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PhD Dissertation

CONTROL OF THE GRAPEVINE MOTH *LOBESIA BOTRANA* THROUGH GENETIC MODIFICATION OF KAIROMONES BIOSYNTHESIS IN THE HOST PLANT

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Abstract

The European grapevine moth *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) is one of the key pests of grape: current control systems are based either on the use of insecticides or on mating disruption, but while the first is not environmentally friendly, the second is not particularly suitable for small and isolated areas, or regions where pest population is high.
Here we explore the possibility to develop a new control strategy of *L. botrana* by interfering with the female host-finding and egg-laying behaviors, which are mostly mediated by the volatile organic compounds (VOCs) emitted by the host plant (kairomones). Wind-tunnel studies have shown that a blend of 3 specific grapevine plant terpenoids, namely *E*-(β)-caryophyllene, *E*-(β)-farnesene and *E*-(4,8)-dimethyl-(1,3,7)-nonatriene (DMNT) elicited attraction comparable to that of the complete plant odor profile.
After characterizing *E*-(β)-caryophyllene synthase, the enzyme that produces the most abundant grapevine sesquiterpene, we chose a genetic engineering approach and produced grapevine and Arabidopsis plants with altered headspace profile. Arabidopsis was used to study the role of *E*-(β)-caryophyllene and *E*-(β)-farnesene in oviposition, and to try to create a model for the emission of DMNT. Grapevine was used to study how a change in kairomones ratio can impact on plant attractiveness from the distance, using the wind tunnel.
Overall, the role of these kairomones in oviposition remains unclear, but we could prove that a modification of the *E*-(β)-caryophyllene / *E*-(β)-farnesene ratio leads to a decreased attraction, creating the rationale for a new pest control method.

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List of Abbreviations

AaβFS = *Artemisia annua* β-farnesene synthase

ANOVA = ANalysis Of Variance

CLSA = Close-Loop Stripping Analysis

DMAPP = Dimethyl Allyl PyroPhosphate

DMNT = *E*-(4,8)-dimethyl-(1,3,7)-nonatriene

FPP = Farnesyl PyroPhosphate

GC-MS = Gas-Chromatography Mass-Spectrometry

GM = Genetically Modified

IPP = Isopentenyl PyroPhopsphate

MD = Mating Disruption

RT-qPCR = Reverse Transcription quantitative Polymerase Chain Reaction

SPME = Solid Phase Micro Extraction

TPS = Terpene Synthase

VOCs = Volatile Organic Compounds

VvCSLinNer = *Vitis vinifera* Cabernet Sauvignon Linalool/Nerolidol synthase

VvGwECar2 = *Vitis vinifera* Gewurtztraminer β-caryophyllene synthase

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Chapter 1

General Introduction

The Grapevine Moth

The European grapevine moth *Lobesia botrana* (Lepidoptera: Tortricidae) (Fig.1) is a known pest of economic significance that originated in Europe and has spread to portions of Africa, the Middle East as well as some wine-growing regions in North and South America (Ioriatti et al., 2011; Lucchi et al, 2014).

