Biosynthesis of monoterpenes, derivatives, and conjugates in plants
Roles in resistance to western flower thrips

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Biosynthesis of monoterpenoid alcohols, derivatives and conjugates in plants
Roles in resistance to western flower thrips

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Contents

Chapter 1  General introduction 3

Chapter 2  Chrysanthemum expressing a linalool synthase gene smells good, but tastes bad to western flower thrips 17

Chapter 3  Expression of geraniol synthase in maize results in accumulation of geranoyl-6-O-malonyl-β-D-glucopyranoside and other alterations in the leaf metabolome 31

Chapter 4  Pyrethrins protect pyrethrum leaves against attack by western flower thrips, Frankliniella occidentalis 55

Chapter 5  Chrysanthemyl diphosphate synthase operates in planta as a bifunctional enzyme with chrysanthemol synthase activity 67

Chapter 6  Chrysanthemic acid:Coenzyme A ligase in pyrethrum—gene cloning and analysis of recombinant enzyme 85

Chapter 7  General discussion 99

Summary 107

Samenvatting 109

Acknowledgements 111

Curriculum vitae 113

Publication list 114

Education statement 115
Chapter 1

General introduction

Ting Yang
1. Western flower thrips as crop pest

Western flower thrips (WFT), *Frankliniella occidentalis*, is a highly polyphagous insect and a vector of several plant viruses of which the Tomato Spotted Wilt Virus and the Impatiens Necrotic Spot Virus are the most important. As a result, WFT has become the most serious pest in several vegetable and flower crops (Daughtrey et al., 1997; Reitz, 2009). It causes damage on a variety of outdoor crops such as tomatoes, lettuce, celery, peppers, peas, onions, apples, grapes, peanuts (Robb, 1989) and maize (Heinrichs et al., 2000) and in greenhouse vegetable and flower crops, including tomatoes, sweet pepper, cucumber, chrysanthemum, rose, impatiens, ivy geranium, petunia, gloxinia, orchids, dahlia, primula, gerbera, fuchsia, and African violet (van Driesche et al., 1999).

The life cycle of western flower thrips consists of an egg, two larval instars, the prepupa, the pupa, and the adult stage (Fig. 1). Both the adult and the larval stages feed by using their mouthparts to pierce plant cells and suck up the contents. Damaged plant cells collapse or fill with air, resulting in “silvery patches” and flecking on expanded leaves (Tommasini and Maini, 1995). Thrips feeding also results in stunted plant growth, and flower and fruit deformation. Oviposition by females causes another type of damage. Females insert eggs under the plant’s epidermis with their saw-like ovipositor, resulting in spots on leaves, petals and fruits (Lewis, 1997). The eggs are fairly well protected and few pesticides are effective against them (McDonough et al., 1999). In addition, plant viruses transmitted by WFT also cause serious economic losses (Reitz, 2009).

Many synthetic pesticides have been used to control WFT. However, their widespread use has led to increasing resistance against the major classes of synthetic insecticides (Broadbent and Pree, 1997; Broughton and Herron, 2009). Therefore, it is desirable to identify natural plant-derived compounds which are effective against WFT to allow breeders to enhance the levels of these compounds in different crop species. Modification in the quantities of these compounds through metabolic engineering has the potential to enable more sustainable agricultural practices by decreasing the use of synthetic insecticides. Besides pesticides, biological control has also been used to control WFT populations, including the use of predatory mites (Sabelis and van Rijn, 1997; Messelink et al., 2006; Wimmer et al., 2008), predatory bugs (Riudavets and Castañé, 1998; Weintraub et al., 2011), fungi (Montserrat et al., 1998; Niassy et al., 2012), and nematodes (Heinz et al., 1996). In addition, host plant resistance has been studied aiming to identify cultivars that are resistant to WFT feeding or oviposition (Fery and Schalk, 1991; De Jager et al., 1995) or to plant viruses transmitted by thrips (Funderburk, 2011). This host resistance may be based on the presence of repelling or deterring metabolites and knowledge of the identity of these chemical resistance factors would further support breeding for WFT-resistance in crop plants. A chemical class of plant metabolites that has been studied frequently in relation to plant resistance to insects are the terpenoids and particularly the monoterpenoids.

2. Monoterpenoids

2.1 Biological functions of monoterpenoids

Monoterpenoids are known as constituents of floral scents and plant resins (Loza-Tavera, 1999). They find extensive industrial application as additives in perfumes, cosmetics,
flavoring agents, medicines and insecticides (Wise and Croteau, 1999; Holstein and Hohl, 2003). Besides their economic value, they have also been found to possess ecological significance. As important constituents of floral scents, monoterpenes function to attract pollinators (Dudareva and Pichersky, 2000). Some floral monoterpenes are also involved in antimicrobial defence, as was shown for linalool and linalool oxide in flowers of *Clarkia* species (Pichersky et al., 1994; Dudareva et al., 1996). In response to herbivory, plants emit blends of volatile compounds which are usually dominated by monoterpenes and sesquiterpenes (Dicke, 1994; Degenhardt et al., 2003). These compounds are involved in direct defence by repelling herbivores, and indirect defence by attracting predators or parasitoids (Paré and Tumlinson, 1999; Dicke and Van Loon, 2000; Dicke and Baldwin, 2010; Clavijo McCormick et al., 2012). For example, among the herbivore-induced volatiles in *Nicotiana attenuata*, the emission of three compounds, including linalool, increased egg predation rates by a generalist predator while at the same time linalool and the complete blend decreased oviposition rates of the herbivore lepidopteran *Manduca sexta* (Kessler and Baldwin, 2001). Furthermore, monoterpenes may play a role in protecting plants against oxidative stress from ozone (Fares et al., 2008). The significance of plant monoterpenes has promoted research on their biosynthesis and the metabolic engineering of their production in plants (Aharoni et al., 2005; Tholl, 2006).

### 2.2 Biosynthesis of monoterpenes

Monoterpenes are predominantly biosynthesized via the methyl erythritol phosphate (MEP) pathway, which is responsible for the production of the two building blocks of terpenes, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), in plant plastids (Fig. 2) (Tholl, 2006). They exist as hydrocarbons or as oxygenated moieties with alcohol, aldehyde/ketone and ester functional groups. Most monoterpenes are produced from geranyl diphosphate (GPP), which is the head-to-tail condensation product of DMAPP and IPP, and these monoterpenes are termed “regular monoterpenes”. The “irregular monoterpenes” are synthesized through non-head-to-tail condensation of two units of DMAPP (Fig. 3) (Thulasiram et al., 2007).

Many different terpene synthases (TPS) have been identified and cloned during the past two decades (Mahmoud and Croteau, 2002; Cheng et al., 2007). Based on protein homology, terpene synthases (TPS) have been divided into 7 subfamilies defined by a minimum of 40% identity between members, and were designated *TPS-a* through *TPS-g* (Bohlmann et al., 1998; Dudareva et al., 2003; Falara et al., 2011). The monoterpane synthases (MTS) are distributed in the subfamilies *TPS-b*, *TPS-d* and *TPS-g* (Bohlmann et al., 1998; Cheng et al., 2007). All
MTS contain N-terminal transit peptides of 50-70 amino acids for plastid targeting (Bohlmann et al., 1998; Dudareva et al., 2003; Martin et al., 2004). All terpene synthases share a common aspartate-rich DDxxD motif which is thought to be involved in the coordination of divalent metal ions for substrate binding (Lesburg et al., 1997). Cloning and characterization of MTS has enabled engineering of monoterpane metabolism in plants, with the objective to improve plant traits, such as plant fragrance or plant resistance against pests (Lange and Ahkami, 2012).

2.3 Metabolic engineering of monoterpenes by MTS in transgenic plants

With the increase in understanding of the biosynthetic pathways of monoterpenes, successful metabolic engineering approaches using MTS have been developed (Lange and Ahkami, 2012). From the large variety of cloned MTS genes, some have been transformed into plants to study the fate of newly expressed monoterpenes and the impact of the metabolic changes on plant traits (Yu and Utsumi, 2009).

2.3.1 Metabolic changes in volatile emission

As monoterpenes are volatiles, the headspace of transgenic plants is normally analyzed for product presence. Often, however, not only the emission of direct products of specific MTS was increased, but also the emission of some other volatiles. For example, limonene synthase was expressed in Mentha arvensis and M. × piperita, and apart from limonene the amounts of two limonene derivatives, pulegone and piperitenone, were significantly increased as well (Diemer et al., 2001). In another example, when geraniol synthase was overexpressed in tomato fruits, not only the content of geraniol increased, but also the content of 11 geraniol derivatives and five unrelated monoterpenes increased (Davidovich-Rikanati et al., 2007). Similar results have been reported for the effect of limonene synthase overexpression in tobacco (Lücker et al., 2004) and lavender (Muñoz-Bertomeu et al., 2008) and linalool synthase overexpression in tomato fruits (Lewinsohn et al., 2001), carnation flowers (Lavy et al., 2002), Arabidopsis leaves (Aharoni et al., 2003) and potato leaves (Aharoni et al., 2006).

2.3.2 Metabolic changes in non-volatile production

Terpenes produced in transgenic plants have also been reported to be conjugated to non-volatile products (Lücker et al., 2001; Aharoni et al., 2003). As (mono)terpene glycosides are widely distributed in plants, it is not unexpected to find glycosides of the introduced monoterpenes in transgenic plants. Glycosylation renders the monoterpenes non-volatile. Thus, it is necessary to analyse the non-volatile metabolites to understand the metabolic fate of the overexpressed monoterpenes. The first glycoside in transgenic plants overexpressing a MTS was reported by Lücker and co-authors (2001). They found that in transgenic petunia expressing linalool synthase, all linalool accumulating in various tissues of the plant was stored as a non-volatile glycoside. Later, the glucosides of linalool and hydroxyl linalool have also been reported in linalool-expressing Arabidopsis and potato leaves (Aharoni et al., 2003; Aharoni et al., 2006).

2.3.3 Impact of metabolic changes on plant traits

Several transgenic plant species with enhanced monoterpane production have been tested for effects on flavour or fragrance to human panels and plant resistance against pest insects. In tomato fruits overexpressing geraniol synthase, a majority of untrained taste panellists preferred the transgenic fruits over controls (Davidovich-Rikanati et al., 2007). However, in the case of carnation flowers overexpressing linalool synthase, the emission of linalool by the transgenic flowers was too low to lead to detectable changes in flower scent for human olfaction (Lavy et al., 2002). Linalool overexpressing Arabidopsis has been tested for resistance to the pest aphid, Myzus persicae, in dual-choice assays, and transgenic plants
significantly repelled or deterred the aphids (Aharoni et al., 2003).

Besides plant fragrance and plant resistance to insects, plant development is also often affected by MTS overexpression. For example, slower plant growth and lighter leaf colour have been reported as a result of linalool synthase expression in Arabidopsis and potato (Aharoni et al., 2003; Aharoni et al., 2006), and upon geraniol synthase expression tomato fruits failed to develop the deep red colour of the control fruits as the lycopene level, a C40 terpenoid produced in plastids, decreased by about 50% (Davidovich-Rikanati et al., 2007). However, overexpressing monoterpenes does not always lead to altered morphological phenotypes. No changes in development were observed in the study of limonene synthase expressing tobacco (Lücker et al., 2004) and lavender (Muñoz-Bertomeu et al., 2008) as well as linalool expressing petunia (Lücker et al., 2001) and tomato (Lewinsohn et al., 2001).

In summary, plants have been successfully metabolically engineered to produce monoterpenes resulting in plants with an altered phenotype and new traits. As yet there is however little control over the level of the transgenic terpenoids produced for example because endogenous enzymes can take the product as a substrate for further conversions and plant development is affected in some cases. Further studies on regulatory features, such as transcription factors, multiple gene clusters and tissue-specific synthesis and storage could probably improve the success of metabolic engineering of monoterpenes in plants (Jirschitzka et al., 2012).

2.4 Several monoterpenes serve as potential deterrent or repellent molecules to WFT

In this thesis, several monoterpenes were chosen to be studied for their potential deterrent or repellent effect on WFT. Linalool is interesting as transgenic Arabidopsis emitting linalool were deterrent to aphids (Aharoni et al., 2003). Geraniol is potentially relevant as it is known to be repellent to insects including mites and ticks (Chen and Viljoen, 2010). Pyrethrins are the most fascinating class of monoterpenoids for WFT control. They have long been used as broad spectrum insecticides and are known to also affect WFT (Casida, 1973). The genes involved in the biosynthesis of linalool (Pichersky et al., 1995; Jia et al., 1999) and geraniol (Iijima et al., 2004; Bantscheff et al., 2007) have been cloned from several plant species, however the biosynthetic pathway of pyrethrins still needs more studies.
3. Pyrethrins-the natural insecticide

3.1 Chemistry of pyrethrins

Pyrethrins comprise a group of 6 closely related esters, derived from two cyclopropane carboxylic acids, chrysanthemic acid (1) and pyrethric acid (2), and three cyclopentanone alcohols, pyrethrolone (3), jasmolone (4) and cinerolone (5) (Fig. 4). The 3 type I esters, pyrethrin I (6), jasmolin I (7) and cinerin I (8) are derived from chrysanthemic acid, while the 3 type II esters, pyrethrin II (9), jasmolin II (10) and cinerin II (11) are derived from pyrethric acid.

Pyrethrins are found in all aboveground parts of pyrethrum (*Tanacetum cinerariifolium*), but predominantly in the ovaries of the flower heads (Brewer, 1973). The concentration of pyrethrins is about 0.1-0.2% (dry weight) in leaves and 1-2% (dry weight) in flowers (Baldwin et al., 1993). Pyrethrin production increases with the development of flowers (Head, 1966). This increase is much more rapid in the first four developmental stages than in the later stages (Fig. 5), suggesting that the enzymes involved in pyrethrin biosynthesis are particularly active in the first four stages when the individual disc florets are still opening.

![Chemical structures of pyrethrins and their acid and alcohol moieties](Matsuda, 2012).

**Fig. 4.** Chemical structures of pyrethrins and their acid and alcohol moieties (Matsuda, 2012).
3.2 Properties of pyrethrins as insecticide

Pyrethrins are the economically most important natural insecticides with broad use in homes, agriculture and stored products for more than 150 years (Casida, 1973). They are neurotoxins and they bind to voltage-gated sodium channels of neuronal cells, causing the channels to remain open (Davies et al., 2007). They can repel, “knock-down” (paralyse flying insects), and kill many different kinds of insects, including crop pests, stored food pests and household pests. Pyrethrins are effective against a broad spectrum of insects and their toxicity for mammals is very low allowing use as a preharvest spray and application during the blooming period (Casida and Quistad, 1995; Schoenig, 1995). All these properties make pyrethrins an ideal organic insecticide. Despite the long historical use of pyrethrins as insecticide, their ecological role in pyrethrum has not been uncovered yet.

3.3 Biosynthesis of pyrethrins

3.3.1 Biosynthesis of acid moieties

Both chrysanthemic acid and pyrethric acid share the cyclopropane ring as a common structural feature. Chrysanthemol is generally considered to be formed first, and successively oxidized to chrysanthemic acid (Donia et al., 1973). The carbon skeleton of chrysanthemic acid was first proposed to be built from two isoprene units (Crowley et al., 1961). To prove this hypothesis, $^{14}$C-labelled mevalonic acid (MVA) was fed to pyrethrum flowers and it was shown to be incorporated into the acid moieties of pyrethrins (Crowley et al., 1961; Crowley et al., 1962). This work confirmed that the acids are monoterpenoids. Forty years after this feeding experiment, the first gene in the pathway was cloned from pyrethrum flowers—chrysanthemyl diphosphate synthase (CDS) (Rivera et al., 2001). CDS is responsible for the first committed step of chrysanthemic acid biosynthesis. It catalyses the condensation of two molecules of DMAPP via an unusual c1'-2-3 linkage to give chrysanthemyl diphosphate (CPP) which has a cyclopropane ring structure (Rivera et al., 2001). The sequence analysis of CDS showed that it has a putative plastidic targeting sequence. In plant plastids, monoterpenes are mostly derived from the MEP pathway (or “non-MVA pathway”), which was discovered around 35 years later than the feeding experiment described above (Rohmer et al., 1996; Dubey et al., 2003). To elucidate from which of these two pathways pyrethrins are derived, Matsuda and co-workers (2005) used $^{13}$C-labelled glucose that they fed to pyrethrum seedlings. They showed that chrysanthemic acid is predominantly derived from the MEP pathway instead of the MVA pathway. The $^{14}$C-labelled MVA incorporation into the acid moieties found in the earlier feeding experiments must hence be regarded as crosstalk.
between the MEP and MVA pathways (Matsuda et al., 2005).

Pyrethric acid is the methyl ester of chrysanthemic dicarboxylic acid. $^{14}$C-Labelled chrysanthemic acid was fed to dissected ovaries, and it was found to be incorporated into both type I and II pyrethrin esters, thus indicating that chrysanthemic acid serves as precursor for pyrethric acid biosynthesis (Donia et al., 1973). Chrysanthemic acid is converted to pyrethric acid through oxidation and methyl esterification. The methyl is transferred from L-methionine by carboxyl methyltransferase (Crowley et al., 1962; Godin et al., 1963). Pyrethric acid may be formed late in a conversion of type I into type II pyrethrins, because the ratio of pyrethrin I to pyrethrin II decreases during flower development and pyrethric acid has not been described as a free compound (Casida and Quistad, 1995).

So far, CDS is the only published gene in the biosynthesis of the acids. For the biosynthesis of chrysanthemic acid, the genes involved in the following steps still need to be discovered—the conversion from CPP to chrysanthemol and the oxidation from chrysanthemol to chrysanthemic acid.

3.3.2 Biosynthesis of alcohol moieties

The three alcohols, pyrethrolone, cinerolone and jasmolone, share a common cyclopentanone structure. They have been shown to be derived from acetate when $^{13}$C-labelled acetate was fed to pyrethrum flowers (Crowley et al., 1961). They were predicted to be derived from the oxylipin pathway (Crombie, 1995), which is responsible for the biosynthesis of the plant hormone jasmonic acid (Feussner and Wasternack, 2002). Later, a feeding experiment using $^{13}$C-labelled glucose further supported this prediction, and the alcohols were proposed to be derived from either cis-jasmone or 7-hydroxy-jasmonic acid (Matsuda et al., 2005). However, the exact biosynthetic route of these alcohols remains to be clarified, and none of the genes involved in the biosynthesis of the pyrethrin precursor-alcohols have been reported.

3.3.3 Biosynthesis of esters

The final step in pyrethrin biosynthesis is the esterification of the acid and alcohol moieties. Currently, there are three different mechanisms to synthesize esters in plants (Fig. 6). In the first one, an acid group activated by coenzyme A (CoA) is transferred to an alcohol (Hobbs et al., 1999; D'Auria et al., 2002; Beekwilder et al., 2004). In the second mechanism, an activated alcohol group (e.g., S-adenosyl methionine) is transferred to an acid (Ibrahim, 1997; Seo et al., 2001). A third option is that an acid group is transferred from an ester to an alcohol to form a new ester (Banas et al., 2005). Pyrethrins are most likely synthesized through the first mechanism, which involves an acyltransferase. Recently, an acyltransferase was cloned from pyrethrum plants, which is capable of transferring the chrysanthemoyl group from the CoA thioester to pyrethrolone to produce pyrethrin I (Kikuta et al., 2012). This enzyme is also able to use CoA-activated pyrethric acid and jasmolone and cinerolone as substrates. The enzyme activity with different acyl-CoA and alcohol combinations varied from 39 to 129%, with 100% representing the activity of the enzyme with chrysanthemoyl-CoA and pyrethrolone. However, it is not clear yet whether the esters are synthesized by independent or sequential steps, or whether the alcohol/acid side chain modifications are completed before or after esterification. The CoA ligase(s) responsible for biosynthesis of the acyl-CoA thioesters also remains to be cloned.
In summary, in the pyrethrin biosynthetic pathway only the enzymes of the first committed step in the acid biosynthesis and the last step of ester biosynthesis have been characterized, leaving the other enzymes to be discovered (Fig. 7).

\[
\begin{align*}
R_1 & \text{O} - S - \text{CoA} + R_2 - \text{OH} \xrightarrow{\text{Acyltransferase}} R_1 - \text{O} - R_2 + \text{CoA} \\
R_1 & \text{OH} + \text{S-adenosyl-L-methionine} \xrightarrow{\text{Carboxyl methyltransferase}} R_1 - \text{O} - \text{CH}_3 + \text{S-adenosyl-L-homocysteine} \\
R_1 - \text{O} - R_2 + R_3 - \text{OH} \xrightarrow{\text{Acyltransferase}} R_1 - \text{O} - R_3 + R_2 - \text{OH}
\end{align*}
\]

**Fig. 6.** General mechanisms to synthesize esters in plants. CoA, coenzyme A; ATP, adenosine triphosphate; AMP, adenosine monophosphate.

### 4. Scope of this thesis

The objective of the research presented in this thesis is to increase the production of monoterpenes that are known to affect insects to improve plant resistance to crop pests such as WFT through metabolic engineering. Monoterpenes are known to play roles in plant resistance against herbivores and have been used before to engineer plant species (Jirschitzka et al., 2012). However, transgenic plants with elevated levels of monoterpenes have rarely been tested for their resistance against insects. Surprisingly, even the most well-known natural monoterpene-derived pesticides — pyrethrins, derived from the monoterpene chrysanthemol, still await a better understanding of the biosynthetic pathway.

**Chapter 2** describes the effect of linalool synthase overexpressing chrysanthemum on WFT. WFT were significantly deterred by the content of leaf discs from transgenic plants. However, the volatiles from leaves of transgenic plants were significantly attractive to WFT. The metabolic changes in volatile and non-volatile profiles of plants were analysed to correlate these to the behaviour of the insects. This study demonstrates complex, combined positive and negative, effects of terpene engineering on plant resistance against herbivores.

**Chapter 3** describes the effect of geraniol synthase overexpressing maize on WFT. In this chapter, the focus is on the metabolic fate of geraniol in maize to explain why no effect on WFT behaviour was observed. Geraniol produced in transgenic maize was efficiently converted to non-volatile glycoside, geranoyl-6-O-malonyl-β-D-glucopyranoside. The results demonstrate that metabolic engineering of geraniol into geranic acid can rely on the existing default substrate delivering pathways, but that competing glycosylation pathways must be controlled if accumulation of the aglycones is desired.
Chapter 4 presents the effect of pyrethrins against WFT, with regard to WFT survival, feeding behaviour, and reproduction. Besides the in vitro assays of pyrethrins against WFT adults, pyrethrins were also infiltrated into chrysanthemum leaves to test their potential role in plant defence against WFT. The results show that the effects of natural concentrations of pyrethrins in pyrethrum leaves can explain the observed high mortality of WFT on pyrethrum leaves.

Chapter 5 presents a second function of the published enzyme, chrysanthemyl diphosphate synthase (CDS), involved in the biosynthesis of the acid moieties of the pyrethrins. CDS has been reported to catalyse the formation of chrysanthemyl diphosphate (CPP); however, here it is demonstrated that CDS will also catalyse the next step of CPP into chrysanthemol both in vitro and in vivo. It is proposed that CDS should be renamed as a chrysanthemol synthase (CHS) using DMAPP as substrate.

Chapter 6 describes the discovery of chrysanthemic acid:CoA ligase, which is involved in the final stage of pyrethrin biosynthesis. The function of this enzyme is characterized in vitro.

Chapter 7 discusses the results from this thesis and integrates them in a wider perspective. Furthermore, some future perspectives are discussed.

**Fig. 7.** Biosynthetic pathway of pyrethrins. The enzymes already identified to be involved in biosynthesis are indicated with circles. Alternative routes are indicated with dashed arrows. DMAPP, dimethylallyl diphosphate; CPP, chrysanthemyl diphosphate; CDS, chrysanthemyl diphosphate synthase; P450, cytochrome P450 enzymes; CoA, coenzyme A.
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Chapter 1
Chapter 2

Chrysanthemum expressing a linalool synthase gene smells good, but tastes bad to western flower thrips

Ting Yang, Geert Stoopen, Gerrie Wiegers, Maarten A. Jongsma

Abstract
Herbivore-induced plant volatiles are often involved in direct and indirect plant defence against herbivores. Linalool is a common floral scent and found to be released from leaves by many plants after herbivore attack. In this study, a linalool/nerolidol synthase, FaNES1, was overexpressed in the plastids of chrysanthemum plants (*Chrysanthemum morifolium*). The volatiles of FaNES1 chrysanthemum leaves were strongly dominated by linalool, but, they also emitted small amount of the C11-homoterpenes, (3E)-4,8-dimethyl-1,3,7-nonatriene, a derivative of nerolidol. Several linalool glycosides were found to be significantly increased in the leaves of FaNES1 plants compared to leaves of wild-type plants. The top four were putatively identified by LC-MS-MS as two linalool-malonyl-hexoses, a linalool-pentose-hexose and a glycoside of hydroxy-linalool. A leaf-disc dual-choice assay with western flower thrips (WFT, *Frankliniella occidentalis*) showed that WFT were significantly deterred by leaf discs from FaNES1 plants 20 h after WFT release. However, in the first 15 min of WFT release, the FaNES1 plants were significantly attractive to WFT. The attractiveness of the odour of FaNES1 plants was confirmed by an olfactory dual-choice assay. Pure linalool at 10% concentration was similarly attractive to WFT. In summary, WFT were attracted by the smell, but over time deterred more by the taste of FaNES1 leaves. This study demonstrates complex effects of terpene engineering on plant resistance against herbivores. Considering the natural distribution of linalool and its glycosides in plant tissues, it suggests that flowers may balance their attractive fragrance with poor taste using the same precursor compound to optimize seed yield.
Effect of linalool chrysanthemum on thrips

Introduction

Plant volatiles play important roles in plant-insect interactions (Pichersky and Gershenzon, 2002; Schoonhoven et al., 2005; Maffei et al., 2011). Volatiles induced by herbivore attack are often found to be involved in direct defence by repelling herbivores, and indirect defence by attracting predators or parasitoids (Paré and Tumlinson, 1999; Dicke and Van Loon, 2000; Dicke and Baldwin, 2010; Clavijo McCormick et al., 2012). Herbivore-induced plant volatiles are usually dominated by mono- and sesquiterpenes (Degenhardt et al., 2003), and enhanced emissions of these terpenes have led to improved plant defence against herbivores (Turlings and Ton, 2006; Dudareva and Pichersky, 2008).

Linalool is a monoterpenic alcohol with a sweet fragrance occurring in the floral scent of a wide variety of plants (Kamatou and Viljoen, 2008). It is also reported to be induced in different plants by damage of a variety of herbivore species, suggesting that linalool may have a role in direct or indirect plant defence against several herbivores. For example, it is induced in crabapple damaged by Japanese beetles (Loughrin et al., 1995), cotton damaged by beet armyworms (Paré and Tumlinson, 1997), maize damaged by caterpillars (Turlings et al., 1998), *Nicotiana attenuata* damaged by caterpillars, leaf bugs or flea beetles (Kessler and Baldwin, 2001), peanut damaged by beet armyworms (Cardoza et al., 2002), spruce damaged by white pine weevils (Miller et al., 2005), lima bean damaged by caterpillars (Mithöfer et al., 2005) or spider mites (Dicke et al., 1990) and tobacco damaged by western flower thrips or caterpillars (Delphia et al., 2007). Constitutive high emission of linalool has been engineered in several transgenic plant species by overexpressing the linalool synthase gene (Lewinsohn et al., 2001; Lavy et al., 2002; Aharoni et al., 2003; Aharoni et al., 2006). Among these, linalool emitting Arabidopsis were found to be less attractive to aphids and diamondback moths than wild-type plants (Aharoni et al., 2003; Yang, 2008).

