-cuticulaire ruimte buiten de celwand van de apicale cellen. In dit proefschrift toon ik echter aan dat deze stoffen ook worden afgegeven aan de plant zelf, in basipetale richting. In pyrethrum wordt chrysanthemylzuur (CA), het monoterpenoide deel van het pyrethrinemolecuul, getransporteerd van de trichomen naar het pericarp, waar het wordt veresterd tot pyrethrines, die daar accumuleren in de intercellulaire ruimte. We laten ook zien dat de pyrethrines tijdens de zaadrijping

vervolgens geabsorbeerd worden door het zich ontwikkelende embryo. Bij de kieming van het zaad worden deze opgeslagen pyrethrines daarna gerecruteerd door de kiemende zaailing, die doordat hij in dat stadium nog geen trichomen heeft niet in staat is zelf afweermoleculen te produceren. Ik toon aan dat in dit jonge stadium sesquiterpeenlactonen op de zaadhuid de grond in diffunderen en net als pyrethrines in de zaailing waarschijnlijk eerder een rol vervullen tegen schimmels dan insecten.

Ondanks de grote vooruitgang die de afgelopen honderd jaar is geboekt ten aanzien van de chemie van pyrethrines, was bij de start van dit proefschrift de moleculaire/biochemische basis van hun biosynthese nog grotendeels onbekend. De zure en alcohol componenten van pyrethrines zijn afgeleid van twee volledig verschillende biosyntheseroutes. De alcohol component wordt verondersteld afgeleid te zijn van linoleenzuur en een groot deel van zijn biosyntheseroute te delen met jasmonzuur. De zure component daarentegen is afgeleid van een ongebruikelijke monoterpeen biosyntheseroute, die leidt tot monoterpenen met een cyclopropaanring. Voor de start van dit project was alleen van het chrysanthemyl difosfaat synthase (TcCDS) gen aangetoond dat het enzym de eerste stap in de biosynthese van CA kon uitvoeren door twee moleculen DMAPP te condenseren tot chrysanthemyl difosfaat. Tijdens het project werd daar door een andere groep een acyltransferase enzym (TcGLIP) aan toegevoegd dat verantwoordelijk was voor de esterficatie van (1R,3R)-chrysanthemoyl-CoA en (S)-pyrethrolone tot pyrethrine I. In dit proefschrift toon ik aan dat dit proces plaatsvindt in het pericarp.

Teneinde additionele pyrethrine biosynthesegenen te identificeren, maakten we drie gen transcript-bibliotheken van ovaria, trichomen en blad. Na annotatie, werden genkandidaten geïdentificeerd door te zoeken op basis van trefwoorden en op basis van homologie met bekende genen uit andere planten betrokken bij soortgelijke omzettingen. Gegeven de waarschijnlijkheid dat cytochroom P450 genen betrokken waren bij de ontbrekende stappen in de biosynthese van de zure component van pyrethrines, werden de pyrethrum transcriptbibliotheken eerst gescreend voor P450 genen (CYP) met een expressiepatroon dat vergelijkbaar was met *TcCDS*, en weefselspecifiek in CA-producerende klierharen. Experimenten met microsomen van gist maakte het mogelijk twee enzymen te selecteren die chrysanthemol konden omzetten in chrysanthemal. Agro-infiltratie van tabaksbladeren toonde vervolgens aan dat deze enzymen niet de achtergrondconcentraties CA konden beinvloeden, maar dat een van de twee (Ct21854) wel in staat was om de emissie van chrysanthemol sterk te verminderen en de concentratie van CA-conjugaat significant te verhogen. Dit toont aan dat Ct21854 chrysanthemol efficient omzet in chrysanthemal en mogelijk ook chrysanthemylzuur.

Rethrolon-verbindingen worden verondersteld afgeleid te zijn van linoleenzuur en een deel van de biosynthese route te delen met jasmonzuur. Dit impliceert dat het eerste enzym in de biosyntheseroute een lipoxygenase enzym moet zijn dat de hydroperoxidatie van linoleenzuur op positie 13 kan uitvoeren. Op basis van deze aanname werden de transcriptbibliotheken van pyrethrum met trefwoorden gescreened om LOX genen te vinden. De expressie van 25 lipoxygenase transcript contigs werd gekarakteriseerd en de genen met een expressiepatroon vergelijkbaar met *TcCDS* en *TcGLIP* werden geselecteerd. Vervolgens werden de complete cDNAs van deze genen geïsoleerd. Van recombinant TcLOX1 enzym kon vervolgens aangetoond worden dat het de peroxidatie van linoleenzuur op positie C13 kon uitvoeren. Het gen bleek bovendien hetzelfde expressiepatroon en weefselspecificiteit in trichomen te hebben als *TcCDS*. Dit suggereert dat trichomen mogelijk eveneens de productie en het transport van de alcoholcomponent van pyrethrines voor hun rekening nemen.