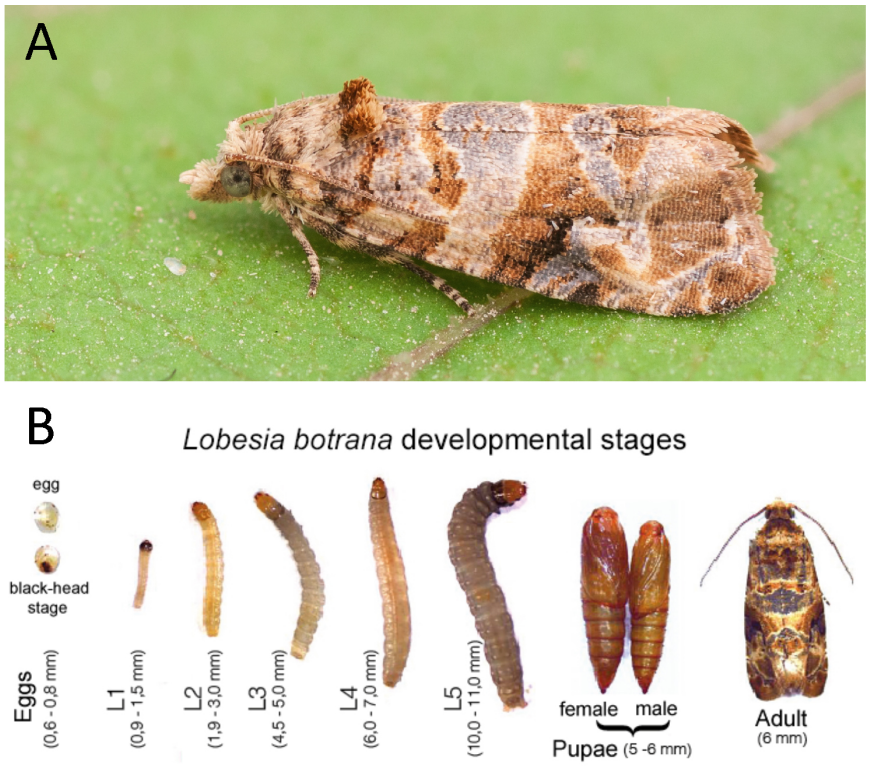

Figure 1. The European grapevine moth *L. botrana*, (A) adult female on a grape leave (B) developmental stages (adapted from www.redagricola.com).

Based on climatic conditions *L. botrana* can have from two to four generations per year: larvae of the first generation feed on inflorescences, damaging reproductive structures and resulting in yield loss. The following generations feed on green and ripe berries, often helping secondary infections caused by other pathogens, (especially the gray mold fungus *Botrytis cinerea*) causing the most significant damage in the vineyards. *L. botrana* is also feeding on more than 20 other species, including economically important plants such as olive, rosemary and raspberries (Thiéry and Moreau, 2005). Two main pest

control strategies are currently in use to limit the infestation of *L. botrana* in vineyards: chemical insecticides and pheromone-based strategies.

In the past decades many insecticides with different mode of actions were traditionally sprayed to control *L. botrana*. Their use has been limited over the years due to several side effects, like deleterious effects of residual chemicals for the consumers or environmental issues like toxicity for other insects. The old pesticides have been gradually replaced by compounds that are more selective and less hazardous for human health, such as new neurotoxic insecticides (e.g. spinosyns and oxadiazines), chitin synthesis inhibitors, molting accelerating compounds and microbial insecticides (Ioriatti et al., 2011). On the other hand, pheromone-based strategies are not based on the toxicity of the released compounds, but they take advantage of the intraspecific olfactory communication between sexes in the moth, discovered in the early 70s. The main compound, (*E*, *Z*)-7,9-dodecadienyl acetate, was the first to be discovered in *L. botrana* (Roelofs et al. 1973, Buser et al. 1974) and is still the active ingredient used nowadays in the technique called Mating Disruption (MD), in which the pheromone is released in the vineyards by hand-applied dispensers, confusing the male and preventing it from finding the mating partner. Although the active compound remains the same, there is a large variation in the load as well as in the physical and chemical features of the dispensers, including size, shape, and thickness of the dispenser walls, leading to differences in release rates and in the dispenser life itself (Anfora et al. 2008). In the field, all the factors that influence concentration, homogeneity and atmospheric distribution of the synthetic pheromone greatly affect the behavioral response of the male and thus the efficacy of MD. These factors include plant spacing, plant canopy and leaf density, and this is mostly because of the ability of the leaves to absorb and release pheromone and to mitigate the effect of wind (Sauer and Karg, 1998). Population density is also a key factor for MD success: above a certain threshold mating is not disrupted, regardless of the ambient pheromone concentrations. For *L. botrana*, 4000 pairs/ha is generally accepted as the critical density above which the efficacy of MD is dramatically reduced (Feldhege et al., 1995), because of the high probability of the male to find the female simply by