However, expression of monoterpenic alcohol synthase genes often results in the synthesis of an array of other volatile and non-volatile derivatives, which may also, or exclusively, be accountable for the observed effects on plant defence. Linalool synthase overexpressing plants have been found, for example, to emit, apart from linalool, also linalool oxide, hydroxy linalool or hydroxy-dihydrolinalool (Lewinsohn et al., 2001; Lavy et al., 2002; Aharoni et al., 2003; Aharoni et al., 2006). By contrast, transgenic petunia expressing linalool synthase, did not produce any volatile linalool or derivative, but instead efficiently converted linalool to a non-volatile glycoside (Lücken et al., 2001). The glycosides of linalool and hydroxy-linalool were also reported in Arabidopsis and potato overexpressing linalool synthase (Aharoni et al., 2003; Aharoni et al., 2006). Transgenic plants overexpressing a similar linear monoterpenic alcohol geraniol by means of a geraniol synthase, produced both volatile and non-volatile derivatives of geraniol such as geraniol (volatile), geranic acid (volatile), geranyl acetate (volatile) in tomato fruits (Davidovich-Rikanati et al., 2007), and geranoyl malonyl glucopyranoside and other derivatives (non-volatile) in maize leaves (Yang et al., 2011). In some cases these derivatives were not found in controls (wild-type or empty vector controls), but often they are also found there, and therefore can be taken to fulfil putative biological roles for the plant species involved. The emerging picture is that terpene derivatives are species and genotype specific and dependent on developmental and tissue- or cell-type specific variations in endogenous modifying enzymes.

Western flower thrips (WFT), *Frankliniella occidentalis*, is a highly polyphagous insect, and has become one of the most serious pests in several vegetable and flower crops world-wide (Reitz, 2009). It feeds by using its mouthparts to pierce plant cells and suck out their contents. Damaged plant cells collapse or fill with air, resulting in stunted plant growth, flower and fruit deformation, or “silver” reflective patches and flecking on expanded leaves (Tommasini and
Maini, 1995). It causes serious damage to a variety of vegetable and flower crops, including chrysanthemum, and transmits Tomato Spotted Wilt Virus (van Driesche et al., 1999).

In this study, the linalool/nerolidol synthase gene from strawberry (Aharoni et al., 2003), FaNES1, was introduced into chrysanthemum plants. Induced volatile and non-volatile metabolites were analysed by GC- and LC-MS, and the effects of linalool overexpression in chrysanthemum were tested against WFT behaviour.

**Results**

**Introduction and expression of linalool synthase gene into chrysanthemum**

Chrysanthemum genotype 1581 was transformed with a linalool/nerolidol synthase gene from strawberry (Aharoni et al., 2004), FaNES1, under the control of the Rubisco small subunit promoter (Ouchkourova, 2003). The FaNES1 protein was targeted to the plastids by fusion with a plastid-targeting signal. Wild-type plants were used as control. Expression levels of FaNES1 in cuttings of 2 T0 transgenic lines, line 28 and 37, were determined by quantitative RT-PCR. The transcript levels of FaNES1 in these two lines were similar ranging from 486 to 1275, relative to the household gene actin (Fig. 1). FaNES1 plants were shorter and lighter in leaf colour compared to wild-type plants (Fig. 2).

**GC-MS analysis of volatile compounds**

Volatile compounds were collected from the headspace of cut leaves at half height of the plants. Linalool, the primary product of FaNES1, was strongly dominant among the detected volatiles (Fig. 3, a-c). The linalool emission was quantified to be 0.33 to 2.91 µg·h⁻¹·g⁻¹-FW (Fig. 4), and not well correlated to the transcript levels of FaNES1 ($R^2=0.64$). Besides linalool, an acyclic C11-homoterpene, (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was also found to be emitted from FaNES1 plants but not from wild-type plants (Fig. 3, d-e). The peak area (total ion current) of DMNT was 30- to 70-fold smaller than that of linalool in transgenic plants.

**LC-MS analysis of non-volatile compounds**

As linalool could also be stored in the form of glycosides, we analyzed the non-volatile metabolites in transgenic (n=6) and control (n=3) plants. Aqueous methanol extracts from young leaves were prepared and analyzed by accurate mass LC-MS in negative mode (Fig. 5). In order to reveal differential compounds, the LC-MS profiles of transgenic plants and control plants were compared in an untargeted manner using Metalign followed by Multivariate Mass
Spectra Reconstruction (MMSR) clustering of extracted signals, as described in Materials and Methods.

In total, 8968 mass signals were extracted, which grouped into 301 clusters of different metabolites. Among all 8968 masses, 2482 masses (i.e. 28%, distributed in 80 clusters) showed at least 2-fold intensity difference ($P < 0.05$) between transgenic and control plants. More masses were found to be significantly increased (2312 masses, distributed in 74 clusters) than decreased (170 masses, distributed in 6 clusters) in the transgenic plants. Differential masses with a signal intensity higher than 500 (i.e. about 50-fold higher than the noise) were subsequently analyzed by LC-MS/MS (Table 1, Fig. 5, Fig. S1). According to their MS/MS spectra, all these compounds were putatively identified as derivatives of linalool and hydroxylinalool: 2 different types of linalool-malonyl-glucose, a linalool-pentose-glucose and a glycoside of hydroxy-linalool.

**Figure 3** GC-MS chromatograms obtained by dynamic headspace trapping of leaves of wild-type chrysanthemum plant Wt-1 (a) and transgenic chrysanthemum plant T37-3 (b). (c), GC-MS chromatogram of an authentic standard of racemic linalool. The product of FaNES1 was determined as (S)-linalool by Aharoni (2003). (d), zoom-in chromatogram of Wt-1 from 8.2 to 8.7 min. (e), zoom-in chromatogram of T37-3 from 8.2 to 8.7 min.

**Figure 4** Linalool emission of cut leaves of wild-type and transgenic chrysanthemum plants. Wt, wild-type; T, transgenic.
Chapter 2

Table 1 Non-volatile metabolites significantly increased in *FaNES1*-Expressing chrysanthemum plants putatively identified by LC-MS-MS.

<table>
<thead>
<tr>
<th>Ret (min)</th>
<th>Av intensity (Wt)</th>
<th>Av intensity (T)</th>
<th>Ratio (T/Wt)</th>
<th>Accurate mass found</th>
<th>Mol form</th>
<th>Δmass (ppm)</th>
<th>MS-MS fragments</th>
<th>Putative ID</th>
<th>MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>47.79</td>
<td>54</td>
<td>24579</td>
<td>457.7</td>
<td>803.3721</td>
<td>C_{19}H_{30}O_{9}</td>
<td>2.5</td>
<td>linalool-malonyl-glucose&lt;sup&gt;a&lt;/sup&gt; (2M-HJ)</td>
<td>linalool-malonyl-glucose&lt;sup&gt;a&lt;/sup&gt; (2M-HJ)</td>
<td>401.1812</td>
</tr>
<tr>
<td>46.07</td>
<td>2678</td>
<td>30161</td>
<td>11.3</td>
<td>803.3718</td>
<td>C_{19}H_{30}O_{9}</td>
<td>2.1</td>
<td>linalool-malonyl-glucose&lt;sup&gt;a&lt;/sup&gt; (2M-HJ)</td>
<td>linalool-malonyl-glucose&lt;sup&gt;a&lt;/sup&gt; (2M-HJ)</td>
<td>401.1812</td>
</tr>
<tr>
<td>37.46</td>
<td>3910</td>
<td>33651</td>
<td>8.6</td>
<td>493.2278</td>
<td>C_{22}H_{38}O_{12}</td>
<td>-1.4</td>
<td>447, 315, 233, 191, 161, 149, 131</td>
<td>493.2285</td>
<td></td>
</tr>
<tr>
<td>36.15</td>
<td>145</td>
<td>22625</td>
<td>155.7</td>
<td>505.2647</td>
<td>C_{22}H_{42}O_{11}</td>
<td>0.4</td>
<td>459, 415, 399, 331, 289, 161</td>
<td>(hydroxy-linalool-hexose&lt;sup&gt;a&lt;/sup&gt;)</td>
<td>505.2649</td>
</tr>
</tbody>
</table>

Significantly different metabolites between transgenic and wild-type plants with mass intensities higher than 500 were selected for analysis by LC-MS/MS.

Ret (min), retention time, in minutes; Av, average; Wt, wild-type control plants; T, transgenic plants; Ratio (T/Wt), ratio of mass signal between transgenic plants (T) and wild-type plants (Wt); Mol form, molecular formula of the metabolite; Δmass (ppm), deviation between the found accurate mass and real accurate mass, in ppm; Putative ID, putative identification of metabolite; MM, monoisotopic molecular mass of the metabolite; <sup>a</sup> The scheme of collision-induced MS/MS-fragmentation is shown in Fig S1; ()FA, formic acid adduct; *, hydroxy-linalool-hexose conjugate to a molecule with formula of C_{7}H_{14}O_{3}, such as hydroxyheptanoic acid, and etc.

Effects of transgenic plants on thrips behaviour

Leaves at similar leaf stage were randomly picked from plants of transgenic line 37, and used to study the effects of *FaNES1* plants on WFT behaviour. The results of repeated dual-choice assays showed that WFT were significantly deterred by *FaNES1* plants 20-28 h after WFT
release with 65% - 70% of WFT settling on wild-type leaf discs (Fig. 6). However, we also noticed that in the first 15 min of WFT release, the FaNES1 plants were significantly attractive to WFT, with 66% WFT initially settling on those leaf discs. We hypothesized that the modified fragrance- linalool emission- of the FaNES1 plants caused the initial attraction of WFT.

To test whether volatile cues determined the initial attraction, we assayed the choice of WFT by placing individual insects on a wire separating two leaf discs, and scoring their choice for either leaf when they left the wire. In this way we compared their response to olfactory cues from wild-type and FaNES1 chrysanthemum leaves. This assay demonstrated that WFT preferred the odour from FaNES1 chrysanthemum leaves (Fig. 7). This olfactory assay was repeated with similar results using test leaf discs from plants of another transgenic line, line 39. As linalool was the major compound in the volatile profile of FaNES1 chrysanthemum leaves, we also tested whether pure linalool dissolved in paraffin oil was attractive to WFT, and found that 10% linalool in paraffin oil was similarly significantly attractive to WFT (Fig. 7) [as demonstrated by Koschier and co-authors (2000)].

**Discussion**

In this study, we aimed to introduce resistance to western flower thrips (WFT) into chrysanthemum by genetic engineering of the emission of the monoterpene alcohol linalool from the aerial parts of the plant. A linalool/nerolidol synthase, FaNES1, was introduced in the plastids of chrysanthemum plants under the control of the Rubisco small subunit promoter. We observed that the FaNES1 plants were significantly attractive to WFT in the first 15 minutes, but in the next 24 hours gradually turned significantly deterrent. To explain these results, we checked the profile of both volatile and non-volatile metabolites in transgenic
plants, and concluded, after different WFT choice assays, that the FaNES1 chrysanthemum volatile emissions are attractive and the non-volatiles are deterrent.

Linalool was found to be released as the major volatile into the headspace of transgenic plants (Fig. 2). Besides linalool, a small amount of DMNT was also released by the transgenic plants, but not by the wild-type plants. In transgenic Arabidopsis overexpressing the same gene in mitochondria, DMNT was also detected, and it was shown to be derived from nerolidol (Kappers et al., 2005). In our transgenic chrysanthemum plants, we did not detect nerolidol emission. However, the DMNT emission indicated that also low levels of nerolidol are produced from presumably low levels of FPP in plastids of FaNES1 chrysanthemum. Besides linalool, hydroxy-linalool and dihydrolinalool were also emitted from FaNES1 Arabidopsis and FaNES1 potato (Aharoni et al., 2003; Aharoni et al., 2005), but those were not detected in the headspace of FaNES1 chrysanthemum.

WFT, as its name describes, in the adult stage, is preferentially a pollen-feeding and flower-inhabiting species. It responds to a number of odours which are commonly found in floral fragrances (Teulon et al., 1993). Linalool is present in the fragrance of many different flowers, and it was tested for the effect on WFT with a Y-tube olfactometer by Koschier and coauthors (2000). In their study, 10% linalool was significantly attractive to WFT, but 1% linalool showed no significant effect. Our results showed that WFT were initially significantly attracted to the volatiles from FaNES1 chrysanthemum leaves which were dominated by linalool with emission rate at 0.33-2.91 µg·h⁻¹·g⁻¹-FW.

The gradual change from attraction to deterrence suggests either an induced linalool-expression related deterrent accumulating over time in response to WFT damage, or the constitutive presence of a deterrent which over time WFT learns to avoid, despite the attractive smell. Already 1 hour after WFT release the attraction is no longer significant, suggesting that a constitutive deterrent may be present in linalool overexpressing plants. In transgenic plants overexpressing different linalool/nerolidol synthase genes, linalool and nerolidol were reported to be stored as glycosides as well (Lücker et al., 2001; Aharoni et al., 2003; Aharoni et al., 2006). The glycone was determined in linalool synthase expressing petunia as glucose (Lücker et al., 2001). In our previous study of transgenic maize expressing a geraniol synthase, which also produces a monoterpen alcohol, the glycone was determined as malonyl-glucose (Yang et al., 2011). In this study, several glycosides of linalool or hydroxy-linalool were putatively identified by LC-MS/MS. The linalyl-glucopyranoside, reported as the only glycoside of linalool in the linalool synthase expressing petunia, was not detected as the major linalool glycoside in FaNES1 chrysanthemum. Among the major linalool glycosides listed in Table 1, two glycosides showed the same molecular mass and mass spectrum. They could be isomers and they were identified as linalool conjugated to malonyl-glucose by comparing their mass spectra to that of geranoyl-6-O-malonyl-β-D-glucopyranoside, which was identified by NMR in geraniol synthase expressing maize. Another linalool glycoside was putatively identified as linalool conjugated to a pentose-glucose. Such a glycoside has been found to be naturally present in raspberry fruit as S-(+)-linalool 3-O-α-L-arabinopyranosyl-(1→6)-β-D-glucopyranoside (Pabst et al., 1991). A glycoside of hydroxy-linalool was also putatively identified in FaNES1 chrysanthemum. The hydroxy linalool glycosides have also been reported in FaNES1 Arabidopsis and potato, however the glycone parts were not determined (Aharoni et al., 2003; Aharoni et al., 2006).

Terpene glycosides are regarded as transport and storage forms of terpenes in plant tissues, and they have been recognized to play important roles as precursors of terpene release (Winterhalter et al., 1997). They may be involved in indirect plant resistance against insects by releasing terpene volatiles as signal compounds attracting predators and parasitoids upon
attack by herbivores, or they may be directly toxic to the herbivores (Zou and Cates, 1997; Pankoke et al., 2010). As linalool was attractive to WFT, we propose that the major glycosides stored in FaNES1 chrysanthemum may explain the deterrence against WFT. An induced linalool-related deterrence is only likely if novel linalool-modifying enzymes are induced by thrips damage, but this was not investigated by us. And this seems less likely considering the rapid loss of attractiveness after 15 minutes. Future research should confirm whether any one or a combination of these glycosides may indeed play a role in WFT deterrence and especially whether this strategy is found in nature. The FaNES1 gene was overexpressed using a Rubisco small subunit promoter and this can lead to glycoside products, such as the malonyl-glucose conjugated products, that are not found in specific species. However, it is interesting to note that a study of the natural distribution of linalool and its glycosides in several linalool-emitting plants showed that linalool glycosides accumulated much more in flowers than in leaves, and that linalool emission was only detected from flowers (Raguso Robert and Pichersky, 1999). Attraction of pollinators by emitted linalool and parallel deterrence of co-attracted herbivores by stored linalool glycosides may, therefore, represent an intricate tactic of flowers to balance attractive fragrance with poor taste to optimize seed yields using the same precursor compound.

**Experimental procedures**

**Plant materials**

The linalool/nerolidol synthase gene from strawberry (Aharoni et al., 2004), FaNES1, driven by the rubisco small subunit promoter from chrysanthemum (Ouchkourov et al., 2003), was cloned into ImpactVector1.1 (www.impactvector.com) and introduced into chrysanthemum plants (Chrysanthemum morifolium Ramat.) cv. 1581. The N terminus of FaNES1 was fused to the plastidic targeting signal derived from FvNES1 to direct FaNES1 from cytosol to the plastids (Aharoni et al., 2004). Wild-type chrysanthemum plants were used as control. Plants were grown in a greenhouse at 25±2 °C under long day conditions (16-h-light/8-h-dark photoperiod).

Two T0 transgenic plant lines 28 and 37 producing the highest levels of linalool and a wild-type control line were propagated by cuttings. Three clones of each plant line were used in the following experiments to determine the transcript levels of FaNES1, total volatiles and total non-volatiles of leaves.

**Transcript analysis**

The RNA transcript levels of FaNES1 were determined by real-time quantitative RT-PCR analysis as described (Schijlen et al., 2007). The actin gene from chrysanthemum was used as reference gene. The sequence of actin gene was obtained from GenBank accession AB205087. All primers were designed by the Beacon Designer software package (Palo Alto, CA, USA). For FaNES1, forward primer 5’-ATCGTCCTCAGCACGCAATCTCTC-3’ and reverse primer 5’- CAGCCTTTCATGTTCCTCTTAAGTAGC-3’ with an expected product size of 116 bp were used, and for the actin gene forward primer 5’- GGATTCTGGTGATGGTGTGAGTC-3’ and reverse primer 5’- GAATCTTCATCAACGCATCAGTC-3’ with an expected product size of 119 bp.

**Volatile analysis by GC-MS and non-volatile analysis by LC-MS**

The 7th leaf from the top of each plant with 14-15 leaves was harvested for headspace trapping. The volatiles were sampled for half an hour, and then analysed by GC-MS as described (Yang et al., 2011). The temperature program of the gas chromatograph was 60 °C for 2.5 min,
rising to 280 °C at 20 °C min\(^{-1}\) and 0.5 min at 280 °C. The mass spectrometer was set to scan from 35 to 300 m/z. The helium flow was constant at 1.0 mL min\(^{-1}\). Ionization potential was set at 70 eV.

For identification, the authentic standard of (R,S)-linalool (Fluka) was run under identical conditions. (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was identified by comparing mass spectra to the Wiley mass spectra library and by calculating the Kovats Index of each peak based on the retention time relative to alkane standards. Linalool emission from transgenic plants was quantified based on calibration curves with the authentic standard.

From the same plants, the 6th leaf from the top of each plant was harvested for non-volatile analysis. The non-volatiles were extracted and analysed by LC-MS as described (Yang et al., 2011).

**GC-MS and LC-MS data processing**

GC-MS data were acquired using Xcalibur 1.4 (Thermo Electron Corporation) and LC-MS data using MassLynx 4.0 (Waters). The data were then processed using MetAlign version 1.0 (www.metAlign.nl) for baseline correction, noise elimination and subsequent spectral data alignment (De Vos et al., 2007). The processing parameters of MetAlign for GC-MS data were set to analyse from scan number 168 to 1929 (corresponding to retention time 4.83 min to 13.62 min) with a maximum amplitude of 4 × 10^7. The parameters for LC-MS data were set to analyse from scan number 84 to 2600 (corresponding to retention time 1.61 min to 49.35 min) with a maximum amplitude of 35000.

In order to elucidate which mass signals originate from the same metabolite, all the detected masses were clustered by an in-house developed software package based on a Multivariate Mass Spectra Reconstruction (MMSR) approach (Tikunov et al., 2005). The mass signal intensities (expressed as peak height using MetAlign) obtained from transgenic plants and wild-type plants were compared using the Student’s \(t\)-test. Masses with a significant (p<0.05) intensity change of at least 2-fold were verified manually in the original chromatograms.

To annotate significantly different compounds, accurate masses were manually calculated, taking into account only those scans with the proper intensity ratios of analyte and lock mass [between 0.25- and 2 (Moco et al., 2006)], and elemental formulae generated within 5 ppm deviation from the observed mass. In addition, mass-directed LC-MS/MS experiments were performed on differential compounds. To obtain proper MS/MS spectra only molecular ions with signal intensities higher than 500 ion counts per scan were selected.

**Dual-choice behavioural studies with thrips**

A population of western flower thrips (WFT), *Frankliniella occidentalis*, was mass-reared on flowering chrysanthemum (*Chrysanthemum morifolium* Ramat.) cv. Sunny Casa in a greenhouse under a photoperiod of L16:D8 at 25±2 °C. In this study, only adult female thrips were used. All bioassays were conducted in a climate room at 20-22 °C with a L16:D8 photoregime as described (Yang et al., 2012).

Leaf discs from wild-type chrysanthemum plants were used as control discs and leaf discs from plants of transgenic line 37 or line 39 were used as test discs. Twelve replicates were used in this experiment. The number of WFT on each leaf disc was recorded 0.25, 1, 2, 4, 20, and 28 h after the release of the WFT. At each time point, a Student's paired \(t\)-test was used to assess the significance of the differences in the mean number of WFT between test and control. The plants of line 39 emitted lower amount of linalool than plants of line 37 and 28.
Thrips olfactory choice assay

To dissect the component of thrips host choice based on olfactory cues only, a metal wire (0.5 mm diameter, ~2.5 cm long) was placed between 2 leaf discs (1.6 cm diameter) embedded, abaxial side up, on a 1.5% (w/v) agar-bed in a Petri dish (7 cm diameter). One of the leaf discs was from wild-type chrysanthemum plants as control disc and the other leaf disc was from transgenic plants of line 37 as test disc. The metal wire was not in contact with any of the leaf discs, and there was about 0.5 cm distance between the end of the metal wire and the leaf disc. Every time, one ice-anaesthetized thrips was released in the middle of the metal wire. Once the thrips became active, it walked along the metal wire without going off it. After one or a couple of rounds of walking, the thrips would finally leave the wire at either end, and walk towards the leaf disc of choice. The number of thrips reaching either leaf disc was recorded. The only available cues were volatiles released from the leaf discs. Every pair of leaf discs was assayed with 10 individual thrips, and this experiment was replicated with 6 pairs of leaf discs. More than 90% WFT made their choices within 2 min in this assay. A Student's paired t-test was used to assess the significance of the differences in the mean number of WFT between test and control.

In the experiment checking the olfactory response of thrips to the linalool standard, WFT were given choices between filter papers (~1.5 cm²) applied with 10 µl paraffin oil or with 10 µl 10% linalool dissolved in paraffin oil.

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Figure S1 Scheme of collision-induced MS/MS-fragmentation of mass 401 eluting at 47.79 and 46.07 min (a), mass 447 eluting at 37.46 min and mass 459 eluting at 36.15 min.
Chapter 3

Expression of geraniol synthase in maize results in accumulation of geranoyl-6-O-malonyl-β-D-glucopyranoside and other alterations in the leaf metabolome

Ting Yang, Geert Stoopen, Nasser Yalpani, Jacques Vervoort, Ric de Vos, Alessandra Voster, Francel W. A. Verstappen, Harro J. Bouwmeester, Maarten A. Jongsma

Abstract

Many terpenoids are known to have antifungal properties and overexpression of these compounds in crops is a potential tool in disease control. In this study, 15 different mono- and sesquiterpenoids were tested \textit{in vitro} against two major pathogenic fungi of maize (\textit{Zea mays}), \textit{Colletotrichum graminicola} and \textit{Fusarium graminearum}. Among all tested terpenoids, geranic acid showed very strong inhibitory activity against both fungi (MIC < 46 µM). To evaluate the possibility of enhancing fungal resistance in maize by overexpressing geranic acid, we generated transgenic plants with the geraniol synthase gene cloned from \textit{Lippia dulcis} under the control of a ubiquitin promoter. The volatile and non-volatile metabolite profiles of leaves from transgenic and control lines were compared. The headspaces collected from intact seedlings of transgenic and control plants were not significantly different, although detached leaves of transgenic plants emitted 5-fold more geranyl acetate compared to control plants. Non-targeted LC-MS profiling and LC-MS-MS identification of extracts from maize leaves revealed that the major significantly different non-volatile compounds were 2 geranic acid derivatives, a geraniol dihexose and 4 different types of hydroxyl-geranic acid-hexoses. A geranic acid glycoside was the most abundant, and identified by NMR as geranoyl-6-O-malonyl-β-D-glucopyranoside with an average concentration of 45 µM. Fungal bioassays with \textit{C. graminicola} and \textit{F. graminearum} did not reveal an effect of these changes in secondary metabolite composition on plant resistance to either fungus. Transgenic maize didn’t show different effect on western flower thrips, a serious pest insect, either. The results demonstrate that metabolic engineering of geraniol into geranic acid can rely on the existing default pathway, but branching glycosylation pathways must be controlled to achieve accumulation of the aglycones.
1. Introduction

Mono- and sesquiterpenoids are the main constituents of essential oils of aromatic plants (Rohloff, 2004). They play major ecological and physiological roles in flower pollination and in responses to biotic or abiotic stress (Yu and Utsumi, 2009). In the search for natural fungicides many antifungal terpenoids were identified, but their role in plant defense remained unclear (Inouye et al., 2001; Kalemba and Kunicka, 2003). Recently, however, the potential role of the antifungal monoterpenes neomenthol and menthol to enhance plant resistance to fungal and bacterial infections has been demonstrated by overexpression and silencing experiments in pepper and Arabidopsis (Choi et al., 2008).

Fusarium graminearum and Colletotrichum graminicola are fungal pathogens of maize (Zea mays) seriously reducing grain yield and quality (Bergstrom and Nicholson, 1999; Vigier et al., 2001). F. graminearum causes maize ear and stalk rot, and seedling blight (Vigier et al., 2001; Presello et al., 2006), and C. graminicola induces stalk rot and anthracnose in most maize tissues (Bergstrom and Nicholson, 1999; Sukno et al., 2008). Several studies have examined the in vitro effects of essential oils against these two pathogens. For F. graminearum, 5 out of 37 examined essential oils inhibited fungal development, and the effects were attributed to the main components of these oils including the monoterpenoids geraniol, citral (oxidation product of geraniol) and carvacrol (Velluti et al., 2004b). For C. graminicola, 4 essential oils from the Cymbopogon genus effectively inhibited its mycelial growth (Somda et al., 2007; Zida et al., 2008), and among the monoterpenes tested citral was found to be the most effective (Dev et al., 2004).

In the past years a large variety of mono- and sesquiterpenoid synthase genes from different plant species have been characterized, and many of them were used for metabolic engineering of plants (Mahmoud and Croteau, 2002; Cheng et al., 2007a; Yu and Utsumi, 2009). Production and increased emissions of target mono- and sesquiterpenoids through heterologous expression of the corresponding terpenoid synthase genes, has been achieved in various plants: geraniol in tomato fruits (Davidovich-Rikanati et al., 2007); linalool in tomato fruits, carnation flowers, Arabidopsis leaves and potato leaves (Lewinsohn et al., 2001; Lavy et al., 2002; Aharoni et al., 2003; Aharoni et al., 2006); β-pinene and γ-terpinene in tobacco (Lücker et al., 2004); limonene in mint, tobacco and lavender (Diemer et al., 2001; Ohara et al., 2003; Lücker et al., 2004; Muñoz-Bertomeu et al., 2008); nerolidol in Arabidopsis (Kappers et al., 2005); (E)-β-caryophyllene in Arabidopsis, rice and maize (Cheng et al., 2007b; Degenhardt et al., 2009); α-zingiberene in tomato fruits (Davidovich-Rikanati et al., 2008); amorphadiene in tobacco and Artemisia annua (Wu et al., 2006; Ma et al., 2009).

Apart from direct products of expressed terpene synthases, volatile or non-volatile derivatives are often produced as well in transgenic plants by the action of endogenous enzymes. Among the many types of different modifications, oxidation, hydroxylation and acetylation of primary terpenoid skeletons are the most common endogenous modifications. For example, when geraniol synthase or linalool synthase were overexpressed, the oxidation products of geraniol (geranal and geranic acid) or linalool (linalool oxide), the hydroxylation product of linalool (hydrxyl-linalool) and the acetylation product of geraniol (geranyl acetate) were found (Lewinsohn et al., 2001; Lavy et al., 2002; Davidovich-Rikanati et al., 2007). Modifications can also take place sequentially resulting in complex transformations. In plants overexpressing amorphadiene synthase for example, amorphadiene is hydroxylated and then oxidized into artemisinc acid and dihydroartemisinc acid (Ma et al., 2009). The volatile terpenoids may be further modified by conjugation to larger moieties such as sugar residues, which usually renders them non-volatile. Among glycosidic conjugations, until now, only glucosylation has been reported. In petunia, Arabidopsis and potato expressing recombinant...
linalool synthase, glycosides of linalool and hydroxyl-linalool were detected (Lücker et al., 2001; Aharoni et al., 2003; Aharoni et al., 2006). Analysis of the non-volatile metabolites is, therefore, important for a more comprehensive understanding of the metabolic fate of terpenes in plants engineered for the production of novel metabolites.

In this study, among 15 mono- and sesquiterpenoids which were tested in vitro against *F. graminearum* and *C. graminicola*, geranic acid was identified as the most effective one against the two maize pathogens. To produce geranic acid in maize, a geraniol synthase was cloned and overexpressed in maize. Both volatile and non-volatile metabolic profiles of transgenic plants were analyzed, and potential effects of the modified terpenoid content against these fungi were assayed.