Pyrethrumplanten zijn naast pyrethrines ook een bron van sesquiterpeenlactonen. Alhoewel er een aanzienlijke hoeveelheid informatie beschikbaar is over de bioactiviteit en het industriele belang van pyrethrum sesquiterpeenlactonen, waren zowel de localisatie als de biosynthetische oorsprong van deze stoffen nog niet bekend. Zoals in andere leden van de Asteraceae familie, worden sesquiterpenen van pyrethrum uitsluitend opgeslagen in de trichomen, en is aangetoond dat germacratrien-12-oic acid (GAA) waarschijnlijk de centrale precursor is van alle bekende sesquiterpeenlactonen die in pyrethrum aangetroffen worden. Kandidaatgenen betrokken bij de eerste twee specifieke stappen van farnesyl difosfaat naar GAA werden in de pyrethrum transcriptbibliotheek geidentificeerd op basis van homologie, gecloneerd en gekarakeriseerd in gist en planten. Bovendien werd een gen gekarakteriseerd coderend voor een enzym dat de C6 hydroxylering van GAA kon uitvoeren. Deze hydroxylering resulteerde in een spontane lactonisering en dit enzym is daarom mogelijk verantwoordelijk voor de C6-C7 costunolide afgeleide sesquiterpeen lactonen die mogelijk voorkomen in pyrethrum. Het is echter ook mogelijk dat het enzym de hydroxylering op de C6 positie van de dominante groep van C7-C8 type sesquiterpeenlactonen uitvoert.

Concluderend leveren de beschreven resultaten van dit proefschrift nieuwe inzichten in de rol van pyrethrumtrichomen in de biosynthese en selectieve secretie van pyrethrine precursors en sesquiterpeen lactonen in tegengestelde richtingen. Het levert een bijdrage aan ons begrip van de rol van deze secundaire metabolieten die worden opgeslagen in de bloemen, maar die een rol hebben in de passieve bescherming van de volgende generatie tegen insecten en pathogenen. Bovendien levert dit onderzoek een belangrijke bijdrage aan ons begrip van de biochemische en moleculaire basis van de biosynthese

van pyrethrines en sesquiterpeen lactonen, de twee meest relevante bioactieve stoffen die in pyrethrum worden aangetroffen.

Acknowledgements

Acknowledgements

Rumor has it that this is the only part of the thesis that people actually read, so I honestly hope I did not forget to mention your name, valued contribution, and priceless support without which today I would not be finding myself writing these pages.

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My MSc thesis supervisor, Dr. Iris Kappers, I would like thank her for giving me the opportunity to work on a subject that was entirely new to me, which constituted the stepping stone to a new career path. I specially thank her for believing in me and supporting me during my application to this PhD position.

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bad, through happiness and sorrow, sickness and health. You are the true heroes of “The Netherlands” chapter of my life, chapter that would not have one single page if it were not for every single one of you. Gracias Totales, love you all!!

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Curriculum Vitae

List of Publications

Education Statement

Curriculum Vitae

Aldana Mariel Ramirez was born in Cordoba, Argentina on the 22nd of June 1979. After completing her secondary education, she initiated a degree in Biology at Universidad Nacional de Cordoba (UNC), Cordoba, Argentina. In 2002, after obtaining her MSc degree in Biology, Aldana moved to The Netherlands where she worked as a junior researcher for the biobased world leader in food preservation, Purac Biochem, Gorinchem, The Netherlands. After almost five years investigating the antimicrobial properties of a wide range of natural compounds against pathogenic microorganisms, which resulted in successful findings and a series of patents, Aldana decided to change the focus of her scientific career by engaging on a MSc program in Plant Biotechnology at Wageningen University, Wageningen, The Netherlands. The MSc program was completed in only one year and culminated with a thesis entitled "Potato Terpene Synthases in Relation to Different Herbivores", which was carried out at the Laboratory of Plant Physiology of the same university, under the supervision of Dr. Iris Kappers and Prof. Dr. Harro Bouwmeester. During her tenure at the Laboratory of Plant Physiology, a PhD position became available on the INterSECT project: "Development of innovative methods for selecting and engineering natural resistance to thrips in ornamental and vegetable crops" of the Foundation Technological Top Institute Green Genetics (TTI-GG). The interdisciplinary project involved investigators from different research groups of Wageningen University; Prof. Dr. Harro Bouwmeester from the Laboratory of Plant Physiology, Prof. Dr. Marcel Dicke from the Laboratory of Entomology and Dr. Maarten Jongsma from Plant Research International. Aldana was selected, and in January 2009 started her PhD research that aimed to elucidating and developing knowledge around the metabolic pathway of the bioactive constituents of pyrethrum flowers (*Tanacetum cinerariifolium*), pyrethrins and sesquiterpene lactones. The outcomes of her research can be found in this thesis. On the 1st of January of 2013, Aldana moved to Paris to start her education towards becoming a pastry chef at the world known culinary arts institute, Le Cordon Bleu.

List of Publications

Aldana M. Ramirez, Ting Yang, Harro J. Bouwmeester, Maarten A. Jongsma (2013). A trichome-specific linoleate 13S-lipoxygenase expressed during pyrethrin biosynthesis in pyrethrum. Lipids. Submitted.