chance and not following the pheromone trace in the air. Moreover, lower pheromone concentrations along borders combined with increased population densities (Louis et al., 1997) account for difficulties in obtaining satisfactory results along vineyard edges (Varner et al., 2001). The only way to reduce this border effect is to apply MD to wide areas, as it is done in the Northern Italy and in some regions of Germany, Switzerland, and Spain (Ioriatti et al., 2008; Ioriatti and Lucchi 2016). When a population density above the critical threshold is reached, it is necessary to apply pesticides again, and reduce the population to a level at which MD is effective again (Louis and Schirra, 1997). For all these reasons, despite the great amount of research during the last twenty years, MD is applied in Europe only in 4% of the total vineyards areas (≈140000 ha), which is quite a small value. A notable exception is the province of Trento, where a *L. botrana* management by MD has been successfully implemented since 1998, reaching in 2010 the 9/10 of the total vineyard area. The main limitation of the MD strategy is that it can affect only males behavior: to have a stronger control it would be desirable to affect also the female host-finding and egg-laying behaviors. These in many insects are mediated by the release of volatile compounds from the host plant (Bruce et al. 2005), which act as kairomones.

Grapevine

Grapevine cultivation is diffused in more than 40 countries and with more than 7 Mha is the fruit crop with the highest harvested surface in the world (www.faostat.org). These plants belong to the family of *Vitaceae* and the genus *Vitis (*Tourn.), which is divided into the subgenera *Muscadinia* and *Euvitis* according to their chromosome number (2n = 40 and 2n = 38 respectively) and to morphological differences. Almost all the cultivated grapes belong to the Eurasian group of the *Euvitis* subgenus, of the species *Vitis vinifera* L. *ssp. vinifera* domesticated from the ssp. *sylvestris* more than 7000 years ago. They are perennial woody plants with deciduous leaves and their inflorescences are composed by a peduncle with a central rachis carrying lateral branches that have from a few tens up to hundreds of flowers

on pedicels. The flowers are small, inconspicuous and perfumed, their petals are fused in a calyptra and flowering corresponds to the loss of calyptra and exposure of stamens and pistil. The process of pollination and fertilization triggers the development of the ovary that becomes fruit (the berry). Although the flowers are self-fertilizing, outbreeding by means of wind and insect pollination is very frequent: as result, all the cultivars are highly heterozygous and carry deleterious recessive mutations. Inbreeding depression is severe, so that sterility often ensues from the second or third generation of selfing.

Terpenes in plants

Terpenoids build up the biggest class of metabolites in plants, involved both in primary (Croteau *et al*., 2000) and secondary metabolism (Zwenger & Basu, 2008). Humans have traditionally used them as fragrances and flavors, since many of them are present in plants essential oils. More recently in human history, they were investigated for their role as high-value chemicals for obtaining materials (e.g. rubbers), pharmaceuticals and even biofuels (Bohlmann & Keeling, 2008). All the classes of terpenes formally derive from the C_5 isomers isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), whose biosynthesis can take place in plants via two distinct pathways: the mevalonate pathway in the cytoplasm, and the MEP/DOXP pathway in the chloroplast (Rohmer, 1999) (Fig.2).