### 2. Materials and methods

#### 2.1. Antifungal compound assays

The pathogenic fungi, *Fusarium graminearum* and *Colletotrichum graminicola*, were Pioneer Hi-Bred isolates from diseased corn. Cultures of each fungus were maintained on potato dextrose agar (PDA) and stored on silica at -20 °C. Fifteen terpenoids which were pre-selected from previous experiments (data not shown) were selected in this study to determine their specific antifungal effect (Table 1).

Antifungal effects were studied using a dilution in agar technique (Santos et al., 2005). Each terpenoid was tested at 7.8, 15.6, 31.3, 62.5, 125, 250 and 500 µg mL⁻¹. The terpenoids were dissolved in 95% ethanol and assayed in 24-well plates. Into each well, 5 µl of terpenoids was added and quickly mixed with 500 µl of agarose for *F. graminearum* and 1/4 Czapek-Dox V8+0.8% SeaPlaque-GTG agarose for *C. graminicola*). Wells containing ethanol, water or only medium, instead of terpenoids, were used as negative control. When the medium cooled down, 3 µl of spore suspension (8000 mL⁻¹) was centrally placed onto it. Wells were then sealed with double-layer of parafilm and double-layer of blotter paper. Then the plates were covered and incubated in the dark at 27 °C. The effects of terpenoids were scored after 48 h by measuring spore germination and hyphal elongation for each colony (Duvick et al., 1992). The minimum inhibitory concentration (MIC) is the concentration inhibiting at least 25% hyphal elongation, and the minimum complete inhibitory concentration (MCIC) is the lowest concentration completely inhibiting spore germination and hyphal growth.

<table>
<thead>
<tr>
<th>Monoterpenoids</th>
<th>MCIC (µg mL⁻¹)</th>
<th>MIC (µg mL⁻¹)</th>
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<th>CGR FGR</th>
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<td>31.3</td>
<td>&lt;7.8</td>
</tr>
<tr>
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<td>125</td>
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</tr>
<tr>
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<td>125</td>
<td>500</td>
<td>62.5</td>
</tr>
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<td>62.5</td>
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<td>125</td>
</tr>
<tr>
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<tr>
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</table>

### 2.2. Cloning of full-length *LdGES* cDNA and overexpressing it in maize

The geraniol synthase gene, *LdGES*, was isolated from *Lippia dulcis*, and the LdGES protein was expressed in *Escherichia coli* to characterize its function (for details, see Supplementary
Overexpressing geraniol synthase in maize

Methods). The LdGES was placed under the control of a maize ubiquitin promoter, and Agrobacterium tumefaciens strain LBA4404 harboring the binary vector (Fig. S3) was used to transform immature embryos of maize (Zea mays) genotype PHWWE (Pioneer Hi-Bred) using protocols described previously (Zhao et al., 2001). Maize plants transformed with a vector lacking LdGES were used as control.

Three T₀ transgenic plants producing the highest level of new compounds (see below) and one empty vector control plant were crossed with untransformed PHWWE to get the T₁ generation. Plants were grown in a greenhouse at 20 °C under 18/6 h light/dark photoperiod. After headspace trapping of intact plants, T₁ Plantlets from 3 independent transgenic lines (10 plants per line) were screened by real-time PCR for the expression of LdGES (for primers, see below). For LC-MS, GC-MS analysis and measurements of phenotypic traits, 5 positive plantlets from each transgenic line and 5 progenies from the control line were used.

2.3. Transcript analysis

The expression level of LdGES was determined by real-time quantitative RT-PCR analysis as described (Schielen et al., 2007). The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (gi22302) was used as reference gene and the primers were designed as reported (Hahnen et al., 2003). For the LdGES, the following primers were designed by software Beacon Designer (Palo Alto, CA, USA): forward primer 5’-AATACCACCAACGAGATATGCTAC-3’ and reverse primer 5’-TCCACCATTGAACCACCTTTGC-3’ with an expected product size of 129 bp.

2.4. Volatile GC-MS analysis

Volatile GC-MS analysis

Volatile GC-MS analysis

Volatile from the headspace of intact plants were collected from 2-week old T₁ and control seedlings in a dynamic headspace trapping system. Hereto, each seedling was placed in a 2-L glass cuvet in a climate chamber at 20 °C. The cuvets were closed with a Teflon-lined lid with a Tenax cartridge (140 × 4 mm; 150 mg Tenax [20/35 mesh; Alltech, Breda, the Netherlands]) to purify incoming air and a second Tenax cartridge (140 × 4 mm) on the outlet to trap the volatiles. Air was pumped by a vacuum pump through the glass cuvet at about 100 mL min⁻¹. The volatiles were sampled for 2 h.

Volatile from cut leaves were collected from 8-week old plants. Leaf tips (about 10 cm long) from the second top leaf were harvested and immediately transferred into a 10-mL glass vial filled with tap water. The vial was placed in a 1-L glass cuvet in the climate chamber and sampled as above.

The volatiles trapped in the outlet cartridges were analyzed by Thermodesorption GC-MS using a thermal desorber (Unity, Markes International Limited) and a Trace GC Ultra (Thermo Electron Corporation) coupled with a DSQ mass spectrometer (Thermo Electron Corporation). The cartridges were first purged to waste for 5 min at room temperature using helium at a flow-rate of 30 mL min⁻¹ to remove free water and oxygen. Trapped volatiles were then released from the Tenax material in the thermal desorber at 250°C for 4 minutes. Volatiles were then collected on a cold trap at 10 °C and desorbed by increasing the temperatures from 10 to 250 °C at 12 °C s⁻¹, and a hold at 250 °C for 2 min. The column used for chromatography was an Rtx-5 ms column (Restek, 30 m x 0.25 mm i.d., 1 μm d.f.). The temperature program of the gas chromatograph was 40 °C for 3.5 min, rising to 280 °C at 10 °C min⁻¹ and final time for 2.5 min. The mass spectrometer was set to scan from 35 to 450 m/z. The helium flow was constant at 1.0 mL min⁻¹. Ionization potential was set at 70 eV.

For identification, the authentic standards of geraniol, geranyl acetate and geranic acid (present in a mixture of geranic acid and its isomer--neric acid) were run under identical conditions.
2.5. Non-volatile LC-MS analysis

Non-volatile compounds were analyzed using a protocol for untargeted metabolomics of plant tissues (De Vos et al., 2007). In brief, 200 mg young leaf tip from each plant was ground in liquid nitrogen and extracted with 0.6 mL methanol:formic acid (1000:1,v/v). The extracts were prepared by brief vortexing and sonication for 15 min. Then the extracts were centrifuged and filtered through 0.2 µm inorganic membrane filters (RC4, Sartorius, Germany). LC-PDA-MS analysis was performed using 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. The column used was an analytical column (2.0 × 150 mm; Phenomenex, USA) attached with a C18 pre-column (2.0 × 4 mm; Phenomenex, USA). Degassed eluent A (ultra pure water:formic acid [1000:1,v/v] ) and eluent B (acetonitril:formic acid [1000:1,v/v]) were pumped at 0.19 mL min\(^{-1}\) into the HPLC system. The gradient started at 5% B and increased linearly to 35% B in 45 min. Then the column was washed and equilibrated for 15 min before the next injection. The injection volume was 5 µl. The MS-MS measurements were done with following collision energies of 10, 15, 25, 35 and 50 eV. Leucine enkaphalin ([M-H]\(^{-}\)=554.2620) was used as a lock mass for on-line accurate mass correction.

2.6. GC-MS and LC-MS data processing

GC-MS data were acquired using Xcalibur 1.4 (Thermo Electron Corporation) and LC-MS data using MassLynx 4.0 (Waters). The data were then processed using MetAlign version 1.0 (www.metAlign.nl) for baseline correction, noise elimination and subsequent spectral data alignment (De Vos et al., 2007). The processing parameters of MetAlign for GC-MS data were set to analyze from scan number 1340 to 16000 (corresponding to retention time 2.32 min to 28.05 min) with a maximum amplitude of 1.4 × 10^8. The parameters for LC-MS data were set to analyze from scan number 70 to 2620 (corresponding to retention time 1.4 min to 49.73 min) with a maximum amplitude of 35000.

In order to elucidate which mass signals originate from the same metabolite, all the detected masses were clustered by an in-house developed software package based on a Multivariate Mass Spectra Reconstruction (MMSR) approach (Tikunov et al., 2005). The mass signal intensities (expressed as peak height using metAlign) obtained from transgenic plants and empty vector control plants were compared using the Student’s t-test. Masses with a significant (p<0.05) intensity change of at least 2-fold were verified manually in the original chromatograms.

To annotate significantly different compounds, accurate masses were manually calculated, taking into account only those scans with the proper intensity ratios of analyte and lock mass (between 0.25- and 2 (Moco et al., 2006)), and elemental formulae generated within 5 ppm deviation from the observed mass. In addition, mass-directed LC-MS/MS experiments were performed on differential compounds. To obtain proper MS/MS spectra only molecular ions with signal intensities higher than 500 ion counts per scan were selected.

2.7. Purification and identification of geranoyl-6-O-malonyl-β-D-glucopyranoside

Plant extracts were prepared as described above, and the most dominant new compound in the transgenic plants, later identified as geranoyl-6-O-malonyl-β-D-glucopyranoside, was initially purified with an analytical HPLC system coupled to a PDA detector. This purification method, however, was not suitable for NMR analysis, as the fractions collected were not sufficiently pure, mainly due to column bleeding and the multiple injections needed to purify sufficient amounts for NMR analysis.
Therefore, the compound was purified with a preparative LC-MS system, consisting of an Agilent 1200 HPLC with a flow splitter and connected to a Bruker MicroTOF MS. The split ratio was set to 1:100. The column used was an Alltima C18 (5 μm) LC column (22x150 mm). Degassed eluent A (ultra pure water:formic acid [1000:1,v/v]) and eluent B (methanol:formic acid [1000:1,v/v]) were pumped at 2 mL min\(^{-1}\) into the LC system. The gradient started at 50% B and increased linearly to 70% B in 40 min. This was the optimal gradient for separation of the mass of interest from the rest of the matrix. Then the column was washed and equilibrated for 30 min before next injection. For each measurement, 500 µl of extract was manually injected. The mass spectrum was monitored with software Hystar (ver. 3.2). The fraction containing the molecular ion of the desired compound ([M-H] = 831.33) was manually collected in a glass tube. The plant extract and the collected fractions were kept on ice or at 4 °C, as the compound in extraction solvent was unstable at room temperature. The fraction collected from one preparative LC-MS run was freeze-dried, re-dissolved in methanol and then re-analyzed by analytical LC-MS as described above. The compound collected in this fraction eluted at the expected retention time (49.1 min) and showed the expected mass spectrum. Then, this fraction was collected from 7 sequential preparative LC-MS runs in the same way. The fractions were pooled, freeze-dried and re-dissolved in deuterated dimethyl sulfoxide (DMSO) to be analyzed by NMR. The amount of the compound purified from these 7 runs (3.5 ml extract injected) was estimated to be 20 µg.

NMR measurements were carried out with a 600 MHz Bruker Avance III NMR spectrometer equipped with a 5 mm cryoprobe. All measurements were performed at 298 K. Gradient enhanced versions were used when applicable. One and two-dimensional NMR spectra were obtained (COSY, HSQC, HMBC,TOCSY). Calibration was done relative to the solvent resonance of DMSO at 2.54 ppm. The amount was checked by integration of the intensity of the signals of the compound. The Avance III NMR instrument was calibrated by measuring the integral of reference molecules at different receiver gain values of the instrument.

### 2.8. Phenotypic trait measurements and fungal infection assays

Plant height and leaf numbers of T\(_1\) maize plants were measured at the tasselling stage. Chlorophyll content of leaves was measured as previously described (Mocquot et al., 1996). The fresh kernel weights were measured directly after harvesting.

The leaf sheaths of leaf 4 or 5 of T1 plants at the V5 stage (plants with 5 fully developed leaves) were inoculated with 50 µL of spore suspension (5 × 10\(^6\) mL\(^{-1}\)) after wounding the leaf sheath on both sides about half way between edge and midrib with a small screwdriver. Plants were grown in the greenhouse. The leaf sheath was covered with plastic wrap for 5 days. Nine days after inoculation the area of lesions was measured. Fifty positive T\(_1\) plants from 10 independent transgenic lines (5 plants per line) and 50 control plants were assayed.

### 2.9. Dual-choice behavioural studies with thrips

A population of western flower thrips (WFT), *Frankliniella occidentalis*, was mass-reared on flowering chrysanthemum (*Chrysanthemum morifolium* Ramat.) cv. Sunny Casa in a greenhouse under a photoperiod of L16:D8 at 25±2 °C. In this study, only adult female thrips were used. All bioassays were conducted in a climate room at 20-22 °C with a L16:D8 photoregime.

The leaf discs (diameter 1.6 cm) were punched from chrysanthemum leaves at similar leaf stage. Leaf discs from wild-type maize plants were used as control discs and leaf discs from plants of transgenic line 13, line 19 or line 20 were used as test discs. After overnight starvation, WFT were anaesthetized on ice and then placed between 2 leaf discs embedded, abaxial side up, on a 1.5% (w/v) agar-bed in a Petri dish (7cm diameter, 10 WFT/dish).
Twelve replicates were used in this experiment. The number of WFT on each leaf disc was recorded 0.25, 1, 2, 4, 20, and 28 h after the release of the WFT. At each time point, a Student’s paired t-test was used to assess the significance of the differences in the mean number of WFT between test and control.

3. Results

3.1. In vitro antifungal effect of mono- and sesquiterpenoids

We examined 12 monoterpenoids and 3 sesquiterpenoids for their antifungal activity against 2 major maize pathogenic fungi, *Fusarium graminearum* and *Colletotrichum graminicola* (Table 1). In this *in vitro* antifungal assay, every terpenoid was tested at a series of concentrations ranging from 7.8 to 500 µg mL\(^{-1}\) for 48 h to determine the minimum complete inhibitory concentration (MCIC) and minimum inhibitory concentration (MIC). The most effective growth inhibitor of both fungi was geranic acid with a MIC of less than 7.8 µg mL\(^{-1}\) and MCIC of 31.3 µg mL\(^{-1}\) for *F. graminearum* and 7.8 µg mL\(^{-1}\) for *C. graminicola* (Table 1).

Previously, concentrations ranging from 7.8 to 31.3 µg mL\(^{-1}\) (i.e. 46 to 186 µM) were easily achieved for monoterpene alcohols in several transgenic plants (Lücker et al., 2001; Aharoni et al., 2003). To investigate this possibility for maize, we cloned a geraniol synthase and overexpressed it in maize.

3.2. Cloning of the *LdGES* cDNA and introduction into maize

*LdGES*, a full length cDNA of a geraniol synthase gene, was cloned from *Lippia dulcis*, which is a strongly aromatic herb of the Lamiales family, also known as *Phyla dulcis* (Trevir.) Moldenke. The *LdGES* gene (GU136162) contains an open reading frame of 1755 nucleotides encoding a protein of 584 amino acids. Nucleotide blast against the Nr database revealed that it shared the highest degree of homology with geraniol synthase from *Ocimum basilicum* (*ObGES*; Iijima et al., 2004) (For alignment of the deduced amino acid sequences of *LdGES* and other geraniol synthases, see Fig. S1).

The entire reading frame of *LdGES* was cloned into a vector for expression in *E. coli*. In the presence of geranyl diphosphate (GDP), the only product produced by the recombinant LdGES protein was geraniol (Fig. S2). No product was detected when the enzyme was supplied with farnesyl diphosphate (data not shown).

The coding region of the cDNA encoding *LdGES* was placed under the control of the strong 35S enhancer-ubiquitin-promoter combination (Fig. S3) and introduced into maize plants. Plants transformed with the vector lacking *LdGES* were used as controls. Expression levels of the *LdGES* gene in T\(_1\) transgenic plants were determined by quantitative

<table>
<thead>
<tr>
<th>Plants</th>
<th>Mean</th>
<th>SD</th>
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<td>n.d.</td>
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<td></td>
</tr>
</tbody>
</table>

Transgene expression levels were determined by quantitative RT-PCR. n.d., not detectable. Expression levels of the reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene, were set to 1. Values for each sample are based on 3 technical replicates.
real-time RT-PCR. Out of 30 analyzed plants (10 plants per line), 15 of them (5 plants per line) were positive with a *LdGES* transcript level from 1.13 (ger 20-1) to 1.83 (ger 19-9), relative to the level of the household gene glyceraldehyde-3-phosphate dehydrogenase (Table 2).

### 3.3. Effect of LdGES on headspace emissions from intact plants and detached leaves

Volatiles were collected from the headspace of intact plants and detached leaves from transgenic and control plants at the same developmental stage and analyzed by GC-MS (Fig. 1, a-d). The primary product of the LdGES protein, geraniol, was not detected in any plant analyzed (Fig. 1, a-d, f). The desired antifungal compound, geranic acid, was not detected either (Fig. 1, a-d, g). Subsequently, an untargeted metabolomics approach and comparison of all mass signals was carried out on intact and detached leaves. Only when comparing the headspace collected from detached leaves of transgenic and control plants were significant differences found. MetAlign software detected 2241 mass signals with a signal-to-noise ratio higher than 3, and that represented 191 metabolites (mass clusters) according to the MMSR clustering script (Tikunov et al., 2005). For each extracted mass, the average mass intensity was used to calculate the intensity ratio of transgenic (n=15) to control plants (n=5), and significant differences between plants were determined by the Student’s *t* test. Among all 2241 detected masses, 120 masses (i.e. 5.4%) showed at least 2-fold intensity difference (*P* < 0.05) between transgenic and control plants. More masses were found to be significantly increased (97) than decreased (23) in the transgenic plants. Differential masses were checked manually for differential peak areas in the original chromatograms, and compounds were identified by comparing to authentic standards. Geranyl acetate was the only compound found to be significantly different, showing 5.05-fold increase. Detailed analysis of the mass clusters revealed that 76 significantly increased masses corresponded to geranyl acetate. For the other 21 significantly increased and 23 decreased masses, at most 1 or 2 masses per compound were different in the *t*-test, while manual inspection of their chromatographic peak areas showed no significant differences. Therefore, these differential masses were regarded as noise and not significantly different between transgenic and control plants. Within transgenic plants, the emission of geranyl acetate displayed a positive correlation with the expression level of LdGES (*R*² = 0.88).

### 3.4. Effect of LdGES on the profile of non-volatile compounds from leaves

As monoterpene alcohols such as geraniol may (partly) be conjugated to other compounds rendering them non-volatile, we also analyzed the non-volatile metabolites in transgenic (n=15) and control plants (n=5). Aqueous methanol extracts from young leaves were prepared and analyzed by accurate mass LC-MS in negative mode (Fig. 2, a-b). In order to reveal differential compounds, the LC-MS profiles of transgenic plants and control plants were compared in an untargeted manner using MetAlign followed by MMSR clustering of extracted signals, as described above for the GC-MS profiles.
Figure 1. GC-MS chromatograms obtained by dynamic headspace trapping of intact plants and cut leaves of control and LdGES expressing plants. (a)-(b), GC-MS chromatograms of headspaces trapped from intact control (a) and LdGES expressing plant 19-2 (b). There was no significant difference between them. (c)-(d), GC-MS chromatograms of headspaces trapped from cut leaves of control (c) and LdGES expressing plant 19-2 (d). Compared to control, cut leaves of LdGES expressing plant emitted 5-fold more geranyl acetate as the only significant difference. (e)-(g), GC-MS chromatograms of authentic standards of geranyl acetate (e), geraniol (f) and geranic acid (g) (neric acid is the isomer of geranic acid). *, the peak eluting at 17.05 min is not geraniol, as the mass spectrum of this peak is different from that of geraniol. Geraniol was not detected in any sample. **, the peak eluting at 18.39 min is not geranic acid, as the mass spectrum of this peak is different from that of geranic acid. Geranic acid was not detected in any sample.
Figure 2. Negative mode LC-QTOF-MS chromatograms of aqueous-methanol extract of leaves of an empty vector control plant (a) and *LdGES* expressing plant 19-2 (b). (c), the accurate mass of the compound eluting at 49.12 min uniquely found in the transgenic lines; (d), the MS/MS spectrum of the 49.12 min compound. (e), scheme of collision-induced MS/MS-fragmentation of mass 415 eluting at 49.12 min, which was later identified by NMR as geranyl-6-O-malonyl-β-D-glucopyranoside. The ion trace of *m/z* 831, i.e. [2M-H] -, was extracted from the chromatograms of the empty vector control (f) and *LdGES* expressing plant (g).
In total, 5869 mass signals were extracted, which grouped into 257 clusters representing 257 metabolites. Among all 5869 masses, 641 masses (i.e. 10.9%) showed at least 2-fold intensity difference (P < 0.05) between transgenic and control plants. More masses were found to be significantly increased (466) than decreased (175) in the transgenic plants. Differential masses with a signal intensity higher than 500 (i.e. about 50-fold higher than the noise) were subsequently analyzed by LC-MS/MS (Table 3). According to their MS/MS spectra, these compounds were putatively identified as derivatives of geraniol: 2 different glycosides of geranic acid, 1 geraniol dihexose, and 4 different isobaric forms (i.e. different retention times, but identical accurate mass) of hydroxyl geranic acid (or neric acid)-hexose. The amounts of these compounds did not correlate with the expression levels of LdGES (data not shown). Without authentic standards mass spectrometry has fundamental limitations in resolving the different possible chemical structures, so that it is not obvious to which carbon the hydroxyl group is attached and to which position the hexose is conjugated.

The newly produced compound that was most abundant, as determined by UV absorption (220 nm) resulting from the double bond of geranyl-compounds, showed up as m/z of 831.3287 (Fig. 2, c) and eluted at a retention time of 49.12 min (Fig. 2, b). In control plants, the amount of this compound is at least 2000 fold lower as it’s lower than the detection limit (Fig. 2, f-g). This compound was selected for further identification by LC-MS/MS and NMR.

3.5. Identification of the most distinct new compound in leaves of transgenic plants by LC-MS/MS and NMR

In order to identify the most abundant new compound in the extracts of transgenic maize plants, the apparent parent mass was fragmented by LC-MS/MS in negative mode. The selected mass 831.3287 appeared to be a [2M-H]+ adduct of 415.1608. Within the MS/MS fragments of 415.1608, we detected an ion with mass 167.1076, i.e. a -0.9 ppm deviation from the elemental formula of geranic acid (C16H26O2). This MS/MS experiment suggested that the selected compound is a derivative of geranic acid. In order to unambiguously identify the

Table 3. Non-volatile metabolite significantly increased in transgenic plants putatively identified by LC-MS-MS.

<table>
<thead>
<tr>
<th>Ret (min)</th>
<th>Av intensity (C)</th>
<th>Av intensity (T)</th>
<th>Ratio (T/C)</th>
<th>Accurate mass found</th>
<th>Mol form</th>
<th>∆mass (ppm)</th>
<th>MS-MS fragments</th>
<th>Putative ID</th>
<th>MM</th>
</tr>
</thead>
<tbody>
<tr>
<td>49.12</td>
<td>n.d.</td>
<td>2255.2</td>
<td>–</td>
<td>831.3298</td>
<td>C19H26O10</td>
<td>1.4</td>
<td>415, 371, 353, 311, 239, 221, 209, 203, 191, 189, 181, 167, 161, 143</td>
<td>geranyl-6-O-malonyl-β-D-glucopyranoside&lt;sup&gt;*&lt;/sup&gt; ([2M-H]&lt;sup&gt;+&lt;/sup&gt;)</td>
<td>416.1683</td>
</tr>
<tr>
<td>48.25</td>
<td>1.5</td>
<td>583.1</td>
<td>388.73</td>
<td>473.2025</td>
<td>C16H25O11</td>
<td>0.4</td>
<td>167</td>
<td>geranic acid&lt;sup&gt;*&lt;/sup&gt; (geraniol-dihexose)FA</td>
<td>474.2101</td>
</tr>
<tr>
<td>36.66</td>
<td>422.8</td>
<td>2204.3</td>
<td>5.21</td>
<td>523.2405</td>
<td>C16H25O13</td>
<td>2.7</td>
<td>477, 316, 161</td>
<td>hydroygeranic acid-hexose&lt;sup&gt;†&lt;/sup&gt;</td>
<td>524.2469</td>
</tr>
<tr>
<td>24.12</td>
<td>155.0</td>
<td>1489.3</td>
<td>9.61</td>
<td>345.1565</td>
<td>C16H25O8</td>
<td>4.5</td>
<td>207, 183, 179</td>
<td>hydroygeranic acid-hexose&lt;sup&gt;†&lt;/sup&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>346.1628</td>
</tr>
<tr>
<td>22.84</td>
<td>5.5</td>
<td>499.5</td>
<td>90.82</td>
<td>391.1621</td>
<td>C16H25O8</td>
<td>4.3</td>
<td>345, 183</td>
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<tr>
<td>19.75</td>
<td>59.5</td>
<td>707.6</td>
<td>11.89</td>
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<td>C16H25O8</td>
<td>4.8</td>
<td>121, 119, 101</td>
<td>hydroygeranic acid-hexose&lt;sup&gt;†&lt;/sup&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
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<td>160.3</td>
<td>1865.8</td>
<td>11.64</td>
<td>345.1559</td>
<td>C16H25O8</td>
<td>2.8</td>
<td>161, 121, 119, 101</td>
<td>hydroygeranic acid-hexose&lt;sup&gt;†&lt;/sup&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>346.1628</td>
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</tbody>
</table>

The significantly changed metabolites with mass intensity higher than 500 in either transgenic or control plants were chosen to be analyzed by LC-MS/MS. Ret (min), retention time, in minutes; Av, average; C, empty vector control plants; T, transgenic plants; Ratio (T/C), ratio of mass signal between transgenic plants (T) and empty vector control plants (C); Mol form, molecular formula of the metabolite; ∆mass (ppm), deviation between the found accurate mass and real accurate mass, in ppm; Putative ID, putative identification of metabolite; MM, monoisotopic molecular mass of the metabolite. n.d., not detectable, compounds with intensity less than 1 are not detectable; <sup>*</sup>The structure of geranic acid-malonyl-glucopyranoside was confirmed by NMR; <sup>†</sup>The compound could also be hydroygeranic acid-hexose; FA, formic acid adduct; Geranic acid<sup>*</sup>, geranic acid conjugated with agarobiose, carrobiose, difructose anhydride or their isomers.

<table>
<thead>
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<th>Ratio (T/C)</th>
<th>Accurate mass found</th>
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<th>∆mass (ppm)</th>
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<td>geranyl-6-O-malonyl-β-D-glucopyranoside&lt;sup&gt;*&lt;/sup&gt; ([2M-H]&lt;sup&gt;+&lt;/sup&gt;)</td>
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<td>167</td>
<td>geranic acid&lt;sup&gt;*&lt;/sup&gt; (geraniol-dihexose)FA</td>
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<td>C16H25O13</td>
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<td>hydroygeranic acid-hexose&lt;sup&gt;†&lt;/sup&gt;</td>
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<td>207, 183, 179</td>
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<td>161, 121, 119, 101</td>
<td>hydroygeranic acid-hexose&lt;sup&gt;†&lt;/sup&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td>346.1628</td>
</tr>
</tbody>
</table>
structure of the compound, we subsequently purified the molecule for NMR analysis using preparative LC-MS. To estimate its average concentration, the molecule was purified from the transgenic plant with average mass intensity of mass 831 (the intensity in this particular plant was 2063, i.e. <10% difference from the accurate average intensity 2255.2; n=15). During purification, it turned out that the molecule was unstable at room temperature, posing some challenges to the purification. This was solved by keeping the plant extracts and collected fractions at 0-4 °C. Based on one and two-dimensional NMR data, we could assign the structure of the molecule to geranoyl-6-O-malonyl-β-D-glucopyranoside (Fig. 3; for detailed 13C NMR and 1H NMR data, see Table S1).

![Figure 3. 600 MHz 1H NMR spectrum of geranoyl-6-O-malonyl-β-D-glucopyranoside.](image)

This compound has not been reported in the plant kingdom before. The NMR analysis allowed us to estimate the average compound concentration in transgenic maize leaves at about 17 µg g⁻¹ FW. Assuming a leaf water content of 90%, this concentration corresponds to 45 µM. We propose that the most likely biosynthetic pathway of geranoyl-6-O-malonyl-β-D-glucopyranoside is the conversion of the product of LdGES, geraniol, into its corresponding acid, geranic acid, by two dehydrogenation/oxidation steps, followed by further step-wise conjugation of the acid moiety with malonyl-glucose (Fig. 4).