Aldana M. Ramirez, Nils Saillard, Ting Yang, Maurice C.R. Franssen, Harro Bouwmeester, Maarten Jongsma (2013). Biosynthesis of Sesquiterpene Lactones in Pyrethrum (*Tanacetum cinerariifolium*). PlosOne. Submitted.

Aldana M. Ramirez, Francel W.A. Verstappen, Ric C.H. de Vos, Liping Gao, Harro J. Bouwmeester, Maarten A. Jongsma (2013). Novel enzyme capable of converting chrysanthemol into chrysanthemic acid. Patent filed.

Aldana M. Ramirez, Geert Stoopen, Tila R. Menzel, Rieta Gols, Harro Bouwmeester, Marcel Dicke, Maarten Jongsma (2012). Bidirectional secretions from glandular trichomes of pyrethrum (Tanacetum cinerariifolium) enable immunization of seedlings. The Plant Cell. 24(10): 4252-65.

Ramirez A., Bontenbal E., Kremer D. and R. Otto (2009). Preservation of acidic beverages. International patent number: WO2009/037274A1

Ramirez A., Kremer D. and R. Otto (2009). Use of fatty acid esters of glycerol combined with polylysine against gram-negative bacteria. International patent number: WO2009/037270A1, and United States patent number: US2009/0082443A1

Ramirez A., Kremer D. and R. Otto (2009). New antimicrobial agents based on fatty acid esters of hydroxy carboxylic acids. International patent number: WO2009/037269A1 and United States patent number: US2009/0082253A1

Ramirez A and R. Otto (2009). Antimicrobial Preparations. International patent number: WO2007/113333.

Ramirez A., Pérez G.T., Ribotta P. and León A.E. (2002) The occurrence of friabilin in triticale and its relationship with grain hardness and baking quality. Journal of Agricultural and Food Chemistry, 51(24) 7176-81.

Education Statement

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Aldana Ramirez
Date: 3 May 2013
Group: Plant Physiology, Wageningen University & Research Centre
Wageningen, The Netherlands

1) Start-up phase	date
► **First presentation of your project**	
Development of innovative methods for selecting and engineering natural resistance to thrips in ornamental and vegetable crops	Apr 08, 2009
► **Writing or rewriting a project proposal**	
Development of innovative methods for selecting and engineering natural resistance to thrips in ornamental and vegetable crops. The Jasmonic Acid Biosynthetic pathway	Sep 29, 2008
► **Writing a review or book chapter**	
► **MSc courses**	
MIB-31806 Systems Biology	Mar-Apr, 2009
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*11,5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day, Leiden University	Feb 26, 2009
EPS PhD student day, Utrecht University	Jun 01, 2010
EPS PhD student days, Wageningen University	May 20, 2011
► **EPS theme symposia**	
EPS Theme 3 Symposium 'Metabolism and Adaptation', University of Amsterdam	Feb 18, 2009
EPS Theme 2 Symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 15, 2010
► **NWO Lunteren days and other National Platforms**	
ALW Meeting Experimental Plant Science-Lunteren	Apr 19-20, 2010
ALW Meeting Experimental Plant Science-Lunteren	Apr 04-05, 2011
ALW Meeting Experimental Plant Science-Lunteren	Apr 02-03, 2012
► **Seminars (series), workshops and symposia**	
5th Plant-Insect Interaction workshop	Nov 11, 2010
TTI Green Genetics networking workshop	Sep 22, 2010
Seminar Series Plant Sciences: Chairs Entomology and Plant Cell biology	Jan 12, 2010
Seminar Series Plant Sciences: Chairs Entomology and Virology	May 11, 2010
Seminar: Genetics and Genomics of Disease resistanse in letuce. Prof. Richard Michelmore	Jun 03, 2010
► **Seminar plus**	
► **International symposia and congresses**	
Terpnet 2011, Linnaeus University, Kalmar, Sweden	May 22-26, 2011
► **Presentations**	
Poster: TTI Green Genetics Workshop, 'Unveiling and engineering the biosynthetic pathway leading to pyrethrins: a potent botanical insecticide'	Sep 22, 2010
Poster: Terpnet 2011, 'Biological function and biosynthesis of sesquiterpene lactones in pyrethrum'	May 22-26, 2011
► **IAB interview**	Feb 19, 2011
► **Excursions**	
Subtotal Scientific Exposure	*8,6 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
PhD summer school on Environmental Signaling, Utrecht University	Aug 24-26, 2009
Metabolomics Course, Leiden University	Apr 12-16, 2010
Bioinformatics, a user's approach, Wageningen University	Aug 30-Sep 03, 2010
► **Journal club**	
Member of a literature discussion group	2009 - 2013
► **Individual research training**	
Subtotal In-Depth Studies	*6,9 credits**

4) Personal development	date
► **Skill training courses**	
Guide to digital Scientific Artwork	Apr 20-21, 2009
Information Literacy including EndNote introduction	Feb 09-10, 2010
Dutch I	Mar 06 - July 05, 2012
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3,2 credits**

TOTAL NUMBER OF CREDIT POINTS*	30.2

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study*

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