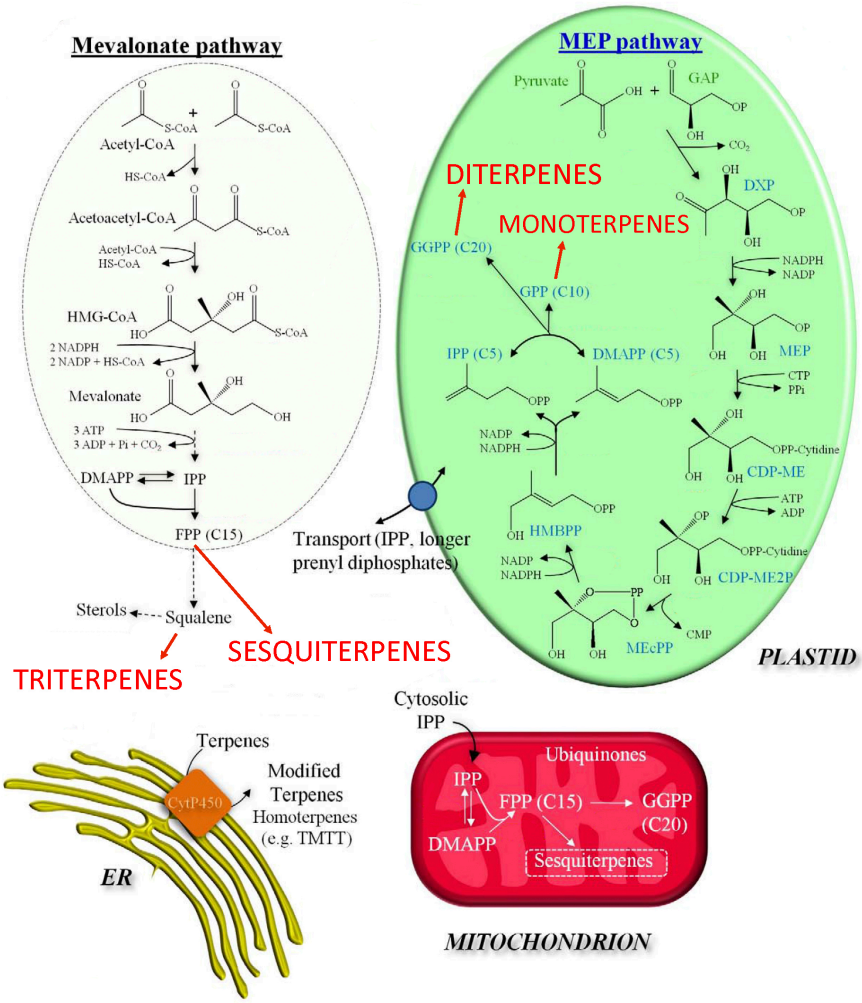

Figure 2. Biosynthetic pathways for mono-, sesqui-, di- and tetra-terpenes in the plant cell (adapted from Tholl and Lee 2011)

The condensation of C_5 isomers by the prenyl-transferase enzymes leads to the C_{10}, C_{15}, C_{20}, C_{30} and C_{40} precursors of mono-, sesqui-, di-, tri- and tetra-terpenes respectively. In the last step of the pathway, the precursors are converted to terpenes by the terpene synthase (*TPS*) enzymes, which are usually coded by gene families of 20-150 members in the genome sequenced so far. Based on the reaction mechanism and products formed, plant TPS enzymes are classified into two groups: class I and class II TPSs. Class I enzymes have an initial step of metal-dependent ionization of the substrate with the formation of a carbocation intermediate, while class II enzymes catalyze the formation of the product through a protonation-induced cyclization of the substrate, with a mechanism that is still unclear. A common feature of class I TPS enzymes is that, because of the stochastic nature of

bond rearrangements of the carbocation intermediate, a single substrate often gives rise to multiple products. TPS gene families also have been divided into seven subfamilies from TPS-a to TPS-h: monoterpene synthases belong to TPS-b and TPS-g subfamilies, while TPS-a subfamily mostly consists of sesquiterpene and diterpene synthase (Chen *et al*., 2011). Many of the direct products of TPS enzymes in specialized metabolism, namely isoprene, mono- and sesquiterpenes, and some diterpenes, are volatile organic compounds (VOCs) under the temperature and atmospheric conditions of the environment in which plants live. Modification of the TPS products by oxidation, peroxidation, methylation, acylation, or cleavage changes their physical properties and may alter their biological activities. In nature in fact, terpenoids are best-known for their multiple ecological roles: from direct defense against pathogens and insects (Hasegawa *et al*., 2010; Heiling *et al*. 2010) to indirect defense attracting enemies of herbivores (Turlings & Ton, 2006; Unsicker *et al.* 2009), but also attraction of pollinators (Dudareva & Pichersky, 2000), mutualistic fungi (Ditengou *et al.,* 2015) and plant-plant communication (Arimura *et al*., 2000). Their role as semiochemicals is also well exploited by phytophagous insects, which commonly use them as kairomones to locate their host plants (Bruce *et al*., 2005) (Fig. 3).