### 3.6. Effect of LdGES on plant phenotype and susceptibility to fungal and thrips attack

Overexpression of monoterpene synthases may be directly toxic and/or lead to reduced steady state levels of terpenoids essential in primary metabolism and plant development such as gibberellic acid and carotenoids, and result in bleached leaves and delayed plant growth (Aharoni et al., 2003; Aharoni et al., 2006; Davidovich-Rikanati et al., 2007). We, therefore, investigated the phenotype and development of the transgenic plants. We observed that the LdGES expressing plants were phenotypically indistinguishable from the control plants (Fig. 5). The plant height, leaf number, chlorophyll content and kernel weight of 3 transgenic lines and a control line were measured (5 plants were used per line). Statistical analysis showed no significant differences between transgenic and control plants for any of the measured traits (Table 4). This indicates that overexpression of LdGES at the reported levels and under the conditions tested does not negatively affect maize plant growth and development.

### Table 4. Plant height, leaf number, chlorophyll content and kernel weight of T1 transgenic plants and control plants.

<table>
<thead>
<tr>
<th>Lines</th>
<th>Plant height (cm)</th>
<th>Leaf number</th>
<th>Chlorophyll (mg/g FW)</th>
<th>Kernel weight (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>c 31</td>
<td>261.21a 30.16</td>
<td>13.8a 0.45</td>
<td>2.96a 0.50</td>
<td>0.46a 0.04</td>
</tr>
<tr>
<td>ger 18</td>
<td>266.20a 8.70</td>
<td>14.4a 0.55</td>
<td>3.19a 0.35</td>
<td>0.45a 0.04</td>
</tr>
<tr>
<td>ger 19</td>
<td>268.82a 13.10</td>
<td>14.2a 0.84</td>
<td>2.59a 0.40</td>
<td>0.41a 0.05</td>
</tr>
<tr>
<td>ger 20</td>
<td>256.64a 15.53</td>
<td>13.6a 0.55</td>
<td>2.67a 0.11</td>
<td>0.47a 0.03</td>
</tr>
</tbody>
</table>

Five plants from each line were measured. Values followed by the same letter within a column are not significantly different (ANOVA test: P > 0.05).
Fifty plants from 10 different T₁ generation transgenic maize lines (5 plants per line) and 50 control plants were tested in the greenhouse for resistance of leaf sheaths to infection by *F. graminearum* and *C. graminicola*. There was no significant difference between transgenic and control plants in lesion area resulting from inoculation by either fungus (Table 5). Leaves at similar leaf stage were randomly picked from plants of transgenic lines and empty vector control line to test the effects of transgenic plants on WFT behaviour. The results of dual-

![Diagram of geraniol metabolism](image)
choice assays showed that WFT did not distinguish transgenic plants from control plants (Fig. 6). The increased content of geraniol and geranic acid derivatives, therefore, did not lead to changes in fungal or thrips resistance under the applied experimental conditions.

4. Discussion

In this study, we aimed to introduce fungal resistance into maize by metabolic engineering. We took a stepwise approach of first testing a wide range of terpenoids and overexpressing the most promising candidate. Among the 15 terpenoids that were tested against two maize pathogenic fungi \textit{F. graminearum} and \textit{C. graminicola}, geranic acid displayed the strongest antifungal activity (Table 1). The MIC of geranic acid was lower than 7.8 µg mL\(^{-1}\) (46 µM) for both fungi, and for these fungi it represents the most potent antifungal activity reported to date. Previously, citral, dimethyloctanol, terpineol and linalyl acetate were tested against \textit{C. graminicola} and citral was the most effective with a MIC of 870 µg mL\(^{-1}\) (5723 µM) (Dev et al., 2004). Only complex essential oils have been screened for their inhibitory effect against \textit{F. graminearum}, however, the MICs of the most effective oils were higher than 1000 µg mL\(^{-1}\) (Velluti et al., 2004a; Singh et al., 2008). The antifungal effects of the individual components of these essential oils are not known, but if they represent more than 1% of the oil, they must be less effective than geranic acid.

Previously, geranic acid has been produced \textit{de novo} in transgenic tomato fruits by overexpressing geraniol synthase (Davidovich-Rikanati et al., 2007). Thus, we cloned and overexpressed a geraniol synthase gene from \textit{Lippia dulcis} (\textit{LdGES}) under control of an ubiquitin promoter in maize plants. In leaves of \textit{LdGES} expressing maize plants, we detected 7 geraniol derivatives with significantly higher concentrations than control plants. Remarkably, the most dominant compound geranoyl-6-O-malonyl-β-D-glucopyranoside in transgenic maize was not detected at all in the control plants, whereas the other 6 geraniol-derived compounds did occur, although at relatively low levels. In view of the presence of these endogenous geraniol derivatives, we expect a geraniol synthase gene to be expressed in maize leaves. So far the only reported candidate gene is the Terpene Synthase 1 (TPS1) gene. This gene has been reported to be capable of producing geraniol and several other mono- and sesquiterpenes in an \textit{in vitro} assay (Schnee et al., 2002). However, as the authors pointed out, it is likely to function as a sesquiterpene synthase \textit{in vivo}, since it lacks an N-terminal signal peptide for chloroplast targeting and it’s more effective to convert farnesyl diphosphate than geranyl diphosphate (Schnee et al., 2002). We presume, therefore, that the operational geraniol synthase gene of maize still needs to be identified (for alignment of all published geraniol synthases, see Fig. S1).

We suggest that the most likely reason for the observed differences in the amounts of accumulating compounds between the transgenic and control plants is the difference between

<table>
<thead>
<tr>
<th>Plants</th>
<th>\textit{C. graminicola}</th>
<th>\textit{F. graminearum}</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>lesion area (cm(^2))</td>
<td>lesion area (cm(^2))</td>
</tr>
<tr>
<td>Control plants</td>
<td>Mean 0.708a 0.23</td>
<td>Mean 2.589a 0.63</td>
</tr>
<tr>
<td>\textit{T\textsubscript{1}} plants</td>
<td>0.670a 0.19</td>
<td>2.778a 0.82</td>
</tr>
</tbody>
</table>

Fifty control or \textit{T\textsubscript{1}} transgenic plants were tested. Values followed by the same letter within a column are not significantly different (ANOVA test: \(P > 0.05\)).

Figure 5. Phenotype of control plant (a) and \textit{LdGES} expressing plant 19-2 (b).
the native promoter and the ubiquitin one used in this study. The maize ubiquitin promoter is known to be active in most cell types of the leaf (Schunmann et al., 2004). The spatial expression pattern of the native geraniol synthase gene remains to be investigated once the gene has been cloned. We would predict that utilizing promoters which are more cell type-specific will reduce the number of different derivatives and conjugates found upon over-expressing terpene synthase genes in plants, and thus that could be one strategy for the metabolic engineering of more specific products.

The identification of the novel compound, geranoyl-6-O-malonyl-β-D-glucopyranoside, was made possible by applying a non-targeted LC-MS approach to analyze any changes in the non-volatile metabolite profile resulting from the expression of terpene synthases. In previous studies analyzing the non-volatile conjugates in plants overexpressing monoterpene alcohols, either indirect methods of detection were used, based on the release of aglycons by glycosidases, or targeted methods based on available standards were used. For example, Lücker et al. (2001) described glucosylation of monoterpenic alcohols with linalool synthase over-expression in petunia, but this was analyzed using a synthetic standard of (R,S)-linalyl β-D-glucopyranoside, and did not exclude the presence of additional, possibly more abundant glycosides. Commercial glycosidase treatments linked with GC-MS have been used to identify non-volatile glycosides of linalool in transgenic Arabidopsis and potato plants (Aharoni et al., 2003; Aharoni et al., 2006), however, both the nature of the original conjugations and the effectiveness of the method to hydrolyze all types of glycosides remained unknown. Thus, the presented non-targeted analysis is basic to know the metabolic fate of heterologously expressed terpenoids, although each non-targeted method is also limited by the extraction method, chromatography behavior and molecular ionization potential. As a result, also here, additional products cannot be excluded.

From a biochemical point of view, glycosyl conjugation reduces chemical reactivity of compounds (Von Rad et al., 2001), while malonyl conjugation can facilitate the transport of glycosylated compounds into the vacuole, a process mediated by ATP-binding-cassette transporters (Liu et al., 2001). Malonylation may also contribute to the water-solubility of the glycosylated compound and prevent glycolysis by glycosidases (Heller and Forkmann, 1994). Reduced susceptibility to glycosidases may have experimental and biological consequences. Firstly, the amount of glycosylated terpenoids reported by others in transgenic plants may have been underestimated, since this amount has been determined by measuring liberated terpenoids after glycolysis with commercial glycosidases (Aharoni et al., 2003; Aharoni et al., 2006), which much less effectively hydrolyze malonylated glycosides (Roscher et al., 1997; Ismael and Hayes, 2005). Secondly, malonylation may also compromise potential release of
Overexpressing geraniol synthase in maize

the aglycon by endogenous or fungal glycosidases and this could potentially result in reduced biological effects under biotic or abiotic stress.

From a physiological point of view, glycosyl and malonyl conjugations are considered to aid in the accumulation, storage or transport of secondary metabolites which may be phytotoxic (Hatzios, 1997; Crouzet and Chassagne, 1999). High concentrations of geraniol, for example, have been shown to be phytotoxic to maize, causing oxidative stress to membranes (Zunino and Zygacllo, 2004), but the compound potentially plays important biological roles in plant communication to attract beneficial insects (James, 2005) and repel some insect pests (Wei et al., 2004; Halbert et al., 2009). Temporary storage and subsequent, stress-induced enzymatic release of geraniol from its glycosides could, therefore, be used in maize as a defense strategy. Enzymatic release may not even be necessary: glycosides can also possess strong bioactivity themselves, as for example resveratrol glycosides against the alfalfa fungal pathogen Phoma medicaginis (Hipskind and Paiva, 2000). Here, we show that geraniol and geranic acid are both stored naturally in maize with additional hydroxylations and/or various glycosylations. This suggests similar potential roles of glycosides of geraniol or its derivatives in plant communication or defense against biotic agents.

The goal of this work was to engineer fungal resistance into transgenic maize by expressing a fungicidal concentration of geranic acid. The average concentration of geranoyl-6-O-malonyl-β-D-glucopyranoside in LdGES expressing maize leaves was around 17 µg g⁻¹ FW (45 µM). This concentration was in the range of previously reported levels of glycosides of heterologous terpenes in transgenic plants. For example, 5 to 10 µg g⁻¹ FW (14-28 µM) linalyl-β-D-glucoside has been found in petunia plants overexpressing a linalool synthase from Clarkia breweri (Lücker et al., 2001), and up to 110 µg g⁻¹ FW (643 µM) hydroxylinalool has been estimated to be present as glycosides in Arabidopsis plants overexpressing a linalool synthase from strawberry (Aharoni et al., 2003). In our experiments, resistance to fungal infections did not significantly improve, however, despite the fact that the average concentration of geranoyl-6-O-malonyl-β-D-glucopyranoside (45 µM) was in the same range as the MIC of geranic acid for both fungi (<46 µM). The reasons why we did not observe significant antibiotic effects in the fungal bioassays (Table 5) may, therefore, be the lack of bioavailability of the geranic acid and/or inappropriate subcellular localization of the compound in relation to the infection strategy of the fungus. The bioavailability could be improved by the down-regulation of genes involved in glycoside biosynthesis (Lochlainn and Caffrey, 2009) or by the up-regulation of genes producing other more bioactive glycosides of geranic acid (Freitag et al., 2006; Pickens and Tang, 2009). However, the corresponding glycon transferases and malonyl transferase genes have not been identified yet. Alternatively, the bioavailability could be improved by co-expression of an appropriate glycosidase either in the same subcellular compartment under an inducible promoter or constitutively in separate compartments if the fungal infection would destroy subcellular compartmentation. As mentioned above the release of geranic acid from malonyl-glucoside may be compromised by the presence of the malonyl group. Some glucosidases have been isolated, however, which can effectively release the aglycone also from malonylated glycosides, such as a malonylsterase from chickpea (Hinderer et al., 1986) and an isoflavone conjugate-hydrolyzing β-glucosidase from soybean (Suzuki et al., 2006). These could be used in future strategies to induce the release of the aglycone upon fungal infections.

No significant difference was detected between the headspaces of intact LdGES expressing and control plants (Fig. 1, a, b). This indicates that in the seedling all products of LdGES were stored in the plant tissues. However, detached leaves of LdGES expressing plants emitted 5-fold more geranyl acetate than control leaves (Fig. 1, c-e), suggesting that the release of geranyl acetate was wound-inducible in both LdGES expressing and control maize plants and
dependent on the relative availability of geraniol as substrate. Geranyl acetate has also been reported in the headspace of maize seedling after infestation by the caterpillars, *Spodoptera littoralis* (D'Alessandro and Turlings, 2006) and *Helicoverpa armigera* (Yan and Wang, 2006), but not in the headspace of undamaged control seedlings (D'Alessandro and Turlings, 2006; Yan and Wang, 2006). Together with other caterpillar-induced volatiles, geranyl acetate was suggested to be involved in plant direct or indirect defense to the caterpillars (D'Alessandro and Turlings, 2006; Yan and Wang, 2006), and *LdGES* expressing plants could be used, therefore, in further studies to establish the ecological function of geranyl acetate in maize-insect interactions. However, for this purpose also other genes involved in the geranyl acetate biosynthetic pathway or transcriptional factors controlling this pathway could help to control the production of geranyl acetate in maize to establish its role (Peebles et al., 2009; Peebles et al., 2010).

*LdGES* expressing maize plants all exhibited a completely normal phenotype and development (Fig. 5, Table 4), despite the fact that in other plant species severe phenotypes have been observed as a result of overexpression of monoterpene synthase genes. Arabidopsis (Aharoni et al., 2003) and potato (Aharoni et al., 2006) expressing a linalool synthase displayed bleached leaves and retarded growth. Tomato fruits expressing geraniol synthase failed to develop the normal deep red color, because of a 50% drop in lycopene content (Davidovich-Rikanati et al., 2007). In our *LdGES* expressing maize plants, several semi-polar compounds were significantly down-regulated, but could not be further identified due to their low signal intensity in the LC-MS analyses. The down-regulation of these compounds could represent the cost of overexpressing *LdGES*. However, the chlorophyll content of the transgenic lines examined was not affected (Table 4) and no obvious phenotype was observed (Fig. 5), suggesting that maize plants have sufficient levels of geranyl diphosphate to support the accumulation of the induced geraniol-derivatives as well as of the endogenous (essential) isoprenoids. Engineering of isoprenoids in maize is, therefore, a feasible option in future research programs.

**Acknowledgments**

We thank Jeff Robson for generating the maize transformation vector, Jay Gilliam for performing *in vitro* antifungal assays, Julie Ritland for plant disease resistance assays, Bert Schipper for assistance in LC-MS analysis, and Yury Tikunov for his help in MS data analysis. Ric de Vos acknowledges additional funding from the Centre for BioSystems Genomics (CBSG), which is part of the Netherlands Genomics Initiative.

**References**


Yan ZG, Wang CZ (2006) Similar attractiveness of maize volatiles induced by Helicoverpa armigera and Pseudaleata separata to the generalist parasitoid Campoletis chlorideae. Entomologia Experimentalis et Applicata 118: 87-96


Supplementary Methods

Cloning of full-length LdGES cDNA

The geraniol synthase gene, LdGES, was isolated from Lippia dulcis. Hereo, terpene synthase PCR-fragments were generated using reverse transcription PCR (RT-PCR) with degenerate primers based on conserved domains of monoterpene and sesquiterpene synthases (forward primer 5’-GAYGARAAYGGIAARTTYAARGA-3’ and reverse primer 5’-CCRTAIGCRTCRAAIGTRTCRTC -3’; indicated with arrows above the sequences in Fig. S1). Products of the expected size were sequenced and one fragment was found to be most similar to the geraniol synthase gene from Ocimum basilicum. This fragment was used as a template to design new gene specific primers for rapid amplification of cDNA ends (RACE). Finally, the full-length cDNA (GU136162) encoding the putative geraniol synthase gene was obtained by PCR based on the information obtained from sequencing of the 3’- and 5’-RACE products.

Heterologous expression of LdGES protein in Escherichia coli

For functional characterization of the LdGES protein, the entire reading frame was subcloned into the pRSET-A expression vector (Invitrogen) fused to an amino-terminal histidine tag, and expressed in Escherichia coli BL-21 under an isopropylthio-β-galactoside inducible promoter. His-tag purified proteins including those from the empty vector control were assayed for monoterpene synthase activity with geranyl diphosphate (GDP) and farnesyl diphosphate (FDP) as substrates, as previously described (Aharoni et al., 2004). The monoterpenes were analyzed by GC-MS using an HP 5890 series II gas chromatograph equipped with an HP5- MS column (30 m × 0.25 mm i.d., 0.25 µm d.f.) and HP 5972A Mass Selective Detector (Hewlett Packard, Agilent Technologies). The injection port (splitless mode), interface and MS source temperatures were 250 °C, 290 °C and 180 °C respectively. The injection volume was 2 µL. The oven was programmed at an initial temperature of 45 °C for 1 min, with a ramp of 10 °C min⁻¹ to 280 °C, and final time of 5 min. Scanning was performed from 39-500 amu. The helium inlet pressure was checked by electronic pressure control to achieve a constant column flow of 1.0 mL min⁻¹. Ionization potential was set at 70 eV.

Products were identified by comparing mass spectra to the Wiley mass spectra library and by calculating the Kovats Index (González and Gagliardi, 2000) of each peak based on the retention time relative to alkane standards.
**Figure S1.** Sequence alignment of LdGES (geraniol synthase from *Lippia dulcis*), ObGES (geraniol synthase from *Ocimum basilicum*; Iijima et al., 2004), CtGES (geraniol synthase from *Cinnamomum tenuipilum*; Yang et al., 2005), PcGES (geraniol synthase from *Perilla citriodora*; Ito and Honda, 2007), PfGES (geraniol synthase from *Perilla frutescens* strain 1864; Ito and Honda, 2007), PsGES (geraniol synthase from *Perilla setoensis*; Masumoto et al., 2010) and VvGES (geraniol synthase from *Vitis vinifera* cultivar Pinot Noir; Martin et al., 2010).
2010) using the ClustalX program. The LdGES gene accession number is GU136162. The GenBank accession numbers of ObGES, CtGES, PcGES, PfGES, PsGES and VvGES are AAR11765, CAD29734, DQ088667, DQ234300, FJ644545 and HM807399, respectively. The LdGES protein sequence shares 67% identity to ObGES, 35% identity to CtGES, 32% identity to PcGES, 32% identity to PfGES, 32% identity to PsGES and 48% identity to VvGES. Besides geraniol synthases mentioned above, they have also been cloned from Perilla frutescens var. crispa strain 79 (GenBank accession DQ897973), Perilla frutescens var. hirtella (GenBank accession FJ644547), Vitis vinifera cultivar Gewurztraminer (GenBank accession HM807398) and Vitis vinifera isolate CS5M2 (GenBank accession HQ326231). As geraniol synthases cloned from the same species share more than 97% homology, only one geraniol synthase from one species was shown in the alignment.

The DDXXD motif which is one of the most characteristic motifs of terpenoid synthases (Bohlmann et al., 1998) is indicated with box. The characteristic RRX₈W motif of monoterpene synthases (Bohlmann et al., 1998; Dudareva et al., 2003) is missing in LdGES, ObGES and VvGES but conserved in CtGES, PcGES, PfGES and PsGES(indicated with box). A horizontal line indicates the putative N-terminal transit peptide region. The domains used for designing degenerated primers to clone LdGES were indicated with arrows above the sequences.

Figure S2. GC-MS profile of the product from geranyl diphosphate catalyzed by LdGES expressed in E. coli. (a), GC chromatogram. (b)-1, Mass spectrum of the chromatographic peak at 11.16 min. (b)-2, Mass spectrum of geraniol in NIST library.

The product was identified by comparing mass spectra to the Wiley mass spectra library and by calculating the Kovats Index of each peak based on the retention time relative to alkane standards.

Figure S3. Structure of the binary vector containing the LdGES cDNA. RB: right border, LB: left border, 35S ENH: cauliflower mosaic virus 35S enhancer, UBIZMPRO: maize ubiquitin promoter, PIN II: potato protease inhibitor-2 terminator, MO PAT: synthetic maize-optimized phosphinothricin acetyltransferase gene (herbicide resistance).
Supplementary Table

Table S1 NMR data for geranoyl-6-O-malonyl-β-D-glucopyranoside.

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References
Chapter 4

Pyrethrins protect pyrethrum leaves against attack by western flower thrips, *Frankliniella occidentalis*

Ting Yang, Geert Stoopen, Gerrie Wiegers, Jing Mao, Caiyun Wang, Marcel Dicke and Maarten A. Jongsma

Abstract

Pyrethrins are active ingredients extracted from pyrethrum flowers (*Tanacetum cinerariifolium*), and are the most widely used botanical insecticide. However, several thrips species are commonly found on pyrethrum flowers in the field, and are the most dominant insects found inside the flowers. Up to 80% of western flower thrips (WFT, *Frankliniella occidentalis*) adults died within 3 days of initiating feeding on leaves of pyrethrum, leading us to evaluate the role of pyrethrins in the defense of pyrethrum leaves against WFT. The effects of pyrethrins on WFT survival, feeding behavior, and reproduction were measured both *in vitro* and *in planta* (infiltrated leaves). The lethal concentration value (LC50) for pyrethrins against WFT adults was 12.9 mg/ml, and pyrethrins at 0.1% (w/v) and 1% (w/v) had significantly negative effects on feeding, embryo development and oviposition. About 20-70% of WFT were killed within 2 days when they were fed chrysanthemum leaves containing 0.01-1% pyrethrins. Chrysanthemum leaves containing 0.1% or 1% pyrethrins were significantly deterrent to WFT. In a no-choice assay the reproduction of WFT was significantly reduced when the insects were fed leaves containing 0.1% pyrethrins, and no eggs were found in leaves containing 1% pyrethrins. Our results suggest that the natural concentrations of pyrethrins in pyrethrum leaves may be responsible for the observed high mortality of WFT on pyrethrum leaves.

**Key Words** Pyrethrum, Pyrethrins, Western flower thrips, *Frankliniella occidentalis*, Natural pesticide, toxicity, *Tanacetum cinerariifolium*.
Introduction

Western flower thrips (WFT), *Frankliniella occidentalis*, is a highly polyphagous insect causing both direct and indirect effects on plant development and health. The adults and larvae feed on epidermal and subepidermal cells of both meristematic and mature leaf and flower tissues, inhibiting plant growth and development and causing necrotic or light-reflective blotches on the tissue. Furthermore, they indirectly damage plants by transmitting tospoviruses such as tomato spotted wilt virus (Reitz, 2009). As a result, WFT has become the most serious pest in several vegetable and flower crops world-wide (Daughtrey et al., 1997; Reitz, 2009). The widespread use of chemical insecticides to control WFT has led to increasing resistance against the major classes of synthetic insecticides (Broadbent and Pree, 1997; Flanders et al., 2000; Broughton and Herron, 2009). The growing awareness and demand for insecticides that are not environmentally hazardous has stimulated the study of plant-derived compounds for pest control (Boeke et al., 2004). Such compounds could be used as natural pesticides, and in theory genes responsible for the biosynthesis of those compounds could be isolated and transferred to other crops to improve plant defense against WFT (Annadana et al., 2002; Ouchkourov et al., 2004).

Among the sources of botanical pesticides, pyrethrins from pyrethrum plants (*Tanacetum cinerariifolium*) represent the economically most important class of compounds with broad usage both in homes and organic agriculture (Casida, 1973). They are neurotoxins which bind to voltage-gated sodium channels of neuronal cells, causing the channels to remain open (Davies et al., 2007). Pyrethrins comprise a group of six closely related esters, named pyrethrin I and II, cinerin I and II, and jasmolin I and II. They are found in all aboveground parts of the pyrethrum plants, but predominantly in the ovaries of the flower heads (Brewer, 1973). On average, the concentration of pyrethrins is about 0.1% (dry weight) in leaves and 1-2% (dry weight) in flowers (Baldwin et al., 1993). Assuming a water content of 90%, pyrethrins account for around 0.01% of the fresh weight of leaves and 0.1-0.2% of the fresh weight of flowers. Pyrethrins are effective against a broad spectrum of insects while their toxicity for mammals is very low, allowing their use as a preharvest spray (Casida and Quistad, 1995; Schoenig, 1995). WFT are sensitive to synthetic pyrethroids (Thalavaisundaram et al., 2008), but there is no report on the effect of natural pyrethrins against WFT. Pyrethrins could provide pyrethrum with a broad range protection against many different insect pests, but the role of pyrethrins in pyrethrum defence has not been studied.

In initial experiments, we observed that WFT adults died within one day when fed pyrethrum leaves, but that they are abundant in open flowers. Here, we test the hypothesis that pyrethrins are responsible for protecting pyrethrum leaves against WFT by assessing adult and embryo toxicity, and by examining feeding and oviposition deterrence both *in vitro* and *in planta*.

Methods and Materials

*Field Observation* A pyrethrum field close to Luxi, Yunnan province, China, was used for surveying thrips populations (24°27'10.34"N-103°32'21.01"E). The field was 0.5 ha in size and the presence of insect species was monitored during the flowering period of spring 2010, when the flowers were predominantly in developmental stages 2-5 [numbered according to Casida (1973)]. To assess populations of small resident insects including thrips, flowers at each developmental stage were collected in each one of three blocks of the field. Each flower was taken by the stem and turned upside down into a jar containing 75% alcohol. Flowers were fully immersed and vigorously stirred. The procedure was repeated until each jar contained the insects from 100 flowers from a single block and at a particular stage. The
number of insects of each species for each stage was scored. In the case of thrips, the number of adults and larvae were scored separately. Among all collected thrips, 30 were randomly picked and identified, where possible to the species level.

*Insects and Plant Material Used in Laboratory Experiments*  
A population of western flower thrips (WFT) was mass-reared on flowering chrysanthemum (*Chrysanthemum morifolium* Ramat.) cv. Sunny Casa in a greenhouse under a photoperiod of L16:D8 at 25±2 °C. In this study, only adult female thrips were used. The chrysanthemum plants used for bioassays were from the same cultivar, but were grown in an insect-free compartment of the greenhouse under the same light and temperature conditions. All bioassays were conducted in a climate room at 20-22 °C with a L16:D8 photo regime.

*Insecticide*  
Pyrethrum oil (70% w/w) was obtained as a gift from Honghe Senju Biological Co. Ltd., Yunnan, China and had been extracted from dried and ground pyrethrum flower heads with liquid CO$_2$ leaving no solvent residue. Butylated hydroxytoluene (BHT) had been added to the oil (1%) to prevent oxidation. We confirmed the concentration and composition of the oil by Gas Chromatography-Mass Spectrometry comparison to a pyrethrin standard (Nguyen et al., 1998). Since the major insecticidal compounds in pyrethrum have long been known as pyrethrins (Casida, 1973), the effect of pyrethrum oil was considered to be the effect of pyrethrins. When calculating the concentrations of pyrethrins in different solutions, the percentage of pyrethrins in the oil (70%) was taken into account. For example, 1% (w/v) pyrethrins was prepared by dissolving 14.3 mg pyrethrum oil in 1 ml solvent.

*In vitro Bioassays-Toxicity Assays*  
The toxicity of pyrethrins was evaluated by topical application to thrips (Robb et al., 1995). Pyrethrum oil was dissolved in acetone to achieve a concentration range of 1 to 30 mg pyrethrins per ml, and the solutions were applied to the thorax with a 10-µl glass syringe at 1 µl per thrips. The droplet briefly covered the thorax of the insect and also the paper support before evaporating in a few seconds, leaving a residue both on the insect and the support. Acetone alone was used as control. After treatment, all thrips were transferred to Petri dishes containing a piece of chrysanthemum leaf embedded in an agar substrate. Mortality was assayed after 24 h by counting the number of insects that did not respond to prodding with a fine brush. Six replicates were used for each concentration and 10 thrips were used per replicate. Percent mortality was corrected for mortality observed in acetone control using Schneider-Orelli’s formula (Schneider-Orelli, 1947). Data were analyzed using probit analysis (Finney, 1977).

*In vitro Bioassays-Choice Assays with Topically Applied Pyrethrins*  
A dual-choice leaf disk assay was used to determine the deterrent effect of pyrethrins on WFT. All leaf disks (diameter 1.6 cm) were punched from chrysanthemum leaves of similar leaf age. Pyrethrum oil was dissolved in 0.2% (v/v) aqueous Tween-80 to achieve 3 concentrations of pyrethrins: 0.01%, 0.1% and 1% (w/v). Control leaf disks were sprayed with solvent solution (0.2% Tween-80) and test leaf disks were sprayed with the pyrethrin solutions using a Potter Precision Laboratory spray tower, which produced a uniform deposit (3 µl/cm$^2$) of solution on the leaf disks. After overnight starvation, WFT were anaesthetized on ice. Groups of 10 WFT were positioned between a control and a test leaf disk placed abaxial side up and 2 cm apart on a 1.5% (w/v) agar-bed in a Petri dish (7 cm diameter). After positioning the thrips, the Petri dish was covered by a 120 µm mesh size nylon mesh lid to prevent condensation. The number of WFT on each leaf disk was recorded 0.25, 1, 2, 4, 20 and 28 h after the release of the WFT. Each concentration was replicated with 12 leaf disks. At each time point, a Student’s paired $t$-test was used to assess the significance of the differences in the mean number of WFT between test and control.
In vitro Bioassays-Oviposition Assays Oviposition-deterrent effects were assayed with a non-choice method slightly modified from Annadana et al. (2002). The assay was conducted in Perspex ring cages (3 cm in length and 3.5 cm diameter), which were closed with a nylon mesh at the bottom. Pollen of Scotch pine (*Pinus sylvestris* L.) was supplied in a small open tube as food source for WFT. After placing 10 WFT in a cage, the top was sealed with two layers of stretched Parafilm, with 300 µl aqueous solution in between the layers. The solutions used were water, 0.2% Tween-80 or pyrethrins at 0.01%, 0.1% or 1% dissolved in 0.2% Tween-80. WFT were allowed to adapt to the diet (pollen and water) for 3 days, and then every day for 5 days fresh test solution was provided. All eggs were deposited in the solutions, and counted daily under a binocular microscope. Each solution was replicated 6 times. Data were analyzed by a one-way ANOVA and a mean separation test was conducted using LSD (α=0.05).

In vitro Bioassays-Embryo Development Assays Around 200 WFT were kept in a Perspex ring cage (7 cm in length and 9 cm diameter) to allow oviposition in a water solution as described above. Eggs laid on the same day were collected with a fine brush under a binocular microscope and then transferred to 2 layers of filter paper in Petri dishes (3.5 cm diameter). The filter papers were drenched in 300 µl of assay solution (water, 0.2% Tween-80 or pyrethrins at 0.01%, 0.1% or 1% in 0.2% Tween-80) so that each paper was fully wetted but had no excess solution. After transferring the eggs, the Petri dishes were closed with lids and sealed with Parafilm. The developmental status of eggs was monitored every day for 6 days. To facilitate the observations, the bottoms of the Petri dishes were marked with lines which could be seen through the filter paper from the top, and the eggs were placed on filter paper along these lines. This facilitated finding the eggs under the microscope and the viability of hatched larvae was assessed in terms of their ability to move away (>0.5 cm) from the hatch position. Four replicates of 10 eggs were used for each assay. Data were analyzed by a one-way ANOVA and mean separation test was conducted using LSD (α=0.05).

In planta Bioassays-Mortality Assays on Pyrethrum Leaves Mature pyrethrum leaves were harvested in November from a field in the Netherlands when they were still flowering (51°59'22.08"N-5°39'44.75"E, Wageningen). Two or three pieces of leaves were placed, abaxial side up, on 1% (w/v) agar in a Petri dish (7 cm diameter). After transferring 10 WFT to each Petri dish, dishes were covered with lids with gauze. Petri dishes with two leaf disks (diameter 1.6 cm) of chrysanthemum leaves, with a total mass similar to the mass of the pyrethrum leaf samples, or with only agar were used as controls. Six replicates were carried out for each treatment. The mortality of WFT was recorded daily for three days.

In planta Bioassays-Choice Assays To test the *in planta* activity of pyrethrins against WFT, pyrethrins were infiltrated into whole chrysanthemum leaves as described by Ratcliff et al. (2001). Leaf disks (diameter 1.6 cm) were punched from the infiltrated leaves, avoiding the infiltration points so that WFT would not contact pyrethrins directly, except at the edge of the disk. In the initial experiments, we infiltrated only water into the leaves and determined that on average 29.1 mg (± 2.1 mg) water could be infiltrated into each leaf disk (6 replicates). As the fresh weight of each leaf disk was on average 45.3 mg (± 1.2 mg), we infiltrated 0.025%, 0.25% or 2.5% pyrethrins solution to bring the concentrations to 0.01%, 0.1% or 1% pyrethrins. Leaf disks infiltrated with 0.2% Tween-80 were used as control. The assay and data analysis were conducted as described above for the choice assays with topically applied pyrethrins. The number of WFT on each leaf disk was recorded 0.25, 1, 2, 4, 20 and 28 h after the release of the WFT.

In planta Bioassays-Reproduction Assays To test the effects of pyrethrins on oviposition and hatching of larvae, WFT were assayed with chrysanthemum leaf disks as described by De Kogel et al. (1997), with slight modifications. Leaf disks were punched from untreated leaves,
from leaves infiltrated with 0.2% Tween-80, or from leaves containing 0.01%, 0.1% or 1% pyrethrins in Tween solution. WFT were placed on leaf disks (1.2 cm diameter, 2 WFT/disk) which were embedded, abaxial side up, on agar in wells of 24-well Greiner plates. Plates were covered with Parafilm and every well was carefully sealed by pressing the Parafilm on the edge of each well. WFT were allowed to oviposit for 48 h and were then removed, with simultaneous assessment of mortality. Subsequently, half of the leaf disks from each plate were used to determine the number of eggs, and the other half of the leaf disks were used to determine the number of hatched larvae. To determine the number of eggs, the leaf disks were boiled in water for 3 min so that the eggs were clearly visible under a binocular microscope with transmitting light. To determine the number of hatched larvae, the leaf disks were transferred to Petri dishes containing water and incubated in a climate chamber (25 °C, L16:D8) for 5 days to allow the larvae to hatch. The hatched larvae were counted under a binocular microscope. The 24 leaf disks in the same plate were received the same treatment. One plate was used for each treatment. Data were analyzed by a one-way ANOVA and mean separation test was conducted using LSD (α=0.05).

Results

**Natural Distribution of Insects in Pyrethrum Fields**

Our field survey in China showed that several thrips species were the most abundant (98%) insects on pyrethrum flowers (Table 1). In addition, a few *Nysius* species (Heteroptera: Lygaeidae) (1.9%) and lacewing larvae (Neuroptera) (0.05%) were found. A total of 30 individuals were identified to species level; the thrips species found were mainly *Thrips tabaci* (44%), *Frankliniella occidentalis* (western flower thrips, or WFT, 25%) and *Thrips flavus* (22%). The number of thrips in flowers was dependent on the flower’s developmental stage (Fig. 1). The number of thrips increased until stage 3 (the first row of disk florets are open), and then decreased in later stages. The thrips found inside flowers were mainly adults. Larvae accounted for 7-26% of the total number of thrips per flower, depending on the flower developmental stage (Fig. 1).

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* A total of 1200 insects were collected to count the frequencies of different insects. * A total of 30 thrips were used to identify species. N.d., not determined.

**Effect of Pyrethrum Leaves on Mortality of WFT**

We assayed the suitability of pyrethrum leaves as a food substrate for WFT. Mortality could be as high as 80% within 3 days, although the degree of mortality depended on the plant source (data not shown). When only water and agar were provided, with no plant-based food, only 20-30% WFT died in 3 days. All WFT feeding on control chrysanthemum leaves remained alive during the 3-day-experiment. This showed that the mortality of WFT was caused by a toxic principle of pyrethrum leaves rather than deterrence or starvation.

The toxic principle of pyrethrum plants against insects has long been known to be a group of 6 pyrethrin esters (Casida, 1973). We were, therefore, interested in specifically testing the effect of pyrethrins against WFT.
**In vitro Insecticidal and Deterrent Effects** To determine the effects of pyrethrins against WFT, pyrethrins were tested *in vitro* at different concentrations on adult mortality, feeding, oviposition, and embryo development.

The mortality of WFT female adults increased with the concentration of topically applied pyrethrins in the range of 1 to 30 mg/ml. Probit analysis showed that the LC$_{50}$ and LC$_{90}$ of pyrethrins are 12.9 mg/ml (with 95% confidence limit of 10.9-14.8 mg/ml) and 39.0 mg/ml (with 95% confidence limit from 30.7 to 50.4 mg/ml).

Thrips were significantly deterred from feeding by 0.1% and 1% pyrethrins (Fig. 2). When given a choice between chrysanthemum leaf disks coated with 0.2% Tween (control) or 0.1% added pyrethrins, after 2 hours significantly more (61-77% of thrips) settled on control leaf disks. Pyrethrins at 1% were more highly deterrent. Within one hour 72-90% of thrips chose control leaf disks. For both concentrations of pyrethrins, the maximum deterrent effect was reached at 4 h. Application of 0.01% pyrethrins on leaf disks did not show significant deterrent effects except at the 4 h time point (Fig. 2).

Pyrethrins negatively affected oviposition by WFT (Fig. 3). The carrier, 0.2% Tween-80, did not affect the oviposition of thrips compared to water throughout the experiment, but WFT oviposited significantly fewer eggs with increasing pyrethrin concentrations during the 5-day experiment (Fig. 3).

The development of eggs was inhibited by 0.1% and 1% pyrethrins. About 80% of larvae hatched when the eggs were incubated with water, 0.2% Tween or 0.01% pyrethrins, while only 28% or 6% of the larvae hatched when the eggs were incubated with 0.1% or 1% pyrethrins.
pyrethrins, respectively (Fig. 4). In the latter two treatments, the embryos that did not develop into larvae had severely stunted and abnormal shapes (Fig. 5), and dried out after a few days.

In planta Insecticidal and Deterrent Effects To study in planta activity of pyrethrins against WFT, thrips were assayed with chrysanthemum leaves that had been infiltrated with pyrethrins to contain 0.01%, 0.1% or 1% pyrethrins. In this experiment, the pyrethrins could not be contacted directly by thrips except by feeding, and the source of nutrition consisted of leaves instead of pollen.

In the reproduction assay, thrips fed with chrysanthemum leaf disks containing pyrethrins exhibited higher mortality and lower reproduction rates compared to those fed with untreated leaf disks or leaf disks containing 0.2% Tween (Table 2).

In the dual-choice assay chrysanthemum leaves containing 0.1% and 1% pyrethrins showed significant deterrent effects on thrips within 15 min of release (Fig. 6). A total of 74-93% of the thrips chose to settle on the control leaf disk when the other leaf disk contained 0.1% pyrethrins, and 85-95% thrips chose to settle on the control leaf disk when the other leaf disk
Pyrethrins against thrips

Table 2: Mortality, number of eggs and hatched western flower thrips larvae per leaf disk on chrysanthemum leaf disks infiltrated with different concentrations of pyrethrins

<table>
<thead>
<tr>
<th>Treatment of leaf disks</th>
<th>Mortality (%)</th>
<th>Eggs</th>
<th>Hatched larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated leaf disks</td>
<td>0 a</td>
<td>2.0±0.4 a</td>
<td>1.4±0.3 a</td>
</tr>
<tr>
<td>Leaf disks containing 0.2% Tween</td>
<td>0 a</td>
<td>1.7±0.5 a</td>
<td>1.3±0.3 ab</td>
</tr>
<tr>
<td>Leaf disks containing 0.01% pyrethrins</td>
<td>25.0±6.7 b</td>
<td>1.3±0.3 ab</td>
<td>0.8±0.2 b</td>
</tr>
<tr>
<td>Leaf disks containing 0.1% pyrethrins</td>
<td>29.2±7.9 b</td>
<td>0.7±0.2 bc</td>
<td>0.1±0.1 c</td>
</tr>
<tr>
<td>Leaf disks containing 1% pyrethrins</td>
<td>68.8±9.3 c</td>
<td>0 c</td>
<td>0 e</td>
</tr>
</tbody>
</table>

Values (mean±SE, n = 48 per treatment) followed by the same letter within a column are not significantly different (ANOVA: P>0.05).

Discussion

Pyrethrins, well-known natural insecticidal compounds, are exclusively found in and extracted from the composite flowers of pyrethrum (*Tanacetum cinerariifolium*), which belongs to Anthemideae tribe within the Astaraceae family (Casida and Quistad, 1995). Remarkably, the potential role of pyrethrins in pyrethrum plant defence has not been studied. Here, we report that western flower thrips (WFT) adults thrive on pyrethrum flowers, but die within a few days on pyrethrum leaves. The hypothesis that pyrethrins are responsible for protecting pyrethrum leaves against WFT was tested by spraying or infiltrating pyrethrins to leaves of chrysanthemum, a related pyrethrins-free species belonging to the same tribe. We assessed toxicity to the adult and embryo stages of WFT, and negative effects on feeding and oviposition both in vitro and in planta, and found that the natural concentrations of pyrethrins present in leaves have strong negative effects on WFT. We speculate that the thrips found on pyrethrum flowers survive on pollen which is devoid of pyrethrins (T. Yang, unpublished data).

For many populations of WFT, resistance has been reported for some synthetic insecticides (Espinosa et al., 2005). Furthermore, many synthetic insecticides are considered harmful for human health and the environment. It is relevant, therefore, to find natural insecticides effective against WFT. Previously, several other plant-derived compounds were tested for their insecticidal effects against WFT adults. For example, carvacrol at 1% and thymol at 0.1% and 1% significantly reduced the oviposition rate of WFT when these compounds were sprayed on leaf disks, but neither compound affected the feeding activity of WFT (Sedy and Kosehier, 2003). Salicylaldehyde (0.1% and 1%) and methyl salicylate (0.1% and 1%) were also tested. Within 24 h of applying 1% methyl salicylate to bean or cucumber, the feeding and oviposition activities of thrips females were significantly reduced (Koschier et al., 2007). The effect on the insect could be a result of changes in the plant induced by methyl salicylate, since it is a plant hormone involved in induced resistance (Pieterse et al., 2009). A series of commercially available plant-derived essential oils tested at recommended concentrations (0.02-0.5%), including neem oil, rosemary oil, peppermint oil, garlic oil, and cottonseed oil, caused less than 30% mortality within 7 days (Cloyd et al., 2009). Compared to other plant-derived compounds, pyrethrins are highly effective against WFT. Our results showed that 0.1% and 1% pyrethrin solutions sprayed on leaf disks significantly deterred WFT at 4 h, and, topically applied pyrethrins were toxic to adults at an LC₅₀ value of 12.9 mg/ml (1.3%). By mimicking the natural site of pyrethrin accumulation by infiltration of leaves, we found that 1% pyrethrins caused 69% mortality and completely inhibited oviposition. Furthermore, 0.1% pyrethrins was strongly deterrent and resulted in abortion of 95% of the embryos while as little as 0.01% pyrethrins caused 25% mortality in 2 days. We propose, therefore, that the...
natural concentrations of pyrethrin in pyrethrum leaves, around 0.01% by fresh weight, accounts for the observed high mortality of thrips adults on this plant.

Insecticides have not previously been reported to affect the development of WFT embryos. WFT eggs are embedded in plant tissues (Childers, 1997), and as a result they are unlikely to be affected by non-systemic chemicals that are applied on the surface of plants. However, pyrethrins naturally accumulate inside pyrethrum tissues, stored in what appear to be unstructured intercellular cavities (M.A. Jongsma, unpublished observations). Therefore, besides feeding and oviposition deterrence, the embryo-toxic effect of pyrethrins is a third component contributing to their effect for plant defence against WFT (Fig. 4 and 5).

Compared to some synthetic insecticides, the toxicity of natural pyrethrins in the absence of synergists against WFT was not high. In previous studies using topical application methods, the LC$_{50}$ values of insecticides tested against susceptible WFT strains ranged from 10 to 83 µg/ml for pyrethroids, 20 to 960 µg/ml for carbamates and 49 to 522 µg/ml for organophosphates (Robb et al., 1995; Espinosa et al., 2005). The LC$_{50}$ value of pyrethrins against WFT by topical application was determined as 12.9 mg/ml, and the action of pyrethrins was, therefore, 10 to 1000-fold weaker than for these synthetic pesticides. On the other hand, pyrethrins did show much stronger negative effects on feeding behaviour and reproduction, which can be explained by the action of pyrethrins on the nervous system, resulting in disordered function of excitable (nerve and muscle) cells (Bradberry et al., 2005). At 0.01% (100 µg/ml), pyrethrins not only caused mortality of adults and embryos, but also significantly reduced oviposition (Table 2). All those factors together cumulatively affect the life history parameters. As a result WFT damage on pyrethrin-containing leaves may be virtually absent and also virus transmission may be strongly reduced. We hypothesize that if plants such as closely related chrysanthemum species which do not contain any pyrethrins were genetically engineered to produce pyrethrins, their resistance to WFT in leaves could be significantly improved.

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Chapter 4
Chapter 5

Chrysanthemyl diphosphate synthase operates in planta as a bifunctional enzyme with chrysanthemol synthase activity

Ting Yang, Liping Gao, Geert Stoopen, Maarten A. Jongsma

Submission in preparation
Abstract

Chrysanthemyl diphosphate synthase (CDS) is the first pathway-specific enzyme in the biosynthesis of pyrethrins, the most widely used plant-derived pesticide. CDS catalyzes 1'-2-3 cyclopropanation reactions of two molecules of dimethylallyl diphosphate (DMAPP) to yield chrysanthemyl diphosphate (CPP). Three proteins are known to catalyze this cyclopropanation reaction of terpene precursors. Two of them, phytoene and squalene synthase, are bifunctional enzymes with both prenyltransferase and terpene synthase activity. CDS, the other member, was reported to perform only the prenyltransferase step. Here, we show that, under lower substrate conditions prevalent in plants, CDS also catalyzes the next step converting CPP into chrysanthemol by hydrolyzing the diphosphate moiety. The enzymatic hydrolysis reaction followed conventional Michaelis-Menten kinetics, with a $K_M$ value for CPP of 196 µM. For the chrysanthemol synthase activity, DMAPP competed with CPP as substrate. The DMAPP concentration required for half-maximal activity to produce chrysanthemol was ~100 µM, and significant substrate inhibition was observed at elevated DMAPP concentrations. The N-terminal peptide of CDS was identified as a plastid targeting peptide. Transgenic tobacco plants overexpressing CDS emitted chrysanthemol at a rate of 0.12 – 0.16 µg·h$^{-1}$·g$^{-1}$ FW. Chrysanthemol glycosides were also detected and the major one was putatively identified as chrysanthemol conjugated to malonyl-glucose. We propose that CDS should be renamed a chrysanthemol synthase (CHS) utilizing DMAPP as substrate.
Introduction

Pyrethrins are found predominantly in pyrethrum flower heads (Tanacetum cinerariifolium), and represent the economically most important natural pesticide (Brewer, 1973; Casida, 1973). They are neurotoxins effective against a wide range of insect species, and broadly applied in private homes, gardens, stables and organic agriculture. Their low toxicity to warm-blooded animals and high degradability under sunlight gives them environmentally friendly properties (Casida and Quistad, 1995).

Pyrethrins comprise a group of 6 closely related esters with either chrysanthemic acid (type I esters) or pyrethric acid (type II esters) as terpene acid moieties. Chrysanthemic and pyrethric acid share a common cyclopropane ring structure, but the latter possesses a terminal carboxymethyl group. The acid moieties were shown to be predominantly derived from the methyl-erythritol phosphate (MEP) pathway (Crowley et al., 1961; Matsuda et al., 2005). The MEP pathway provides two universal C5 terpene building blocks, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), in the plastids of plants. Monoterpenes (C10), diterpenes (C20) and carotenoids (C40) are synthesized via this pathway (Wu et al., 2006a). The first pathway-specific step for pyrethrin biosynthesis is the condensation of 2 molecules of DMAPP yielding chrysanthemyl diphosphate (CPP) (Fig 1A) (Epstein and Poulter, 1973). The gene responsible for this step was cloned and the recombinant enzyme was characterized as chrysanthemyl diphosphate synthase (CDS; EC 2.5.1.67) (Rivera et al., 2001). The genes and enzymes involved in the next steps converting CPP into the monoterpene alcohol, chrysanthemol, and the further oxidation to chrysanthemic acid have not been identified yet, but recently the reactions were shown to occur in the glandular trichomes (Ramirez et al., 2012). The final step of the esterification of chrysanthemic acid with one of the lipid alcohols is performed by a recently cloned GDSL lipase from pyrethrum. This enzyme transfers the chrysanthemoyl group from the CoA thioester to pyrethrolone to produce pyrethrin I, but does so in the pericarp of the seeds and not in the glandular trichomes (Kikuta et al., 2012; Ramirez et al., 2012).

CDS, the enzyme catalyzing the first pathway-specific step, evolved from farnesyl diphosphate synthase (FDS) (Rivera et al., 2001; Thulasiram et al., 2007; Liu et al., 2012). It takes two molecules of DMAPP as substrate to produce CPP as major product and lavandulyl diphosphate (LPP) as minor product (Fig. 1). CDS has been cloned and characterized from pyrethrum (Tanacetum cinerariifolium) (Rivera et al., 2001) and sagebrush (Artemisia tridentata ssp. spiciformis) (Hammerlin et al., 2003), but recently CDS-like genes were also reported from Tanacetum coccineum, Achillea asiatica, chrysanthemum lavandulifolium, Aster ageratoides, Helianthus exilis and Helianthus annuus (Liu et al., 2012). For sagebrush, the CDS protein sequence shares 75% identity (and 96% similarity) with the FDS protein sequence from the same species. FDS catalyses the sequential c1’-4 condensation of isopentenyl pyrophosphate with DMAPP to form the intermediate geranyl diphosphate (GPP, C10), and with another IPP, the product farnesyl diphosphate (FPP, C15), or it catalyses c1’-4 condensation of GPP with IPP to yield FPP (Szkopińska and Płochocka, 2005). CDS catalyses the c1’-2-3 condensation of 2 units of DMAPP yielding CPP (C10) (Rivera et al., 2001). Thus, compared to FDS, the product size of CDS has shifted from C15 to C10, and the enzyme activity of CDS has changed from chain elongation to cyclopropanation.

The c1’-2-3 cyclopropane ring structure in CPP is also found in the C30 and C40 terpenoids, presqualene diphosphate (PSPP) and prephytoene diphosphate (PPPP). PSPP and PPPP are intermediates in reactions producing squalene and phytoene, which are precursors for biosynthesis of sterols and carotenoids. These reactions are catalyzed by squalene synthase (EC 2.5.1.21) and phytoene synthase (EC 2.5.1.32), respectively (Dogbo et al., 1988).
Squalene synthase catalyzes the condensation of two molecules of farnesyl diphosphate (FPP) to give PSPP, and subsequent reductive rearrangement of this intermediate generates squalene (Nakashima et al., 1995). Phytoene synthase catalyzes a similar reaction, taking geranylgeranyl diphosphate (GGPP) as substrate to give PPPP as intermediate and phytoene as final product (Dogbo et al., 1988). Notably, both these terpenoid synthases are bifunctional enzymes catalyzing consecutive prenylation and dephosphorylation steps (Radisky and Poulter, 2000; Iwata-Reuyl et al., 2003).

Our preliminary experiments with cell lysate of E. coli expressing CDS showed that the lysate was able to produce chrysanthemol directly from DMAPP, while cell lysate of E. coli transformed with the empty vector was not able to convert CPP to chrysanthemol. This suggested that CDS could also be a bifunctional enzyme catalyzing 2 consecutive reactions from DMAPP to CPP and then to chrysanthemol (Fig 1A). We now report the characterization of CDS to synthesize chrysanthemol from CPP and DMAPP in vitro, and the confirmation of this claim in planta using transgenic tobacco plants.

Results

Sequence determination of full-length cDNA of CDS. In earlier work the start codon of pyrethrum CDS was not determined (Rivera et al., 2001). This motivated us to study the 5'-end sequence of CDS cDNA using RACE PCR. Eight 5'-end sequences (Fig. S1) obtained by us were quite different from the 5'-end sequence was previously reported CDS [Accession number: I13995, (Rivera et al., 2001)]. We then determined the genomic DNA (gDNA) sequence of CDS (Accession number: JX913537) from pyrethrum to see whether alternative splicing could explain these differences. By comparing the cDNA and gDNA sequences, an intron was easily identified at the 5'-end. The putative start codon proposed by Rivera and coauthors is actually in this intron region. Twenty-four nucleotides corresponding to the first 8 amino acids of CDS reported by Rivera were the intron sequence. The rest of the coding sequence of CDS from Rivera is nearly 100% the same as obtained by us, with only 1 bp difference and no effect on the protein sequence. We also found that one out of our eight sequenced 5'RACE products was not correctly spliced either (Fig. S1). Apparently, Rivera and co-authors cloned this low frequency mRNA product. The CDS used in this study was our own clone isolated from pyrethrum ovaries (Accession number JX913536).

Enzymatic characterization of CDS with CPP. CDS takes dimethylallyl diphosphate (DMAPP) as substrate to produce chrysanthemyl diphosphate (CPP) (Rivera et al., 2001). Surprisingly, when CPP was provided, CDS converted CPP into chrysanthemol (Fig. 1B). The reaction followed standard Michaelis-Menten kinetics (R = 0.997) with apparent $K_M$ and $k_{cat}$ values of $196 \pm 23 \ \mu M$ and $3.3 \pm 0.2 \times 10^{-3} \ \text{min}^{-1}$, respectively (Fig. 2A). Chrysanthemol was not detected in the control assay incubated with CPP for 20 h (Fig. 1B).
**Product analysis of CDS with DMAPP.** The conversion of CPP into chrysanthemol suggested that CDS supplied with DMAPP should also produce chrysanthemol. However, no formation of chrysanthemol was detected when CDS was assayed with 2 mM DMAPP for 2 h (Rivera et al., 2001) or upon longer incubation for 6, 12 and 24 h.

We then decided to prolong the assay time to 96 h and lower the DMAPP concentration. First we made sure that CDS was active during the 96-h assay. CDS was incubated with 400 µM CPP and chrysanthemol production was checked every 24 h. A linear correlation was found between chrysanthemol production and assay hours with $R^2=0.995$ (Fig. S2), indicating that CDS was fully active during this 96-h assay. The DMAPP concentration was then lowered from 2000 to 600 µM, which is the previously published $K_M$ value of CDS for DMAPP to produce CPP (Rivera et al., 2001). Figure 1B shows that under those modified assay conditions chrysanthemol, as well as lavandulol, were detected in the 96-h assay. However, by incubating the same water phase with alkaline phosphatase, also significant concentrations of CPP and lavandulyl diphosphate (LPP) were found in the assays. Clearly, a quantitative approach was necessary to establish the balance in the production of chrysanthemyl and lavandulyl diphosphates and alcohols. For this, a series of DMAPP concentrations ranging from 10 to 600 µM were assayed with CDS for 96 h and both terpene alcohol and phosphate production was determined (Fig. 2B). Chrysanthemol production increased with DMAPP concentration up to 300 µM (Fig. 2B, top). Further increase of DMAPP concentration led to a decrease of chrysanthemol, but strong further increase of CPP production. More chrysanthemol than CPP was detected with DMAPP concentrations ranging from 40 to 150 µM (Fig. 2B, top). The DMAPP concentration required for half-maximal activity to produce chrysanthemol was ~100 µM and at that concentration on a molar basis at least twice as much chrysanthemol was formed compared to CPP. On the other hand, CPP production increased.
with DMAPP concentrations up to the tested maximum of 600 µM (Fig. 2B, top). To check non-enzymatic conversion of CPP into chrysanthemol, control reactions without enzymes were performed at CPP concentrations ranging from 2 to 80 µM. This range covered the CPP production in 96-h assays, which produced a maximum of 25 µM CPP with DMAPP. However, no chrysanthemol was detected in any of the control reactions.