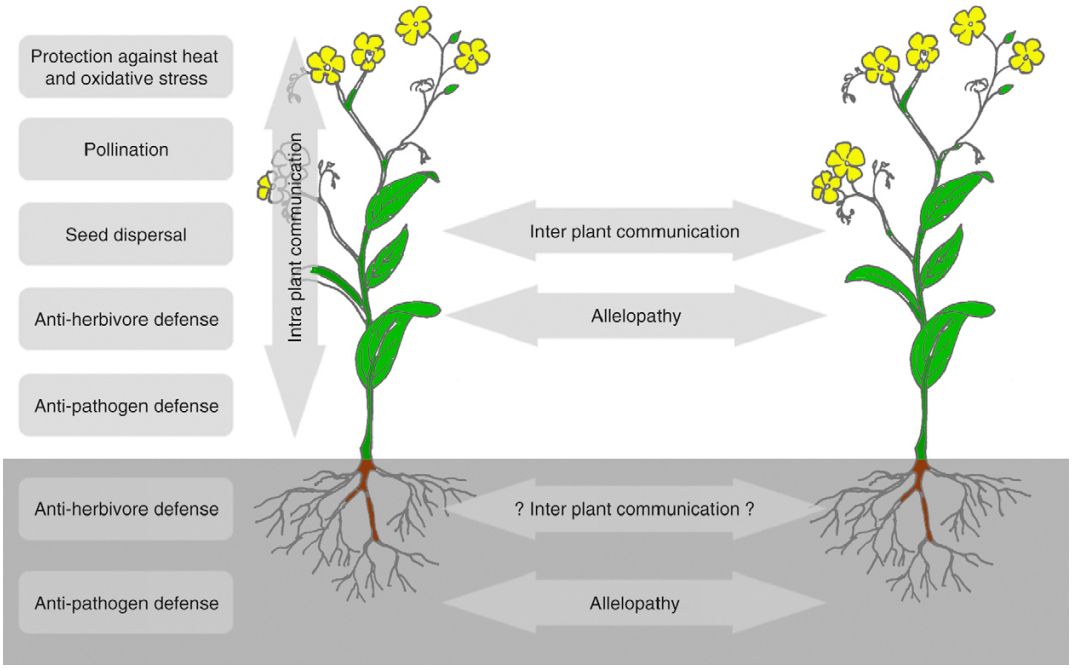

Figure 3. Scheme of the role of VOCs in the life of plants (adapted from Unsicker et al. 2009)

Grapevine terpenoids and interaction with *L. botrana*

Grapevine emits a great variety of VOCs belonging to several chemical categories (fig. 4). The most abundant group derives from the terpenoid pathways, which give rise to mono-, sesqui- and diterpenes, apocarotenoids, and other irregular volatile terpenes. Among all the genomes sequenced so far, grapevine has the biggest TPS gene family, with 69 putatively functional TPS genes, 20 partial genes and 63 probable pseudogenes (Martin et al., 2011). In the form of free volatiles and as glycoside conjugates, terpenoids are indeed amongst the most important aroma compounds of grape berries and wine bouquet components (Lund and Bohlmann, 2006).