The relationship between DMAPP substrate concentration and lavandulol or LPP production was very similar to chrysanthemol and CPP (Fig. 2B, bottom). Lavandulol production was highest with 150 µM DMAPP and decreased with higher concentrations of DMAPP, while LPP production increased with DMAPP concentrations up to 600 µM.

**Chrysanthemol production with CPP is inhibited by DMAPP.** The above experiments suggested that higher concentrations of DMAPP substrate caused inhibition of chrysanthemol production. We, therefore, assayed CDS activity with both CPP and DMAPP as substrates in the same assay tube. In all assays, CPP concentration was kept constant at 150 µM (around the $K_M$ value of CDS for CPP) while DMAPP concentrations were varied from 0 to 600 µM. In general, addition of DMAPP to the assays caused a decrease of chrysanthemol production, and this effect was significant from 75 µM upward (Fig. 2C). The chrysanthemol production was not inhibited when 10 to 40 µM DMAPP was added.
**Subcellular localization of CDS.** The N-terminal sequence of CDS was predicted to serve as a targeting signal to plastids (Rivera et al., 2001). To test the prediction, we analyzed the targeting with gene fusions to green fluorescent protein (GFP). The gene fragment corresponding to the first 54 amino acids or the whole cDNA without stop codon were fused to GFP and transferred to tobacco (*Nicotiana tabacum*) protoplasts. Transient expression of the fused GFP in tobacco cells showed that both the N-terminal signal peptide and the complete CDS protein targeted GFP to plastids (Fig. 3).

**Headspace emissions of transgenic tobacco overexpressing CDS.** To verify the function of CDS *in vivo*, its cDNA was cloned under the regulation of the chrysanthemum RbcS1 promoter into a binary vector and used to transform tobacco plants. Strong emissions of chrysanthemol and lower emissions of lavandulol, similar to the *in vitro* ratios of volatile products of CDS, were detected in the headspace of transgenic tobacco plants expressing *CDS* (n=9), but not in control tobacco plants (n=3) which were transformed with the empty vector lacking *CDS* (Fig. 4). Chrysanthemol was emitted at levels of 0.12 – 0.16 µg·h⁻¹·g⁻¹ FW. Transgenic tobacco plants were shorter and leaf color was lighter compared to empty vector control plants.

**Non-volatile profile of transgenic tobacco overexpressing CDS.** To study whether there is also accumulation of non-volatile derivative(s) of chrysanthemol and/or lavandulol, we analyzed the non-volatile metabolites in transgenic (n=3) and control (n=3) plants using a non-targeted approach (Fig. S3). The newly produced compound that was most abundant, as determined by UV absorption (220 nm) resulting from the double bond of the compounds, showed up as m/z of 803.3498 and eluted at a retention time of 45.93 min. In control plants, this compound was not detected. The non-volatile product was putatively identified as...
chrysanthemol conjugated to malonyl-glucose by comparing its mass spectrum to that of geranoyl-6-O-malonyl-β-D-glucopyranoside, which was identified by NMR in geraniol synthase expressing maize [Fig. S4; (Yang et al., 2011)].

Discussion

Although pyrethrins represent the economically most important and widely used natural pesticide, not much is known about their biosynthesis at the genetic or enzymatic level. Only the genes involved in the first and last step of the biosynthetic pathway have been cloned (Rivera et al., 2001; Kikuta et al., 2012). CDS has been reported to catalyze the first committed step of pyrethrin biosynthesis by joining two molecules of DMAPP to produce CPP (Rivera et al., 2001), but the recently evolved enzyme serves roles also in other composite plant species that do not produce pyrethrins (Hemmerlin et al., 2003; Liu et al., 2012). Interestingly, our in vitro and in vivo results demonstrate that CDS also catalyzes the subsequent conversion of CPP to chrysanthemol, which furthers our understanding of the monoterpene branch of pyrethrin biosynthesis that occurs in pyrethrum glandular trichomes (Ramirez et al., 2012).

CDS was active with DMAPP and CPP as substrates. In both cases, the production of the monoterpene alcohols, chrysanthemol and lavandulol, was detected. Rivera et al. (Rivera et al., 2001) reported earlier that CPP and LPP were the final products of CDS. We used a typical protocol for activity analysis of terpene synthases which produce volatile and hydrophobic products (Bouwmeester et al., 2002b; Aharoni et al., 2004), while earlier protocols were based on the activity analysis of diphosphate synthases, which produce non-volatile and hydrophilic products (Burke et al., 1999; Hemmerlin et al., 2003). Though we used similar reaction conditions, the combination of the following factors in our assays could contribute to the observed differences: (a) a pentane overlay to capture the volatile alcohol; (b) the addition of detergent, Tween-20, to promote release of the hydrophobic product; (c) a low substrate concentration, common to plants; and (d) a long incubation time to observe products in this notoriously slow reaction in vitro. In former assays, the lack of an organic layer to capture the volatile product may have prevented detecting chrysanthemol formation. The presence of Tween-20 could increase the CDS activity to produce chrysanthemol as reported for SQS and PSY. For SQS, Tween-80 stimulated its activity 10-20 fold, and Tween-20 showed similar stimulating effects (Zhang et al., 1993). PSY also requires a detergent for maximal activity, as the presence of Tween-80 was reported to result in almost 20,000-fold higher activity compared to previously reported PSY activities (Iwata-Reuyl et al., 2003).

Rivera and co-authors used 2 mM DMAPP to assay the CDS activity. However, our results (Fig. 2C) showed that DMAPP concentrations higher than 0.3 mM inhibited chrysanthemol production, but not CPP production. In addition, we established that using low substrate concentrations, incubation times up to 96 h were needed to produce significant amounts of chrysanthemol. The turnover number \( (k_{cat}) \) of CPP hydrolysis was determined as \( 3.3 \pm 0.2 \times 10^{-3} \text{ min}^{-1} \), which is about 150-fold slower than that of the initial step \( (k_{cat} = 0.5 \text{ min}^{-1}, \text{(Rivera et al., 2001)}) \). This low velocity explains the long incubation time needed for the monoterpene alcohol production from DMAPP, but at low substrate conditions the rate of producing chrysanthemol was twice higher than for CPP.

Though the turnover from CPP \( (3.3 \times 10^{-3} \text{ min}^{-1}) \) or DMAPP \( (\sim 1 \times 10^{-4} \text{ min}^{-1}) \) to chrysanthemol was inefficient in the in vitro assays, the production of chrysanthemol by CDS was efficient in vivo. Chrysanthemol was emitted from leaves of transgenic tobacco overexpressing CDS (Fig. 4), at rates of 120-160 ng·h⁻¹·g⁻¹ FW. This rate was on the high end of the emission rates of other overexpressed terpenes in tobacco leaves. In tobacco plants
overexpressing three monoterpenes synthases (CaMV35S promoter), the total level of additional monoterpenes emitted from leaves reached up to 30 ng·h⁻¹·g⁻¹ FW (Lücker et al., 2004), and plants overexpressing patchoulol synthase, emitted patchoulol at levels of 50-100 ng·h⁻¹·g⁻¹ FW (Wu et al., 2006b). Our preliminary enzyme assays using cell lysate instead of purified CDS also showed much faster turnover rates. The enzyme activity of the lysate was about 10-40 fold higher than that of purified CDS. This suggests that the enzyme loses activity during purification. We tested the effects of adding protein (BSA), all known mineral co-factors (Murashige and Skoog salts) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) to the enzyme assays (Radisky and Poulter, 2000), but the activity of purified CDS was not improved. We suspect, therefore, that another organic co-factor is responsible for promoting CDS enzyme activity in vivo.

Our in vitro results showed that CDS favored the synthesis of chrysanthemol when the DMAPP concentration was lower than 300 µM (Fig. 2C). This DMAPP concentration range is most likely the range encountered by CDS in vivo. DMAPP concentrations in leaves have been determined for several plant species. Compared to the isoprene-emitting species cottonwood (Fisher et al., 2001) and oak (Brüggemann and Schnitzler, 2002), leaf DMAPP concentrations in non-emitting species, such as Arabidopsis, are 6-10 times lower (Loivamäki et al., 2007). Considering that tobacco is also a non-emitting species (Vickers et al., 2011), leaf DMAPP concentrations in tobacco may be similar to those in Arabidopsis at an estimated concentration of around 10 pmol·mg⁻¹ FW (Loivamäki et al., 2007). Then, the DMAPP level in tobacco plastids, the cell compartment where CDS is targeted to (Fig. 3; (Hemmerlin et al., 2003)), was estimated to be around 30 µM based on the assumption that leaf fresh weight is usually linearly correlated to leaf volume with a regression coefficient of 1 g·ml⁻¹ (Huxley, 1971), and assuming that chloroplasts typically occupy 20% of the total cell volume in mature leaf cells (Ellis and Leech, 1985), and that 60% of DMAPP occurs in the chloroplast (Rosenstiel et al., 2002). Chrysanthemol emission and glycosides could also be detected in transgenic Arabidopsis and chrysanthemum plants overexpressing CDS (Fig. S5 and S6).

CDS shares several characteristics with SQS and PSY, which both catalyze intermolecular 1,2,3-cyclopropanation reactions. All three synthases are bifunctional enzymes, functioning as prenyltransferases in the first reaction and as terpene synthases in the second reaction. However, the cyclopropane ring structure remains in the final product of CDS, but not in those of SQS or PSY. The overall enzyme activities of all three enzymes are inhibited by high substrate concentrations, but the production of the intermediate diphosphates is not. For example, the production of squalene by SQS has been shown to be inhibited by FPP concentrations higher than 100 µM (Zhang et al., 1993; Radisky and Poulter, 2000), but the production of the intermediate PSPP is not inhibited by high FPP concentrations (Radisky and Poulter, 2000). Similarly, PSY was reported to be significantly inhibited at GGPP concentrations above 100 µM (Iwata-Reuyl et al., 2003), though each of the separate reactions of PSY showed Michaelis-Menten behavior (Dogbo et al., 1988). In our experiments, DMAPP concentrations higher than 300 µM reduced total chrysanthemol production in favour of CPP (Fig. 2B, top), while lavandulol production was reduced by DMAPP at concentrations higher than 150 µM (Fig. 2B, bottom). CPP production was not inhibited by DMAPP concentrations up to of 600 µM (Fig. 2C, top), in good agreement with earlier data (Rivera et al., 2001). This indicated that DMAPP inhibits the overall CDS activity by inhibiting the second, but not the first reaction. This was confirmed by providing both DMAPP and CPP to CDS resulting in a significant reduction of chrysanthemol production when DMAPP concentration was higher than 75 µM (Fig. 2C). These results suggest that DMAPP and CPP are converted in the same active site.
Previously, chimaeric enzymes have been made in the group of Poulter (Thulasiram et al., 2007) which showed the evolution between the four fundamental coupling reactions: chain elongation, cyclopropanation, branching, and cyclobutanation using farnesyl diphosphate synthase (FDS) and CDS as building blocks. In those experiments the bifunctional activity of the CDS enzyme was not known, but our findings allow an additional perspective on the evolution of FDS, with diphosphate synthase activity only, into a chrysanthemol synthase with bifunctional activity. Future research could reveal the structural and mechanical basis of the two-step reaction and verify this process also in the other members of this new terpene synthase gene family which potentially yields not only chrysanthemol, but also lavandulol, maconelliol or more ancestral C15 alcohols in other plant species (Thulasiram et al., 2007; Liu et al., 2012).

In summary, we have shown both in vitro and in vivo that the previously reported prenyltransferase, (CDS), also functions as a terpene synthase. CDS is therefore the first bifunctional monoterpene synthase showing both prenyltransferase and terpene synthase activity. Considering its overall ability to preferentially catalyze both consecutive reactions in vivo where low substrate concentrations prevail, we propose to rename CDS a chrysanthemol synthase EC 2.5.1.XX. This identification moves forward our understanding of a recently evolved branch of irregular monoterpene biosynthesis which is not involved in primary metabolism like phytoene and squalene syntheses. Normally, there is an ancient and strict task division in terpene biosynthesis between synthases of terpene diphosphates and terpenoid products. Terpene synthases, at the expense of the diphosphate, can modify the skeleton in highly diverse ways to accommodate the required chemical variation in both primary and secondary metabolism of organisms. As an example geranyl diphosphate synthases exist separately from geraniol synthases and are evolutionarily selected not to hydrolyze their own GPP product. CDS, however, evolved only very recently, and there is an evolutionary bottleneck if two enzymes are needed simultaneously to generate a useful product like chrysanthemol. The bifunctional activity solves this issue and serves the needs of a plant which has not yet evolved a secondary purpose for CPP substrates.

Materials and Methods

Plant materials and chemicals. Pyrethrum plants (Tanacetum cinerariifolium) were grown in the field of Wageningen, the Netherlands. Chrysanthemyl diphosphate (CPP) and chrysanthemol were purchased from Isoprenoids Lc. (USA). Dimethylallyl diphosphate (DMAPP) was obtained from Sigma. CPP was a mixture of trans- and cis-racemic forms in a ratio around 9:1, and so was the chrysanthemol.

Cloning full length cDNA and genomic DNA of CDS. To clone the full length cDNA of CDS, following primers were used in 3’ and 5’ RACE PCRs: TcCDS_F 5’- CATCTTCTGGACCTCTTCAATGAG and TcCDS_R 5’-GTACTGAACATCCGACCGTGAAG. RACE libraries were synthesized with 1µg of total RNA from pyrethrum ovary using Supercript® II Reverse Transcriptase (invitrogen), and the Smart RACE kit (Clontech). In a final step based on the complete sequence information, primers were designed to obtain the full length cDNA (Accession number JX913536). Eight clones were sequenced. Based on the full length cDNA information, primers were designed for amplification of the genomic DNA corresponding to the gene with introns (Accession number JX913537). The sequence of CDS obtained by us slightly differs from the one reported by Rivera, et. al. (Accession number I13995)(Rivera et al., 2001). The detailed differences are explained in the results. The CDS sequence used in this study is the one obtained by us (Accession number JX913536).
Protein production in *Escherichia coli* and purification. The open reading frame of *CDS* without the putative targeting sequence was subcloned into the pRSET-A expression vector (Invitrogen) fused to an amino terminal histidine tag, and expressed in *E. coli* BL21(DE3) (forward primer 5'- TTATGGATCCACTACGACATTGAGCGAATCTTAG-3' and reverse primer 5'- TTATGATTCTTACATTGTCCCTATACATCTTTTC-3'; restriction sites are underlined). *E. coli* cells were grown to an OD<sub>600</sub> of 0.6 and then induced with 0.02% L-arabinose at 18 °C for 16 h. After harvesting, the cells were resuspended in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 300 mM NaCl and 1.4 mM β-mercaptoethanol, 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 Ni<sup>2+</sup>-NTA agarose (Qiagen) according to the manufacturer’s protocol. Proteins were concentrated and desalted in 50 mM Tris-HCl (pH 7.5, containing 1.4 mM β-mercaptoethanol) using Amicon Centrifugal Filters for Protein Purification and Concentration (Millipore), checked for purity by SDS/PAGE, and quantified by Bradford method using bovine serum albumin as a standard (Bradford, 1976). The protein purity was greater than 90%.

**Reaction of CDS with CPP.** The activity of CDS to produce chrysanthemol from CPP was assayed by incubating 35 µg purified enzyme in a final volume of 100 µl assay buffer (pH 7.0) containing 15 mM MOPS, 2 mM dithioerythreitol, 12.5% (v/v) glycerol, 1 mM MgCl<sub>2</sub>, 1 mM ascorbic acid, 0.1% (v/v) Tween 20 and various concentrations of CPP. After addition of a 100 µl pentane overlay, the assays were incubated for 20 h at 30 °C. Then, the assay mixture was extracted as previously described (Bouwmeester et al., 2002a) and the organic phase was concentrated to 200 µl under nitrogen flow. A 1-µl portion of the concentrated organic phase was analyzed by GC-MS using a GC (Agilent 7890A) equipped with an HP5-MS column (30 m × 0.25 mm i.d., 0.25 µm d.f.) and a mass-selective detector (Agilent 5975C, Hewlett Packard, Agilent Technologies). The injection port (splitless mode), interface and MS source temperatures were 250 °C, 290 °C and 180 °C respectively. The oven was programmed at an initial temperature of 45 °C for 1 min, with a ramp of 15 °C min<sup>-1</sup> to 280 °C, and final time of 3.5 min. Proteins extracted from *E. coli* lysate cells harboring the empty vector were assayed with CPP as control. For identification, the authentic standard of chrysanthemol was run under identical conditions.

To determine the apparent K<sub>M</sub> value of CDS for CPP, the Mg<sup>2+</sup> concentration (1.0 mM) and pH value (7.0) were set at optimal level as described before (Rivera et al., 2001), and the CPP concentration was varied between 10 and 600 µM with 8 different data points. Triplicate sets of assays were performed at each CPP concentration. The apparent K<sub>M</sub> value was obtained by fitting the data to the Michaelis-Menten equation, using KaleidaGraph (Synergy Software).

**Reaction of CDS with DMAPP.** The activity of CDS to produce diphosphates (CPP and lavandulyl diphosphate, LPP) and terpene alcohols (chrysanthemol and lavandulol) from DMAPP was assayed by incubating 35 µg purified enzyme in a final volume of 100 µl assay buffer described above, but with 600 µM DMAPP instead of CPP. After addition of a 100 µl pentane overlay, the assays were incubate for 96 h at 30 °C. The chrysanthemol production was determined by analyzing the organic phase as described above. CPP production was determined by analyzing the water phase of the same reaction tube. For that, 50 µl water phase was transferred to a fresh reaction tube, treated with calf alkaline phosphatase (Sigma). Liberated compounds were extracted as described before (Rivera et al., 2001). The extracted compounds were then analyzed by GC-MS as described above. For K<sub>M</sub> determination, assays were performed with different DMAPP concentrations from 10 to 600 µM with 8 different data points. Triplicate sets of assays were performed at each DMAPP concentration. To check non-enzymatic conversion from CPP to chrysanthemol in this 96-h assay, control reactions without enzyme were performed with CPP concentrations ranging from 2 to 80 µM. This
range covered a maximum concentration of 25 µM CPP produced by CDS under different DMAPP concentrations.

For identification of chrysanthemol, the authentic standard of chrysanthemol was run under identical conditions. For identification of lavandulol, mass spectra of eluted peak fractions were compared with the published mass spectrum for lavandulol (Thulasiram et al., 2007).

**Determination of DMAPP substrate inhibition in the reaction of CDS with CPP.** In order to check whether chrysanthemol production by CDS from CPP is inhibited by DMAPP, different amounts of DMAPP were added into CDS enzyme assays containing 150 µM CPP (the apparent $K_M$ value of CPP determined above). The enzyme assays were conducted as described above (20-h incubation). DMAPP concentrations were tested in a range of 0 to 600 µM with 8 different data points. The determinations were replicated 3 times. Terpene alcohol and diphosphate products were analyzed by GC-MS as described above.

**Subcellular localization of CDS using GFP.** The gene fragment corresponding to the first 54 amino acids of CDS or the whole CDS gene without stop codon were cloned into the binary vector pBINPLUS-1.eGFP (Seiichi et al., 1995) as an in frame protein fusion to a green fluorescent protein (GFP) reporter gene using the KpnI and XbaI restriction sites. These constructs were named pCDSTAR-GFP and pCDS-GFP, respectively. Tobacco plants (*Nicotiana tabacum* Samsun) were transformed with these constructs as described (Jongsma et al., 1995). Transgenic tobacco mesophyll protoplasts were isolated according to Sheen (2001). Images were taken using a confocal laser scanning microscope (Zeiss) with fluorescence bandwith filters of 620 to 750 nm for chlorophyll imaging, and 500 to 530 nm for GFP.

**Generation of transgenic tobacco.** The complete cDNA of CDS was placed under the control of chrysanthemum RbcS1 promoter, and *Agrobacterium tumefaciens* strain AGL-0 harboring the binary vector was used to transform the tobacco plants using protocols described previously (Jongsma et al., 1995). Tobacco plants transformed with a vector lacking CDS were used as control. Transgenic plants were grown in a greenhouse at 25±2 °C under 18/6 h light/dark photoperiod. The presence of CDS was checked by PCR. All PCR positive plantlets of the T₀ transgenic line were analyzed by GC-MS to check the presence of chrysanthemol in the headspace. The three plantlets with highest production of chrysanthemol were analyzed by LC-MS. Three plantlets from the empty vector control line were also analyzed by GC-MS and LC-MS.

**Volatile GC-MS and non-volatile LC-MS analysis of transgenic tobacco.** Volatiles from cut leaves were collected from 4-week old plants. The second leaf from the top was harvested for headspace trapping. The volatiles were sampled for 2 h, and then analysed by GC-MS as described (Yang et al., 2011). The temperature program of the gas chromatograph was 40 °C for 3 min, rising to 280 °C at 10 °C min⁻¹ and final time for 2 min. The mass spectrometer was set to scan from 35 to 450 m/z. The helium flow was constant at 1.0 mL min⁻¹. Ionization potential was set at 70 eV. For identification of chrysanthemol, the authentic standard of chrysanthemol was run under identical conditions. For identification of lavandulol, the mass spectrum of the eluted peak was compared with the published mass spectrum of lavandulol (Thulasiram et al., 2007).

The third leaf from the top was used for non-volatile analysis according to a protocol for untargeted metabolomics of plant tissues (De Vos et al., 2007) as described in detail before (Yang et al., 2011).

Statistical analysis of GC-MS and LC-MS data was conducted as reported (Yang et al., 2011). The processing parameters of MetAlign for GC-MS data were set to analyze scans 1340 to
16000 (corresponding to retention time 2.32 min to 28.05 min) with a maximum amplitude of $3.5 \times 10^8$. The parameters for LC-MS data were set to analyze scans 70 to 2620 (corresponding to retention time 1.4 min to 49.73 min) with a maximum amplitude of 35000.

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Supporting information

Bifunctional chrysanthemyl diphosphate/chrysanthemol synthase

Fig. S1. Comparison of 5’ genomic DNA sequence with cDNA sequences of CDS gene. TcCDS-1 to TcCDS-8: 5’ cDNA sequences of CDS gene in pyrethrum cloned in this study. I13995 is the CDS reported by Rivera et al. (Rivera et al., 2001). TcCDS gDNA: 5’ genomic DNA sequence of CDS gene in pyrethrum cloned in this study (Accession number JX913537). AtCDS cDNA: 5’ cDNA sequence of CDS gene in sagebrush (Artemisia tridentata) (Hemmerlin et al., 2003). Blue color shows the intron, pink color shows the start codon, and green color shows the start codon.

**Table:**

| TcCDS-1          | ATGAGGACCTTTCTTCTTAAATGATGATGATATATGCGTCTACATATGAAATACTC |
| TcCDS-2          | ATGAGGACCTTTCTTCTTAAATGATGATGATATATGCGTCTACATATGAAATACTC |
| TcCDS-3          | ATGAGGACCTTTCTTCTTAAATGATGATGATATATGCGTCTACATATGAAATACTC |
| TcCDS-4          | ATGAGGACCTTTCTTCTTAAATGATGATGATATATGCGTCTACATATGAAATACTC |
| TcCDS-5          | ATGAGGACCTTTCTTCTTAAATGATGATGATATATGCGTCTACATATGAAATACTC |
| TcCDS-6          | ATGAGGACCTTTCTTCTTAAATGATGATGATATATGCGTCTACATATGAAATACTC |
| TcCDS-7          | ATGAGGACCTTTCTTCTTAAATGATGATGATATATGCGTCTACATATGAAATACTC |
| TcCDS-8          | ATGAGGACCTTTCTTCTTAAATGATGATGATATATGCGTCTACATATGAAATACTC |

I13995: CTAGATCGATGTGCATTTAGTAGAAATATACTTATTTAAAGATATAAAAAATGTCCGCAC

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**Fig. S1.** Comparison of 5’ genomic DNA sequence with cDNA sequences of CDS gene. TcCDS-1 to TcCDS-8: 5’ cDNA sequences of CDS gene in pyrethrum cloned in this study. I13995 is the CDS reported by Rivera et al. (Rivera et al., 2001). TcCDS gDNA: 5’ genomic DNA sequence of CDS gene in pyrethrum cloned in this study (Accession number JX913537). AtCDS cDNA: 5’ cDNA sequence of CDS gene in sagebrush (Artemisia tridentata) (Hemmerlin et al., 2003). Blue color shows the intron, pink color shows the start codon, and green color shows the start codon.
Chapter 5

Fig. S2. CDS activity indicated by chrysanthemol production from CPP for 96 h. Chrysanthemol production was checked every 24 h.

Fig. S3. Negative mode LC–MS chromatograms of aqueous-methanol extract of leaves of an empty vector tobacco plant (a) and CDS overexpressing tobacco plant (b).
**Fig. S4.** The MS spectrum of the compound eluting at 45.93 min (a), which was only detected in CDS overexpressing tobacco plants but not in empty vector control plants. (b), the scheme of its collision-induced fragmentation. The compound was putatively identified as chrysanthemol conjugated to malonyl-glucose.

**Fig. S5.** GC-MS chromatograms obtained by dynamic headspace trapping of whole plant of empty vector control (a) and CDS expressing (b) Arabidopsis plants.
Fig. S6. GC-MS chromatograms obtained by dynamic headspace trapping of leaves of empty vector control (a) and $CDS$ expressing (b) chrysanthemum plants.
Chapter 6

Chrysanthemic acid:Coenzyme A ligase in pyrethrum—gene cloning and analysis of recombinant enzyme

Ting Yang, Maarten A. Jongsma
Abstract

Pyrethrins are the most widely used natural pesticides. In pyrethrum they are synthesized by the transfer of alcohol moieties to activated acid moieties, such as chrysanthemic acid. Chrysanthemic acid is activated with coenzyme A (CoA) by CoA ligases. A chrysanthemic acid:CoA ligase, named *Tanacetum cinerariifolium* Acyl Activating Enzyme 1 (TcAAE1) was cloned from ovaries of pyrethrum plants based on the similarity of its expression pattern with chrysanthemyl diphosphate synthase, the first pathway-specific gene of pyrethrin biosynthesis. TcAAE1 is specifically activating chrysanthemic acid and not benzoic acid, as an alternative short chain aromatic acid, and that benzoic acid ligase has no activity towards chrysanthemic acid. TcAAE1 possesses a putative N-terminal plastid targeting sequence. The potential role of CoA ligation also in facilitating transport of chrysanthemic acid between plant tissues is discussed.
Introduction

Pyrethrins are natural insecticides that are synthesized by the chrysanthemum species, *Tanacetum cinerariifolium* (earlier species name: *Chrysanthemum cinerariaefolium*) of the Asteraceae family and have been used for pest control since medieval times (Crombie, 1995). Although the market for pyrethrins has been taken over by synthetic pyrethroids, there is still worldwide demand for natural pyrethrins as organic pesticides. Like synthetic pyrethroids, natural pyrethrins knock-down, repel and kill many different kinds of insects, including crop pests, stored food pests and household pests. However, in contrast to the synthetic pyrethroids, they are considered environmental friendly as they have low toxicity to mammals and low persistence after use which leads to weak development of resistant strains (Casida and Quistad, 1995). Hence, it would be highly beneficial to breed *T. cinerariifolium* cultivars with enhanced pyrethrin production or otherwise produce pyrethrins by fermentation. However, to achieve this, knowledge of the biosynthetic pathway is essential.

Pyrethrins are biosynthesized by ester condensation of the acid and alcohol moieties. The two acid moieties (chrysanthemic acid and pyrethric acid) possess a cyclopropane ring, while the three alcohol moieties – pyrethrolone, jasmololone and cinerolone – contain a cyclopentenone ring. The pathway-specific enzyme and gene inducing cyclopropane ring formation, chrysanthemyl diphosphate synthase, has been characterized (Rivera et al., 2001). Chrysanthemic acid was recently demonstrated to be synthesized in the glandular trichomes present on most aerial parts of the plants but in high densities on the flower achenes (Ramirez, et al., 2012). It is transported in a basipetal direction into the achene pericarp tissue where it is esterified into pyrethrins. The basis of ester linkage formation by an acyltransferases has also been elucidated recently based on (1R,3R)-chrysanthemoyl CoA and (S)-pyrethrolone. The purified and cloned enzyme was a member of the GDSL lipase family and named TcGLIP. This GDSL lipase showed high substrate specificity for the chrysanthemic and pyrethric acid CoA and rethrolones (Matsuda, 2012).

The monoterpenoid precursors of TcGLIP are Co-enzyme A activated chrysanthemic acid and pyrethric acid molecules, chrysanthemoyl-CoA or pyrethrol-CoA. The activation of chrysanthemic acid and pyrethric acid with CoA is expected to occur through the action of an enzyme family known as CoA ligase utilizing ATP as an energy carrier. In enzymological terms, a chrysanthemoyl-CoA ligase (EC 6.2.1.xx) is an enzyme that catalyzes the chemical reaction that takes ATP, chrysanthemic acid or pyrethric acid, and CoA as substrates and give ATP, chrysanthemic acid or pyrethric acid, and CoA as products.

Acyl-CoA thioesters such as chrysanthemoyl-CoA are formed by members of the acyl-activating enzyme (AAE) superfamily that activate carboxylic acids through an adenylate intermediate (Schmelz and Naismith, 2009). In plants the substrates of AAEs include phenylpropanoids, fatty acids, terpenes and jasmonate precursors. 4-Coumarate:CoA ligase (4CL), an enzyme involved in phenylpropanoid metabolism, and the long-chain acyl CoA synthetases, are perhaps the best characterized of the plant AAEs (Shockey et al., 2002; Hu et al., 2010). The genomic organization and biochemical diversity of AAEs has been recently reviewed (De Azevedo Souza et al., 2008; Shockey and Browse, 2011).

Here we report the cloning and identification of chrysanthemic acid:CoA ligase (AMP-forming). The abbreviated name is TcAAE1 for *Tanacetum cinerariifolium* Acyl Activating Enzyme 1.
Materials and Methods

Isolation of RNA from ovary, leaf and trichome

The ovaries were carefully separated from the flower base and the corollas were removed. Trichomes were isolated from ovaries by vortexing the ovaries in liquid nitrogen and filtering the vortexed ovaries with a pre-cooled 150 μm mesh. The trichomes that passed through the mesh were collected. The young leaves which were not fully developed were used to isolate RNA. To avoid inference of trichome content on RNA isolation, all RNA samples were isolated with hexadecyltrimethylammonium bromide (CTAB).

454-EST sequencing and processing

Three to twenty-five μg of RNA of each tissue were sequenced with 454 sequencing technology (Vertis GmbH, Germany). Briefly, the cDNAs from 3 different tissues, obtained with random hexamer primers, were labelled with different adapters to generate 3 cDNA libraries. The libraries were normalized and then sequenced in a single 454 run. A single 454 run on the 3 libraries 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 lengths 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. In every library, about 40-50% reads could not be assembled into contigs and remained as singlets. Since all the plant materials were harvested from the same genotype, the reads from different libraries were pooled together to be assembled. 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.

Gene expression analysis by qPCR

The expression levels of different contigs were determined by real-time quantitative RT-PCR analysis (qPCR). The sequences of primers were listed in Table S1. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as reference gene (Ramirez et al 2012). The qPCR primers were designed by the software Beacon Designer (Palo Alto, CA, USA) with expected product sizes of around 120 bp.

Cloning of full length AAE1 cDNA

The contigs were used as templates to design gene specific primers for rapid amplification of cDNA ends (RACE). The sequences of primers were listed in Table S2. Finally, the full-length cDNA encoding the putative AAE1 was obtained by PCR based on the information obtained from sequencing of the 3’- and 5’-RACE products.

Heterologous expression of AAE1 protein in E. coli

For functional characterization of the AAE1 protein, the entire reading frame was subcloned into the pRSET-A expression vector (Invitrogen) fused to an amino-terminal histidine tag, and expressed in Escherichia coli BL-21 under an isopropylthio-β-galactoside inducible promoter. E. coli cells were grown to an OD600 of 0.6 and then induced with 0.02% L-arabinose at 25 °C for 20 h. After harvesting, the cells were resuspended in lysis buffer containing 50 mM Tris-HCl (pH 7.5), 300 mM NaCl and 1.4 mM β-mecaptoethanol, 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.
AAE1 activity assay

His-tag purified proteins were assayed for chrysanthemic acid:CoA ligase activity in a final volume of 1 ml assay buffer containing 0.2 M Tris (pH8.0), 0.2mM chrysanthemic acid, 0.2 mM CoA, 2.5 mM ATP and 10 mM MgCl₂. The assay without enzyme was used as control. The assays were incubated for 15 min at room temperature. Then the reaction products were filtered through 0.2 µm inorganic membrane filters (RC4, Sartorius, Germany) before HPLC analysis.

Product analysis in HPLC

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 (10 mM ammonium acetate, pH 5.3) and eluent B (70% acetonitrile in 10 mM ammonium acetate, pH 5.3) 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.

Results

Screening of candidate genes

To obtain transcripts from different pyrethrin producing tissues, the cDNAs of pyrethrum ovaries, leaves and trichomes were sequenced with 454 DNA sequencing technology. The 454-ESTs were BLASTN-aligned to the NCBI database to obtain a putative annotation for each EST. In total, 54 contigs were annotated as acid: CoA ligase, with 52 contigs detected in ovaries, 37 contigs in leaves and 31 contigs in trichomes. There were 31 contigs detected in at least 2 tissues and those were selected for further screening based on gene expression profiles in different tissues.

![Fig 1](image1.png)  
Fig 1. Expression patterns of CDS and 3 CoA ligase contigs in ovaries at different developmental stages of flowers measured by qRT-PCR in three experimental replicates (error bars are SE). The expression level of house-keeping gene (GADPH) was set to 1. St, stage of flowers.
Chapter 6

90

In the first round of screening using qRT-PCR, we analysed the gene expression patterns of those 31 contigs in 3 tissues: young ovary (from stage 3 flowers), old ovary (from stage 5 flowers) and 3-d old seedlings. The patterns were compared to the pattern of the CDS gene, which expressed 20 times higher in young versus old ovaries and did not express in the seedling. Three contigs showed highly similar expression patterns to that of CDS (Table S3). These 3 contigs, Contig 1490, 4402 and 5325, were selected for the second round of screening.

In the second round of screening, we analysed the gene expression patterns of those 3 contigs in the ovaries from 6 different developmental stage flowers. All 3 contigs showed similar expression patterns to that of the CDS gene in different developmental stages (Fig. 1).

Fig 2. Alignment of candidate TcAAE1 and top 2 BLASTN hits of the gene, AMP dependent ligase (EEF40686) and acyl:coA ligase acetate-coA synthetase like protein (EEE94198).
These 3 contigs were each used as template to design gene specific primers for rapid amplification of cDNA ends (RACE) to obtain the full length. For Contig 1490, we failed to get 5’ RACE product, despite the use of 3 different gene specific primers. For Contig 4402 and 5325, the RACE results turned out to yield exactly the same gene. This gene then represented the only remaining candidate for chrysanthemic acid:CoA ligase activity.

The cDNA sequence of the candidate AAE1 (Fig. S1) was BLASTN-aligned to NCBI database. The top 2 hits were shown to be AMP-dependent ligase (EEF40686) from Ricinus communis and Acyl:CoA ligase Acetate-CoA synthetase-like protein from Populus trichocarpa (EEE94198; Fig. 2). However, the functions of these 2 enzymes have not been determined. Among different tissues with or without trichomes, this gene showed similar pattern as CDS suggesting it is also exclusively expressed in the trichomes (Fig. 3).

Enzyme assay for chrysanthemic acid:CoA ligase activity

The recombinant his-tagged chrysanthemic acid:CoA ligase candidate (Contig 4402&5325) was purified from cell-free homogenates. Chrysanthemic acid, ATP and coenzyme A were provided as substrates according to standard protocols. The reaction without any enzyme was used as control. The peak eluting at 6.702 min showed up in the reaction with TcAAE1 candidate, but not in the control reaction (Fig. 4 a-c). The UV spectrum of this peak showed a characteristic absorption spectrum for CoA thioesters. Benzoic acid:CoA ligase (BZL) from Clarkia breweri (Beuerle and Pichersky, 2002) was also tested, and it was not able take chrysanthemic acid as substrate (Fig. 4 d). BZL can take benzoic acid as substrate (Fig. S2). TcAAE1 was not able to take benzoic acid as substrate either (Fig 4 e).

Discussion

In this study, we describe the cloning and characterization of an Acyl Activating Enzyme capable of activating chrysanthemic acid with CoA (TcAAE1). Among different developmental stages of pyrethrum flowers, this gene showed an expression pattern similar to CDS, the first pathway-specific gene of pyrethrin biosynthesis. Both CDS and AAE1 were highly expressed in the ovaries of flowers at stage 2 to 4, then their expression levels decreased 10- to 20-fold at stage 5, and further decreased in later stages (Fig. 1). This expression pattern followed the accumulation pattern of pyrethrins. Pyrethrin content increased steadily in early stages (stage 1 to 4), then it reached the maximum at stage 5 and stayed stable afterwards (Casida and Quistad, 1995). It indicated that the biosynthesis of pyrethrins was highest at early stages (stage 1 to 4), when there were still closed disc florets.

The alignment of the deduced protein sequence of TcAAE1 with other acyl activation enzymes (Fig. 2) indicated that it could possess a targeting signal. AAEs have been reported
to localize to several cell compartments, including chloroplast membranes (Schnurr et al., 2002), ER membranes (Zhao et al., 2010) and peroxisomes (Fulda et al., 2002). The biosynthesis of the acid moiety of pyrethrins was demonstrated to be in chloroplasts by feeding $^{13}$C-labelled glucose to pyrethrum seedlings (Matsuda et al., 2005). CDS has also been demonstrated to localize in chloroplasts by fusing its target signal peptides to GFP (Szkopińska and Płochocka, 2005)(Yang et al., Chapter 5). Thus, we propose that TcAAE1 may also be targeted to chloroplasts.

TcAAE1 showed highest homology to an AMP-dependent ligase from Ricinus communis, however, this enzyme was not further studied. The most well studied AAE family is from Arabidopsis. Their protein sequences were compared phylogenetically and grouped into 7 distinct categories (Shockey et al., 2003; Shockey and Browse, 2011). Clade I contains the long-chain acyl-CoA synthetases (LACS). Clade II contains 3 members with limited sequence similarity to acetyl-CoA synthetase. Clade III represents the largest clade containing adenylases participating plant hormone signalling pathways. Clade IV contains 4-coumarate:CoA ligases (4CLs). Clade V contains 4CL-like proteins. Clade VI is composed of plant-specific branch of AAE, but not yet characterized. Clade VII contains genes with unknown functions. Considering the narrow specificities of the other clades, it was expected that TcAAE1 belongs to the last two clades, as pyrethrins are secondary metabolites requiring specialized unique enzymes specific to plants, but not shared by all. Indeed, among all Arabidopsis AAEs, TcAAE1 showed the highest homology to Arabidopsis AAE2 belonging to Clade VI.

Recently, a trichome acyl-activating enzyme, CsAAE1 from Cannabis sativa was characterized (Stout et al., 2012). This enzyme takes hexanoate to synthesize hexanoyl-CoA,
which is the precursor for the psychoactive and analgesic cannabinoids (e.g. Δ⁹-tetrahydrocannabinol (THC)). Cannabinoids are known to be synthesized in glandular trichomes on female flowers, and Stout and co-authors showed that CsAAE1 like TcAAE1 is highly expressed in trichomes, and exhibits similar expression pattern as other genes involved in cannabinoid biosynthesis.

In pyrethin biosynthesis, the acid precursor of pyrethrins, chrysanthemic acid, was shown to be synthesized in trichomes and subsequently transported to the pericarp (Ramirez et al., 2012). The concentrations of chrysanthemic acid in trichomes and pericarp were very low however (100-1000 fold), compared to the pyrethin concentrations. It may be therefore that the free acid represents a breakdown product relative to a much higher concentration of CoA-ligated product produced in the trichomes and transported out. AAES have been reported to be involved in transportation of activated acids. For example, LACS was shown to be a necessary component for export of free fatty acids using kinetic labelling experiments (Koo et al., 2004). We propose that TcAAE1 may facilitate this transport of activated chrysanthemic acid.

Acknowledgements

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References


Zhao L, Katavic V, Li F, Haughn GW, Kunst L (2010) Insertional mutant analysis reveals that long-chain acyl-CoA synthetase 1 (LACS1), but not LACS8, functionally overlaps with LACS9 in Arabidopsis seed oil biosynthesis. The Plant Journal 64: 1048-1058
## Supplementary tables

| Table S1. Quantitative RT-PCR Primer sequences of 31 CoA ligase contigs. |
|---|---|---|---|
| primer name | primer sequence | primer name | primer sequence |
| Contig24997_Fw | AGCACACTCCTCATCACACTCC | Contig24997_Rv | GTTGGCCGCTGTGATCTTG |
| Contig12880_Fw | CATCCAAAAGATCTACGAGCAG | Contig12880_Rv | AACCAAATCTAGACGCATC |
| Contig2189_Fw | TCACAAATCGAGGCACGATG | Contig2189_Rv | TGAAGCTTGGTTCAGGAC |
| Contig1662_Fw | TGGCAGGACATGATAAACG | Contig1662_Rv | CAAGGCAGTGGATCAAG |
| Contig1490_Fw | GAGAGGACAGTGGACACAG | Contig1490_Rv | TCGTCCACACTCTGGGATC |
| Contig8412_Fw | ACCAGGTGACAGACACTAC | Contig8412_Rv | TGGGATGCTGGTTCAGT |
| Contig5540_Fw | CTCCTCCATCCAGCAAGAC | Contig5540_Rv | GCCTGTTGCTGTTGTGAG |
| Contig4402_Fw | ACCACGCTTGACCATACTCC | Contig4402_Rv | ACTCCGACACACACTCCC |
| Contig27122_Fw | TCGTGGCTTCTGTCGTTCC | Contig27122_Rv | AAGATTCGTCGGGACCA |
| Contig5325_Fw | GATTCTGCTTGGTCGTGC | Contig5325_Rv | ACCTGTCGACTGTAAGG |
| Contig388_Fw | GCAATGGGCTCGTCGGAGAT | Contig388_Rv | ATGCTCAGGTCACACTT |
| Contig2030_Fw | ATCTGAGTGTCACACTCATC | Contig2030_Rv | TCTCTGCTGTTGCTGATC |
| Contig24254_Fw | GGAGGCAAGCGGTGATCA | Contig24254_Rv | CCGTGAAGTGGTGTGAG |
| Contig98_Fw | AATGCGGCGCTTCTCAGT | Contig98_Rv | AACAGCACTGCTGGTAC |
| Contig522_Fw | ATTCGCTCGACACACTTACC | Contig522_Rv | AACTCGACACTACATC |
| Contig16153_Fw | ATGGCCTACTGCAACTTAC | Contig16153_Rv | TCCACACTCCGGAAGATCC |
| Contig22542_Fw | GCCAGGCAGCCTTACAATGTG | Contig22542_Rv | GCCAAGGAGGATGGTAC |
| Contig27133_Fw | TGCCCTTTTGCGCAAG | Contig27133_Rv | CGTGTCCTTCTTCCAC |
| Contig4387_Fw | GGGTGAGAACATGAGTACG | Contig4387_Rv | TTCCGGTCTGTTGCTGATC |
| Contig22693_Fw | TGAGGAGATCTCCTCATGAG | Contig22693_Rv | TGGGATGCTGGTTCAGT |
| Contig549_Fw | AACTGCTCAGGAGCAG | Contig549_Rv | AACTGCTGAGCTGGTAC |
| Contig1483_Fw | TGGCAAGGACAGGTTGGAG | Contig1483_Rv | CAGATGACGTTCCACATC |
| Contig189_Fw | TGGCAACAAAGGACCAACG | Contig189_Rv | TCAAGAAAGGCTGTTGTC |
| Contig19635_Fw | CTTAAGTAGCCGGCAAGACG | Contig19635_Rv | GCATCCAGCAGTACATAG |
| Contig7785_Fw | GGATGGTGGAGTGGTG | Contig7785_Rv | CGGGTGCTTCTTCCAC |
| Contig276_Fw | GTATGGCAATCTGCCACAG | Contig276_Rv | TGGTCCACTCAACATC |
| Contig8656_Fw | GCCACCTACTTGCTGCTG | Contig8656_Rv | TGGTCCACTCAACATC |
| Contig15865_Fw | TGCCACATCAGCTTCTTGG | Contig15865_Rv | TGAGCGGAGATGATGCG |

| Table S2. RACE primer sequences of 3 contigs, contig 1490, 4402 and 5325. |
|---|---|
| primer name | primer sequence |
| Contig1490_GSP2_1 | CCTCTCAACTTAATCAAGGAAGCCTCCA |
| Contig1490_GSP1_1 | AGGGCGGAGGCAAGAATAGACCA |
| Contig1490_GSP2_2 | AACAACCGGCATCCGCTGCCTAGC |
| Contig1490_GSP1_2 | CGCGAATGAAATCGGACTCGTGAC |
| Contig1490_GSP2_3 | GAAGGGCGAGGACAGATGACCA |
| Contig1490_GSP1_3 | CCTCTCAACTTACAGAGAAGCCTCCA |
| Contig4402_GSP2 | TGGTTCAGGGTCCTGATCC |
| Contig4402_GSP1 | TAGGGCGTCTCCGCTCAAAGACTAAT |
| Contig5325_GSP2 | GGATGGAACACACTGCAACCTAG |
| Contig5325_GSP1 | GGATTGGGTTTCACTGCTCCAC |
Table S3. Gene expression level of 31 CoA ligase contigs in stage 3 ovary, stage 5 ovary and seedling.

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<tr>
<th>Contig No.</th>
<th>RGE ov3</th>
<th>RGE ov5</th>
<th>RGE seedling</th>
<th>ratio of RGE ov3/ov5</th>
<th>ratio of RGE ov3/seedling</th>
</tr>
</thead>
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<td>CDS</td>
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<td>0.0559</td>
<td>0.0001</td>
<td>23.30</td>
<td>15195.81</td>
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<tr>
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<td>0.0013</td>
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<td>6.18</td>
<td>N/A</td>
</tr>
<tr>
<td>Contig5325</td>
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<td>0.0037</td>
<td>N/A</td>
<td>13.89</td>
<td>N/A</td>
</tr>
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<td>5.00</td>
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<td>3.35</td>
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<td>0.0697</td>
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</table>

RGE, relative gene expression. The expression level of house-keeping gene (GADPH) was set to 1. Ov3, ovaries from stage 3 flowers. Ov5, ovaries from stage 5 flowers.
Supplementary Figures

>TcAAE1

TTTGAGTTGAGCTCTCCGAAAATATTACATTATAAGGTATGGGATCATATGGTGCTTTGTT
TTACACTTCAAGCTGCTATCCACTATACAAATTACCTTCAAAAACAGGCGCCATCAAGACT
ATGGTAAAAGAGCTGCTGTTGAGCTCTGTTGAGAGAAACACGCCACAGCTGCTGCTGCTGCT
TTCTCTCTATGGTCAAGTCTGATCCTTCG

>ORF:

44..1915 Frame +2 623AA

MDHMVVLHSKSFYSYHNLKSFYSLNSNATNFSVEKLKKSNTRLDHNPKTKLANSFSKVQCKSSQE
SDPSFESRQPMEGVVRVSYVNYPLPSIFLRAAKVYVRDRSVVYGSIKYTWEEHRCVKL
ASSLNLGVRSGVDVAILANPVMIAELHFVPMAGAIIIPNLTRLDSMTTLLLHSETKILF
VDYQLHKAELYMNLKKHSERSRPPLLWVISEVSQSSLNLANEHVEQRLVEAGDFTP0IPR
NDECDPSLNYTSGTTKPKGVIYHRGAYLNLGSGFVMHGMREMTYLVSWPLFHCNGWC
LSWGIAIVGTNVLRRSDKPDINVHRKHTHGMGAPTVMNLSANVQNKPLHRVIMEIM
TAGAPPPSILSKIKLGFMVSHAYGLETVYGLSTWCLWKPEDDLPMEEQGKLKARQGVNH
FGVQDVMKDPVPTEVSKSDRSTGEIMLRGNTVMSAYLKDNPDKATEDFAAGWFSGDGI
KHDPGIEVKDRKLDIVSIEGGINETIEVFVIYHRQAVLEVAVVARPPDYWYGQTCAVFV0L
KEGYHADAEQIIQYCRDHMYMSRPTRVIFQDLPRNSTMKGKEVKSLREKANGLSHHKNV*

Prediction algorithms of signal peptides all predict the absence of targeting. However, it seems that there might still be a targeting signal because most homologies in the protein Nr database start after
amino acid 74 (MEGVV...) with proteins which themselves start with that conserved sequence and therefore lack such a targeting peptide. Marked underlined is therefore the predicted targeting signal.

**Fig S1.** The cDNA sequence of chrysanthenic acid:CoA ligase, TcAAE1.

**Fig. S2** HPLC chromatograms of enzyme assays with benzoic acid:CoA ligase (b) or without it (a). The chromatograms were obtained at wavelength 260 nm. The UV spectrum of the peak eluting at 6.675 min was shown in the insert (c).
Chapter 7

General discussion

Ting Yang
Plant-insect interactions are receiving increasing attention because of their importance in crop production. Among the many factors involved in these interactions, monoterpenes play an important role as they may mediate attraction of pollinators, repellence or deterrence of herbivores and attraction of natural enemies of herbivores. Overexpression of monoterpenes with repellent or deterrent effects could improve plant resistance to pest insects, such as western flower thrips (WFT), the most serious pest in several vegetable and flower crops worldwide. The aim of the study presented in this thesis was to increase the production of monoterpenes that are known to affect herbivorous insects to improve plant resistance to WFT through metabolic engineering.

Production and metabolism of monoterpenes in transgenic plants

The production of monoterpenes in plants can be achieved by overexpressing the corresponding monoterpenic synthases. However, it is known, that the monoterpenes produced in such transgenic plants are often further converted to volatile or non-volatile derivatives or conjugates (Lücker et al., 2001; Aharoni et al., 2003). In our study, three monoterpenic alcohol synthases were studied in different transgenic plants, i.e. linalool synthase (LIS) in transgenic chrysanthemum (Chrysanthemum morifolium Ramat.) (Chapter 2), geraniol synthase (GES) in transgenic maize (Zea mays) (Chapter 3) and chrysanthemol synthase (CHS) in transgenic tobacco (Nicotiana tabacum) (Chapter 5). LIS-overexpressing chrysanthemum and CHS-overexpressing tobacco plants emitted large amounts of linalool and chrysanthemol, respectively. However, in GES-overexpressing maize no geraniol emission was detected. LC-MS and NMR analysis showed that the geraniol produced in the transgenic plants was further oxidised to geranic acid and then conjugated to malonyl-glucose by endogenous enzymes of maize. This is the first time that glycosylation with malonyl-glucose is reported for transgenic plants overexpressing terpene synthases. However, similar conjugation was also detected in LIS-overexpressing chrysanthemum and CHS-overexpressing tobacco. These results suggest that glycosylation with malonyl-glucose is a common strategy for plants to deal with excess terpenes. Lücker and co-authors (Lücker et al., 2001) reported for the first time the presence of a terpenoid glycoside conjugate in a transgenic plant. They showed that overexpression of a linalool synthase in petunia resulted in (S)-linalyl-β-d-glucopyranoside formation. The metabolic changes resulting from transformation with terpene synthases in terms of non-volatile derivatives and conjugates were studied in more detail by others. In LIS Arabidopsis and LIS potato, for example, a range of glucosides of linalool and linalool-derivatives were reported (Aharoni et al., 2003; Aharoni et al., 2006). The identification of entirely new glycosylation types, such as terpenoid conjugations with malonyl-glucose, makes non-targeted LC-MS analysis a necessary analytical complement to GC-MS to fully characterize and interpret the metabolic phenotype of transgenic, terpene synthase overexpressing plants.

Also in the headspace of transgenic plants, volatile derivatives of the primary monoterpenic alcohol products have been detected. Small amounts of (3E)-4,8-dimethyl-1,3,7-nonatriene (DMNT) were detected in the headspace of LIS chrysanthemum plants, but not in wild-type plants (Chapter 2). DMNT is derived from the sesquiterpene nerolidol, which is also a product of LIS when FPP is provided as substrate (Kappers et al., 2005). In our study, LIS was targeted to the plastids in transgenic chrysanthemum. The low emission of DMNT suggests that small amounts of FPP are available in the plastids. When LIS was targeted to the mitochondria, where larger amounts of FPP are available, substantial amounts of DMNT were emitted by Arabidopsis (Kappers et al., 2005). In plastid-targeted LIS-overexpressing Arabidopsis and potato, in addition to linalool, hydroxy-linalool and dihydrolinalool were detected in the headspace (Aharoni et al., 2003; Aharoni et al., 2006). However, these two volatiles were not detected in the headspace of LIS chrysanthemum (Chapter 2). Apparently, the hydroxylation and/or double-bond reduction of linalool are common but not universal in
different plant species. For GES, the overexpression in tomato fruits led to an increase in the already endogenously present geraniol as well as in ten volatile derivatives including geranyl acetate, geranial, geranic acid (Davidovich-Rikanati et al., 2007). In the GES-overexpressing maize the headspace of intact plants was not different from control maize, indicating that geraniol produced in the transgenic maize is efficiently converted to non-volatile derivatives. However, the detached leaves of GES maize emitted 5-fold more geranyl acetate than control leaves, suggesting that the release of geranyl acetate is wound-inducible in both GES-expressing and control maize plants and dependent on the availability of geraniol as substrate, which is higher in the transgenic plants. Based on the above observations, it seems that glycosylation or malonyl-glycosylation of terpene alcohols is the rule rather than the exception in transgenic plants overexpressing these terpenes. Both the primary terpenes and their derivatives even when already present in the plant species will tend to increase when the corresponding terpene synthases are overexpressed using constitutive promoters. However, the nature and quantity of any new derivatives will depend on the difference in tissue-specific expression between the endogenous and ectopic promoter. The study on the terpene glycosyl transferases will be helpful if the accumulation of terpene glycosides would need to be controlled. Co-overexpressing these enzymes with terpene synthases could, for example, further increase the accumulation of terpene glycosides. On the other hand, when the emission of terpenes should be increased, the glycosyl transferases could be suppressed, or the terpene synthases could be introduced in cultivars/mutants that lack them.

**Growth and development of transgenic plants overexpressing monoterpenes**

Overexpression of monoterpenes, especially monoterpenic alcohols, was sometimes shown to lead to strong effects on growth and development. For example, LIS-overexpressing Arabidopsis and potato were smaller and lighter green than wild-type control plants (Aharoni et al., 2003; Aharoni et al., 2006). GES-overexpressing tomato also showed lighter fruit colour because of a 50% decrease in lycopene content (Davidovich-Rikanati et al., 2007). In the present study, LIS chrysanthemum (Chapter 2) and CHS-overexpressing tobacco (Chapter 5) also showed slower growth rate and lighter leaf colour compared to wild-type or empty vector control plants. However, GES-overexpressing maize plants (Chapter 3) did not differ from control plants in plant height, leaf colour and grain weight. Similarly, LIS-overexpressing petunia plants were also visually indistinguishable from control plants (Lücker et al., 2001). In both latter cases, the products of the monoterpenic synthases were completely converted to non-volatile glycosides in the transgenic plants as described above. The strong growth and colour phenotypes of transgenic plants with high levels of gene expression could be due to direct phytotoxicity of the produced monoterpenic alcohols (Lewinsohn et al., 2001), or to insufficient availability of isoprenoid precursors for other essential metabolites such as carotenoids, chlorophylls and gibberellins (Aharoni et al., 2003). In this context, it is remarkable, that when monoterpenic alcohols were not released in free form, but fully converted to non-volatile glycosides or when the monoterpenes did not have an alcohol functional group and immediately emitted, the transgenic plants maintained their wildtype appearance. For example, the growth and development was not affected in transgenic tobacco releasing limonene, β-pinene and γ-terpinene (Lücker et al., 2004), or in transgenic spike lavender (Lavandula latifolia) releasing limonene (Muñoz-Bertomeu et al., 2008). Direct phytotoxicity therefore arguably contributes most to the sometimes observed growth and colour phenotypes.