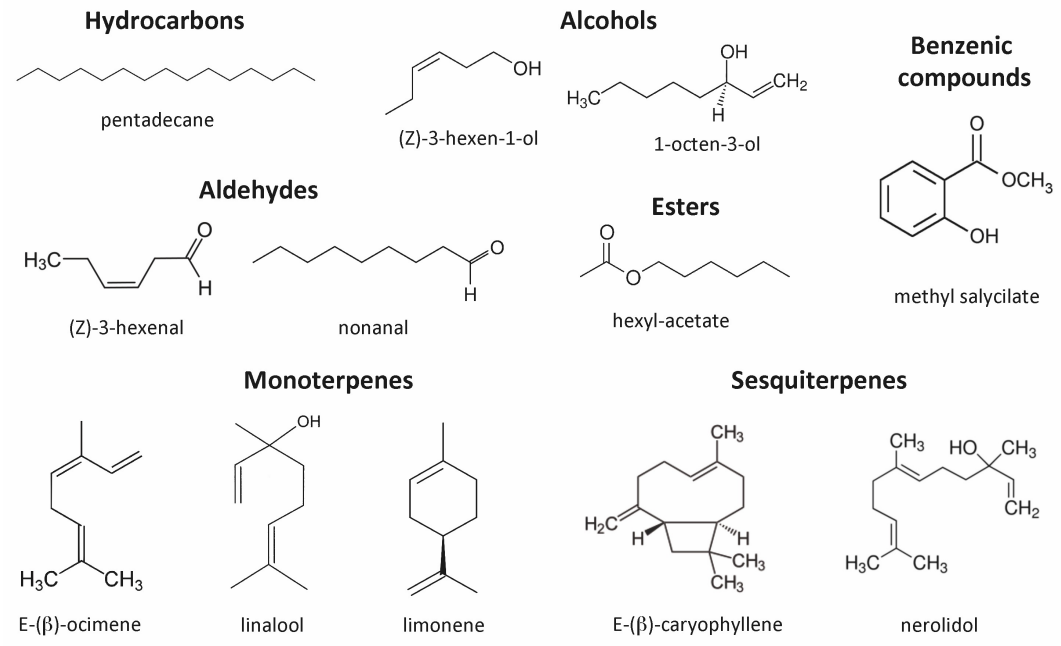

Figure 4. Examples of common VOCs identified in grapevine headspace collection (Tasin et al. 2005).

L. botrana females are attracted by grape odor for egg-laying on flower buds and berries at different phenological stages (Tasin et al. 2005, Masante-Roca et al., 2007). Volatiles from grape have been found to be behaviorally active

on *L. botrana* larvae (Becher and Guerin 2009) and also on adult males, probably because of the higher chance to meet females close to the host plants (Von Arx et al. 2011).

Studies on the perception and the processing of plant volatiles in *L. botrana* at the level of both the central and peripheral nervous system led to the identification of a number of physiologically active compounds (table I), and wind tunnel bioassays enabled the determination of the behavioral relevance of these compounds (Masante-Roca et al. 2002; Masante-Roca et al. 2005; Tasin et al. 2005, 2010, Anfora et al. 2009). In these experiments, it was reported also that *L. botrana* females discriminated among host plants, and attraction was greater for plants with a higher suitability for the development of offspring (*Vitis* versus *Daphne gnidium*, considered the first host). The behavioral plasticity of the moth, which oviposits on a large number of host plants with a different volatile profiles, is due to the redundancy of the attractive olfactory signals between different plants (Tasin et al. 2010).

A synthetic blend of three terpenoids, namely *E*-(β)-caryophyllene, *E*-(β)-farnesene and DMNT, was shown to be as attractive as headspace collections from grapes or volatiles emitted by green grapes under lab conditions (fig. 5). These findings further support that *L. botrana* uses a subset of volatile terpenes emitted by grapes for both host finding and oviposition site selection. In a field experiment, the same blend released from a septum attracted mated females and stimulated oviposition on vegetation near the lure (Anfora et al. 2009). In laboratory assays, blend proportion was also critical: female attraction was not different from control when the three compounds ratio was very different form 100 : 9 : 78.