**Roles of monoterpenes and their derivatives in WFT resistance**

In earlier work, several monoterpenes have been tested as pure compounds for their effects on WFT. While some of them, including linalool, geraniol, nerol and citronellol, induced
significant attraction of WFT at 10% (v/v) (Koschier et al., 2000), other monoterpenes, including thymol and carvacrol, showed anti-oviposition effects on WFT at 0.1% and 1% (v/v) (Sedy and Koschier, 2003). In the research presented in this thesis, the response of WFT was tested using LIS-expressing chrysanthemum and GES-expressing maize (Chapters 2 and 3) as well as on chrysanthemum leaves infiltrated with pyrethrins (Chapter 4). The data show that WFT are attracted by the smell of linalool, but deterred by the taste of linalool glycosides, putatively identified as conjugates of linalool to malonyl-glucose and pentose-hexoses. In GES-overexpressing maize, in which geraniol was efficiently converted to non-volatile geranoyl-6-0-malonyl-β-D-glucopyranoside, the behaviour of WFT was not affected.

Terpene glycosides are regarded as transport and storage forms of terpenes in plant tissues (Winterhalter et al., 1997). They may be involved in plant resistance against insects by direct deterrence (Pankoke et al., 2010), which is illustrated by the deterrence of linalool glycosides to WFT in our LIS-overexpressing chrysanthemum. Flowers of various plant species accumulate many terpenes as glycosides to high levels (Watanabe, 1993). In some rose cultivars, nearly half the petal monoterpenes are present as glycosides (Ackermann, 1989). In the case of LIS-overexpressing chrysanthemum, it is not clear yet, which specific linalool glycoside is responsible for the deterrence to WFT, but the finding suggests that terpene glycosides present in flowers may be involved in protecting them from pest insects while the volatile flower terpenoids attract pollinators. Another interesting example of terpene glycosides with insect resistance property is iridoid glycosides, a group of glycosides of monoterpen derivative that are found in over 50 plant families (Jensen, 1991). They have been demonstrated to have deterrent effects toward generalist insect herbivores (Bowers and Puttick, 1988; Biere et al., 2004). The proposed mechanism of this effect is that the iridoid glycosides are hydrolysed upon feeding and the resulting iridoid aglycone can denature amino acids, proteins and nucleic acids (Konno et al., 1999). We assume that the mechanism of deterrent effects of linalool glycosides on WFT may be similar to that of iridoid glycosides. The lack of effect in GES-overexpressing maize, therefore, may be due to the malonyl group in the accumulated non-volatile geranoyl-6-0-malonyl-β-D-glucopyranoside, as malonyl conjugation can prevent the glycosides from glycolysis (Heller and Forkmann, 1994).

Pyrethrins are ester derivatives of monoterpenes and show strong effects against many insects (Casida, 1995). The primary mode of action of pyrethrins is keeping the voltage-gated ion channels open in insect neurons (Davies et al., 2007). Up to 80% of WFT adults died within 3 days when feeding on leaves of pyrethrum. Both in vitro and in planta assays showed that pyrethrins exhibit a strong negative effect on WFT (Chapter 4). As pyrethrins not only deter WFT from feeding from the plants but also negatively affect WFT reproduction, producing pyrethrins in transgenic plants could be an effective way to improve plant resistance to WFT.

Potential to create WFT-resistant plants by overexpressing pyrethrins

The engineering of WFT resistance by overexpression of pyrethrins requires a thorough understanding of the biosynthesis and regulation of pyrethrin biosynthesis in pyrethrum. Knowledge of the biosynthesis of the acid moiety has strongly increased since the genes catalysing the biosynthesis of chrysanthemol (chrysanthemol synthase, CHS, Chapter 5) and chrysanthemic acid (chrysanthemic acid synthase, CAS; Ramirez, 2013) have been characterized recently. CHS is able to catalyse two consecutive reactions from DMAPP to chrysanthemoyl diphosphate and then to chrysanthemol. Then, chrysanthemol is oxidized to chrysanthemal and chrysanthemic acid, catalysed by a single cytochrome P450 enzyme, chrysanthemic acid synthase (CAS). The modification from chrysanthemic acid to pyrethric acid is through oxidation and methyl esterification. It may require anywhere between one to three enzymes to perform the hydroxylation and subsequent oxidation to the acid of the vinyl
methyl group and subsequently 1 methyl transferase to perform the methyl esterification (Godin et al., 1963).

The alcohol moieties - jasmolone, cinerolone and pyrethrolone - with which the resulting pyrethric acid esterify resemble the plant hormone jasmonic acid. They were proposed to be derived from cis-jasmone or 7-hydroxy-jasmonic acid (Matsuda et al., 2005; Matsuo et al., 2012). Jasmonic acid is suggested to be the precursor of cis-jasmone through dehydrogenation, double bond migration and decarboxylation as a vinylogous β-keto acid (Crombie, 1999). In the next step, jasmonate is supposed to be hydroxylated into jasmolone, which might be the precursor of cinerolone and pyrethrolone through modifications of the side-chain (Crombie, 1999). 7-Hydroxy-jasmonic acid, the hydroxylation product of jasmonic acid, could be converted to jasmolone through decarboxylation and dehydrogenation. Therefore, depending on the biosynthesis route, at least 3 or 4 enzymes involved in the conversion from jasmonic acid to jasmolone need to be further characterized to understand the biosynthesis of the alcohol moieties.

The ester linkage between acid and alcohol moieties is finally formed by sequential action of two enzymes, an acyl-CoA ligase (Chapter 6) and an acyl-transferase (Kikuta et al., 2012). So, in total at least 8 enzymes are required to produce the most basic one of the six pyrethrin esters, jasminol I, from the precursors DMAPP and jasmonic acid which are universally present in plants (Fig. 1). For the formation of the major pyrethrin esters, pyrethrin I and II, at least 1 and 3 extra enzymes are required, respectively. Once all these genes are characterized, a major task remains to introduce them into a different plant species and to express them in such a way that indeed pyrethrins are produced and properly sequestered. Such a project may have more success if one stays close to the original source of the pathway, pyrethrum. Introduction into the commercial ornamental crop chrysanthemum is a logical choice, but also sunflower might be possible. The transformation of multiple genes has been achieved in several plants. The genes were either introduced in a sequential manner in subsequent generations of plants or by crossing (Zhang et al., 2011), or more efficiently, they were introduced at once and on one locus via a multi-gene vector (Farhi et al., 2011). This new system recently even enabled transformation of up to nine genes at once (Zeevi et al., 2012).

Other candidate compounds for host-plant resistance against WFT

Considering the complexity of overexpressing pyrethrins in transgenic plants, overexpression of compounds involved in host-plant resistance against WFT might be an alternative approach to improve plant resistance to WFT. Host-plant resistance against WFT has been identified in many wild or cultivated accessions, including cotton (Trichilo and Leigh, 1988), pepper (Fery and Schalk, 1991), rose (Gaum et al., 1994), tomato (Krishna Kumar et al., 1995), cucumber (Soria and Mollema, 1995) and chrysanthemum (De Jager et al., 1995). Non-targeted metabolic analysis (metabolomics) of thrips-resistant and -susceptible plants has been employed to reveal which compounds correlate with host-plant resistance. In Senecio hybrids, two pyrrolizidine alkaloids jaconine and jacobine-N-oxide and a flavonoid, kaempferol glucoside, were identified to be related to thrips resistance in this way (Leiss et al., 2009). In chrysanthemum, WFT-resistant plants contained higher amounts of the phenylpropanoids chlorogenic acid and feruloyl quinic acid, and bioassays with artificial diets showed that chlorogenic acid at 1% and 5% significantly deterred WFT (Leiss et al., 2009). In tomato, acylsugars were identified as a resistance factor against WFT (Mirnezhad et al., 2010). In pepper, seven compounds were identified to be correlated with resistance to WFT, including tocopherols, an unknown sequiterpene, an unknown phytosterol and three other unknown compounds (Maharijaya et al., 2012). The identification of these metabolites involved in host-plant resistance could facilitate the relative straightforward breeding of WFT resistant crops.
However, the effectiveness of these compounds against WFT still requires more detailed studies.

Outlook

Metabolic engineering of monoterpenes in plants is a promising way to manipulate plant-insect interactions. However, the production of monoterpenes and their derivatives in plants is controlled by many factors besides the overexpressed monoterpene synthases. In this thesis, glycosylation of monoterpenes was identified as an important factor since it does not only affect the monoterpene emission but is also directly involved in insect resistance. This suggests that more attention should be given to the biosynthesis of specific monoterpene glycosides which may have deterrent effects against pest insects. In addition, metabolic engineering of monoterpenes will also benefit from studies on other factors, such as enzyme targeting, regulatory elements (promoters), upstream signalling pathways, multi-enzyme complexes, substrate supply and transporters (Jirschitzka et al., 2012).

Pyrethrins are ideal compounds for host-plant resistance against WFT because of their strong negative effect on feeding, reproduction and survival of WFT. Identification of the genes involved in pyrethrin biosynthesis, as described in this thesis, will enable the metabolic
engineering of pyrethrins in transgenic plants. However, a number of missing genes still need to be identified. The regulation of pyrethrin biosynthesis also awaits further study. Once pyrethrins are introduced into transgenic plants, these plants could be resistant to a broad spectrum of insects, as shown in in vitro studies with pyrethrins (Casida, 1995). For WFT control, it could also be interesting to co-express linalool, an attractant of WFT, and pyrethrins, as such plants could serve as a trap to kill WFT and reduce WFT reproduction.

References


Summary

Western flower thrips (WFT), *Frankliniella occidentalis*, is one of the most serious pests in several vegetable and flower crops worldwide. It is a highly polyphagous insect and a vector of several plant viruses of which the Tomato Spotted Wilt Virus and the Impatiens Necrotic Spot Virus are the most important. Feeding by WFT causes light coloured patches on leaves, petals and fruits, stunted plant growth, and flower and fruit deformation. Synthetic pesticides has been widely used to control WFT. However, the frequent use of these pesticides leads to rapid resistance in WFT, and they are a threat to the environment. Therefore, it is desirable to identify natural sources of resistance effective against WFT to allow breeders to improve resistance in crop species.

Monoterpenes, as constituents of floral scents and plant resins, play an important role in pollinator attraction and in direct and indirect plant defence against pest insects and pathogens. For example, linalool is a common floral scent constituent and found to be emitted from the leaves by many plant species after herbivore attack. In earlier work, linalool-overexpressing Arabidopsis has been tested for resistance to the pest aphid, *Myzus persicae*, in dual-choice assays, and transgenic plants significantly repelled or deterred the aphids. A linalool synthase (LIS) was overexpressed in chrysanthemum plants and studied the effect of transgenic plants on WFT (Chapter 2). The volatiles from leaves of transgenic plants were significantly attractive to WFT, however, WFT were significantly deterred by the content of leaf discs from transgenic plants. The headspace analysis showed that the volatiles of LIS chrysanthemum leaves were strongly dominated by linalool, but, they also emitted small amounts of the C11-homoterpene, (3E)-4,8-dimethyl-1,3,7-nonatriene, a derivative of nerolidol. In addition, LC-MS analysis showed that several non-volatile linalool glycosides were significantly increased in the leaves of LIS chrysanthemum compared with leaves of wild-type plants. A geraniol synthase (GES) was overexpressed in maize to see whether WFT could be affected by geraniol or its derivatives (Chapter 3). However, geraniol produced in transgenic maize was all efficiently converted to non-volatile glycoside, geranoyl-6-O-malonyl-β-D-glucopyranoside, and GES maize had no effect on WFT behaviour. These studies demonstrate complex effects of terpene engineering on the metabolic changes in transgenic plants. These results suggest that the release/glycosylation of terpenes should be controlled to improve plant resistance against WFT upon metabolic engineering with terpene synthases.

The research subsequently focused on a well-known natural pesticide—pyrethrins. Pyrethrins comprise a group of six closely related esters, derived from the monoterpenes alcohol chrysanthemol. Pyrethrins are the economically most important natural insecticide with broad uses in homes, agriculture and stored products for more than 150 years. The effect of pyrethrins against WFT was evaluated on its survival, feeding behaviour, and reproduction both *in vitro* and *in planta* (infiltrated chrysanthemum leaves) (Chapter 4). Pyrethrins at 0.1% (w/v) and 1% (w/v) exhibited a significantly negative effect on feeding, and the effects of natural concentrations of pyrethrins in pyrethrum leaves can explain the observed high mortality of WFT feeding on pyrethrum leaves. After the finding of this strong effect of pyrethrins on WFT, the study on the biosynthetic pathway of pyrethrins was continued in order to introduce pyrethrin biosynthesis in transgenic plants. A second function of the published enzyme, chrysanthemyl diphosphate synthase (CDS) was identified (Chapter 5). CDS has been reported to catalyse the formation of chrysanthemyl diphosphate (CPP). However, CDS was demonstrated to also catalyse the next step of CPP into chrysanthemol both *in vitro* and *in vivo*. CDS was proposed to be renamed as a chrysanthemol synthase (CHS) using DMAPP as substrate. The gene involved in the next step converting chrysanthemol to chrysanthemic acid has also been characterized (Ramirez, 2013). A chrysanthemic acid:CoA
ligase, which is involved in the final stage of pyrethrin biosynthesis was also studied (Chapter 6). The function of this enzyme was confirmed in vitro and the encoding gene showed a similar expression pattern as CHS in several different tissues and flower developmental stages. The gene responsible for making the final esters is a GDSL-lipase-like acyltransferase (Kikuta et al., 2012). We assume still three to four enzymes are required for the biosynthesis of the basic one of the six pyrethrin esters, jasmolin I, from the precursors DMAPP and jasmonic acid which are universal in plants. And four to five extra genes are required for the complete biosynthesis of all six pyrethrin esters.

In this study, new insights were gained for the biosynthesis of monoterpenes and their derivatives and conjugates, as well as for plant resistance to WFT mediated by these compounds. The characterization of genes involved in pyrethrin biosynthesis paves the way for metabolic engineering of this natural pesticide in other crops.

References
Samenvatting

Californische trips (CT), Frankliniella occidentalis, is wereldwijd een van de meest problematische plaaginsecten in verscheidene groente en bloemengewassen. Het is een uitzonderlijk polyfaag insect en vector van verscheidene plantenvirussen, waarvan het tomatenbromsvlekkenvirus TSWV en het Impatiens-vlekkenvirus INSV de meest belangrijke zijn. Het voedingspatroon van CT veroorzaakt licht gekleurde vlekjes op bladeren, petalen en vruchten, alsook verminderde groei en vervorming van bloemen en vruchten. Synthetische pesticiden worden breed ingezet tegen CT. De frequente inzet van deze pesticiden leidt echter tot snelle ontwikkeling van resistentie in CT en ze vormen een bedreiging voor het milieu. Om die reden is het wenselijk om natuurlijk bronnen van tripsresistentie te identificeren teneinde veredelaars in staat te stellen de resistentie in hun gewassen te verbeteren.

Monoterpenen zijn componenten van de geur van bloemen en secreties van planten, en in die hoedanigheid spelen ze een belangrijke rol in de aantrekking van bestuivers en in de directe en indirecte afweer tegen plaaginsecten en pathogenen. Linalool is bijvoorbeeld een algemeen voorkomende component van de geur van bloemen, maar wordt ook afgegeven door de bladeren van veel plantensoorten wanneer ze worden belaagd door herbivoren. In eerder werk werden Arabidopsisplanten die linalool produceren getest op hun resistentieniveau tegen de perzikluis, Myzus persicae. Keuze-experimenten toonden aan dat de bladluizen significant afgestoten werden door de transgene linaloolplanten. Een linalool synthase (LIS) werd in dit promotieonderzoek tot expressie gebracht in chrysant en het effect op CT werd bestudeerd (Hoofdstuk 2). De vluchtige stoffen uit bladeren van transgene planten bleken significant aantrekkelijk voor CT, maar CT werd afgestoten door de inhoudsstoffen van de geteste bladponsen van transgene planten. Analyse van plantengeur toonde aan dat geur van LISR chrysanten sterk gedomineerd werd door linalool, alhoewel er ook kleine hoeveelheden van de C11-homoterpeen, (3E)-4,8-dimethyl-1,3,7-nonatriene, een derivaat van nerolidol, aangetroffen werden. Bovendien werd met LCMS analyse aangetoond dat verschillende niet-vluchtige linaloolglycosiden significant hoger waren in de bladeren van LISR chrysanten vergeleken met de wildtype-planten. Een geraniol synthase (GES) werd tot overexpressie gebracht in mais in een andere studie om te zien of CT beïnvloed zou worden door geraniol of derivaten daarvan (Hoofdstuk 3). In transgene mais werd alle geraniol echter efficient omgezet in het niet-vluchtige glycoside, geranoyl-6-O-malonyl-β-D-glucopyranoside, en GES-mais had in dit geval geen effect op het gedrag van CT. Deze studies tonen aan dat er complexe effecten zijn van de genetische modificatie van terpeen biosyntheseroutes op de inhoudsstoffen van transgene planten. De resultaten suggereren dat, bij de genetische modificatie van de terpeen biosynthese, de emissie, dan wel de glycosylering van terpenen beter gereguleerd moeten worden om CT-resistentie in planten te bewerkstelligen.

Het onderzoek hield zich vervolgens bezig met een bekend natuurlijk pesticide, pyrethrine genaamd. Pyrethrines omvatten een groep van zes nauw verwante esters, die afgeleid zijn van de monoterpeel alcohol chrysanthenol. Pyrethrines vormen al meer dan 150 jaar het economisch meest belangrijke natuurlijke insecticide met brede toepassingen in huishoudens, de land- en tuinbouw en in de bescherming van de oogst. Het effect van pyrethrines op de overleving, het gedrag en de productie van CT werd zowel in vitro als in planta (geïnfiltreerde chrysantenbladeren) bestudeerd (Hoofdstuk 4). Concentraties van pyrethrines van 0.1% (w/v) en 1% (w/v) bleken een significant negatief effect op het voedingsgedrag te hebben en de natuurlijke concentraties van pyrethrines in pyrethrumblad zelf konden de hoge mortaliteit van CT op pyrethrumblad verklaren. Op basis van dit sterke effect van pyrethrines op CT, werd vervolgens een onderzoek uitgevoerd met de doelstelling om de pyrethrine biosyntheseroute op te helderen en in transgene planten te introduceren. Een tweede functie van het bekende enzym, chrysanthenyl difosfaat synthase (CDS) werd geïdentificeerd.
(Hoofdstuk 5). Van CDS was bekend dat het de vorming van chrysanthemyl difosfaat (CPP) katalyseerde. Ik toonde echter aan dat CDS ook de volgende omzetting van CPP in chrysanthemol zowel in vitro als in vivo uitvoert. Het CDS enzym werd daarom hernoemd tot een chrysanthemol synthase (CHS) enzym dat dimethylallyldifosfaat (DMAPP) als substraat gebruikt. Het gen dat chrysanthemol in chrysanthemylzuur omzet werd ook gekarakteriseerd (Ramirez, 2013). Een chrysanthemylzuur:CoA ligase, betrokken in de laatste stap van de pyrethrine biosynthese werd ook geïsoleerd (Hoofdstuk 6). De functie van dat enzym werd bevestigd in vitro en van het gen werd aangetoond dat het een vergelijkbaar expressiepatroon vertoonde als het CHS gen in verschillende weefsels en ontwikkelingsstadia van de bloemen van pyrethrum. Het gen verantwoordelijk voor de synthese van de pyrethrine esters is een GDSL-lipase met een ongebruikelijke acyltransferase activiteit (Kikuta et al., 2012). We veronderstellen dat er nu nog drie tot vier tot nog toe onbekende enzymen nodig zullen zijn om met de universele precursors DMAPP en jasmonzuur, jasmoline I te kunnen maken. Jasmoline I wordt gezien als de precursor van de overige pyrethrines. Synthese van die overige pyrethrines vereist de opheldering van mogelijk nog eens vier tot vijf enzymen.

In dit onderzoek werden niet alleen nieuwe inzichten verworven in de biosynthese van monoterpenen en hun derivaten en conjugaten, maar ook in de effecten daarvan op de resistentie van planten tegen californische trips. De karakterisering van verscheidene genen betrokken bij de biosynthese van pyrethrines heeft de mogelijkheid om de biosynthese van deze insecticiden te introduceren in andere gewassen een stuk dichterbij gebracht.

Referenties
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Curriculum Vitae

Ting Yang was born on December 9th, 1981 in Huangshi, Hubei province, China. Ting performed her bachelor study between 1999 and 2003 with a major in Horticulture in Huazhong Agricultural University and with an additional major in English in Huazhong University of Science and Technology. Due to her excellent performance during the bachelor study, Ting was recommended for admission for an MSc study in Huazhong Agricultural University in 2003. For her MSc study, she chose to study Vegetable Science with specialization in Molecular Biology. In 2006, she obtained her MSc degree and graduated with distinction. Afterwards, she temporarily worked as a research assistant in the group of Dr. Maarten A. Jongsma, Plant Research International, Wageningen University and Research Center, the Netherlands. In 2008, she became a PhD student in the same group under the supervision of Prof. Marcel Dicke, Prof. Harro Bouwmeester and Dr. Maarten A. Jongsma. This thesis summarizes the research results of her PhD study in metabolic engineering of monoterpenoids in plants to improve plant resistance to insects.
Publication list

Publication related to this thesis


T Yang, L Gao, G Stoopen, MA Jongsma. 2013. Chrysanthemyl diphosphate synthase operates in planta as a bifunctional enzyme with chrysanthemol synthase activity (under review)

Ramirez AM, Yang T, Bouwmeester HJ, Jongsma MA: A trichome-specific 13-lipoxygenase expressed during pyrethrin biosynthesis in pyrethrum (submitted)


Ramirez AM, Verstappen FWA, de Vos RCH, Yang T, Gao L, Bouwmeester HJ, Jongsma MA: A single Cytochrome P450 Enzyme Catalyzes the Formation of Chrysanthemic Acid from Chrysanthemol in Pyrethrin Biosynthesis (patent application filed)

Other publication

**Education Statement of the Graduate School**

**Experimental Plant Sciences**

**Issued to:** Ting Yang  
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<td>EPS PhD student day, Utrecht University</td>
<td>Jun 01, 2010</td>
</tr>
<tr>
<td>EPS PhD student day, Wageningen University</td>
<td>May 20, 2011</td>
</tr>
<tr>
<td>► EPS theme symposia</td>
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<tr>
<td>EPS theme 3 “Metabolism and Adaptation”, Wageningen University</td>
<td>Nov 06, 2007</td>
</tr>
<tr>
<td>EPS theme 3 “Metabolism and Adaptation”, Amsterdam University</td>
<td>Feb 18, 2009</td>
</tr>
<tr>
<td>EPS theme 2 “Interactions between Plants and Biotic Agents”, Utrecht University</td>
<td>Jan 15, 2010</td>
</tr>
<tr>
<td>EPS theme 2 “Interactions between Plants and Biotic Agents”, Amsterdam University</td>
<td>Feb 03, 2011</td>
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<tr>
<td>EPS theme 3 “Metabolism and Adaptation”, Wageningen</td>
<td>Feb 10, 2011</td>
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<tr>
<td>► NWO Lunteren days and other National Platforms</td>
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<tr>
<td>ALW meeting ‘Experimental Plant Sciences’, Lunteren</td>
<td>Apr 19–20, 2010</td>
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<td>ALW meeting ‘Experimental Plant Sciences’, Lunteren</td>
<td>Apr 04–05, 2011</td>
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<tr>
<td>ALW meeting ‘Experimental Plant Sciences’, Lunteren</td>
<td>Apr 02–03, 2012</td>
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<tr>
<td>► Seminars (series), workshops and symposia</td>
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<tr>
<td>EPS symposium ‘From Molecules to Multitrophic Interactions’, Wageningen University</td>
<td>Mar 23, 2007</td>
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<tr>
<td>The Plant and Insect Interaction Workshop, Amsterdam University</td>
<td>Oct 27, 2007</td>
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<tr>
<td>► Seminar plus</td>
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<tr>
<td>► International symposia and congresses</td>
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<tr>
<td>TERPNET 2009: 9th International meeting, Tokyo, Japan</td>
<td>May 25–29, 2009</td>
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<tr>
<td>Second Joint Retreat of PhD students in Plant Sciences, Cologn, Germany</td>
<td>Apr 15–17, 2010</td>
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<tr>
<td>14th Symposium on Insect-Plant Interactions, Wageningen</td>
<td>Aug 13–18, 2011</td>
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<td>► Presentations</td>
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<tr>
<td>EPS PhD student day, Leiden (oral)</td>
<td>Feb 26, 2009</td>
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<tr>
<td>Bioscience thematic meeting, Wageningen (oral)</td>
<td>Apr 07, 2009</td>
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<td>Bioscience thematic meeting, Wageningen (oral)</td>
<td>Jan 12, 2010</td>
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<tr>
<td>Bioscience thematic meeting, Wageningen (oral)</td>
<td>Jun 13, 2012</td>
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<tr>
<td>INterSECT project meeting, Wageningen (oral)</td>
<td>Oct 30, 2008</td>
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<td>INterSECT project meeting, Wageningen (oral)</td>
<td>Nov 24, 2009</td>
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<td>INterSECT project meeting, Wageningen (oral)</td>
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<td>INterSECT project meeting, Wageningen (oral)</td>
<td>Dec 02, 2010</td>
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<tr>
<td>INterSECT project meeting, Wageningen (oral)</td>
<td>Jun 21, 2011</td>
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<tr>
<td>INterSECT project meeting, Wageningen (oral)</td>
<td>Nov 25, 2011</td>
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<tr>
<td>Metabolic Regulation cluster meeting, Wageningen (oral)</td>
<td>Apr 18, 2007</td>
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<tr>
<td>Metabolic Regulation cluster meeting, Wageningen (oral)</td>
<td>Oct 08, 2008</td>
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<td>Metabolic Regulation cluster meeting, Wageningen (oral)</td>
<td>Oct 14, 2009</td>
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<td>Metabolic Regulation cluster meeting, Wageningen (oral)</td>
<td>Mar 17, 2010</td>
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<td>Metabolic Regulation cluster meeting, Wageningen (oral)</td>
<td>Mar 09, 2011</td>
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<tr>
<td>Metabolic Regulation cluster meeting, Wageningen (oral)</td>
<td>Sep 26, 2012</td>
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<tr>
<td>Symposium ‘Joint Retreat of PhD students in Plant Sciences’, Cologn (oral)</td>
<td>Apr 15, 2010</td>
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<tr>
<td>TERPNET 2009: 9th International meeting, Tokyo (poster)</td>
<td>May 26, 2009</td>
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<tr>
<td>ALW meeting ‘Experimental Plant Sciences’, Lunteren (poster)</td>
<td>Apr 04, 2011</td>
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<tr>
<td>14th Symposium on Insect-Plant Interactions, Wageningen (poster)</td>
<td>Aug 17, 2011</td>
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<tr>
<td>► IAB interview</td>
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<tr>
<td>► Excursions</td>
<td></td>
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</table>

**Subtotal Scientific Exposure** 31.6 credits*
### 3) In-Depth Studies

<table>
<thead>
<tr>
<th>Date/Date Range</th>
<th>Courses/Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jun 06-08, 2007</td>
<td>Workshop &quot;Metabolomics&quot;, Wageningen University</td>
</tr>
<tr>
<td>Apr 21-23 &amp; 28-29, 2008</td>
<td>PhD course &quot;Metabolomics&quot;, Leiden University</td>
</tr>
<tr>
<td>Dec 10, 2008</td>
<td>PhD competence assessments, Wageningen University</td>
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<tr>
<td>Aug 30-31 &amp; Sep 01-03, 2010</td>
<td>PhD course &quot;Bioinformatics-A user’s approach&quot;, Wageningen University</td>
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</table>

**Subtotal In-Depth Studies**: 8.7 credits*

### 4) Personal Development

<table>
<thead>
<tr>
<th>Date/Date Range</th>
<th>Courses/Activities</th>
</tr>
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<tbody>
<tr>
<td>Feb 04, 11, 25 &amp; Mar 11, 2009</td>
<td>EPS courses or other PhD courses (highly recommended)</td>
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<tr>
<td>May 06-Jun 24, 2010</td>
<td>The Art of Writing</td>
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<tr>
<td>Feb 09-10, 2010</td>
<td>Scientific Writing</td>
</tr>
<tr>
<td></td>
<td>Information Literacy, including Introduction Endnote</td>
</tr>
<tr>
<td></td>
<td>Organisation of PhD students day, course or conference</td>
</tr>
<tr>
<td></td>
<td>Membership of Board, Committee or PhD council</td>
</tr>
</tbody>
</table>

**Subtotal Personal Development**: 4.2 credits*

**TOTAL NUMBER OF CREDIT POINTS**: 52.0

* Herewith the Graduate School declares that the PhD candidate has compiled 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.