Table I GC-EAD antennal response of *L. botrana* mated females to compounds of grapevine (cv. Chardonnay) headspace (adapted from Tasin et al. 2005)

Compound	Antennal Response ± SD ($mV \times 10^4$ ng)	Relative response % vs. *E,E*-(α)-farnesene
Hydrocarbons		
pentadecane	2.03 ± 0.91	12

1-pentadecene	0.39 ± 0.04	2
Heptadecane	5.48 ± 0.93	33
1-heptadecene	1.08 ± 0.12	6
octadecane	1.99 ± 0.61	12
nonadecane	6.51 ± 1.52	39
1-nonadecene	0.95 ± 0.28	6
heneicosane	7.16 ± 1.71	43
Alcohols		
(*Z*)-3-hexenol	2.22 ± 1.16	13
1-hexadecanol	2.23 ± 1.91	13
1-heptadecanol	4.76 ± 2.21	28
1-octadecanol	6.59 ± 2.14	39
Aldehydes		
(*Z*)-3-hexenal	4.76 ± 0.32	28
nonanal	4.98 ± 4.18	30
Ketones		
2-undecanone	1.86 ± 1.04	11
2-dodecanone	6.30 ± 5.03	38
tridecanone	1.03 ± 0.67	6
Esters		
(*Z*)-3-hexenyl acetate	2.50 ± 1,27	15
Aromatic compounds		
methyl salicylate	5.37 ± 3.12	32
Terpenoids		
(*E*)-4,8-dimethyl-1,3,7-nonatriene	66.02 ± 39.14	393
limonene	5.61 ± 4.55	33
(*E*)-β-ocimene	3.25 ± 2.55	19
linalool	3.33 ± 1.68	20
(*E*)-β-caryophyllene	7.03 ± 4.50	42
(*E*)-β -farnesene	2.95 ± 1.07	18
(*E*,*E*)-α-farnesene	16.79 ± 3.53	100

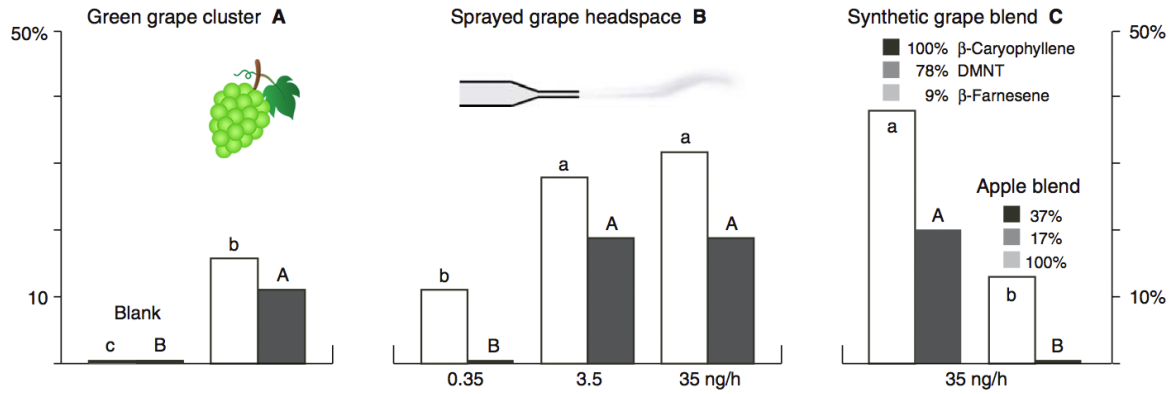

Figure 5. Upwind attraction of grapevine moth mated females. White and dark bars indicate the percentage of upwind flights and landing respectively. Results with (A) a cluster of green grapes (B) sprayed headspace collection from grapes cluster (C) blends of synthetic plant volatiles (adapted from Tasin et al. 2006)

Genetic engineering of terpenes biosynthesis in plants for biological control

The potential of plant Volatile Organic Compounds (VOCs) in pest control has been investigated for a long time, looking for new perspectives for managing insects pests (Degenhardt et al., 2003). In particular, terpenes biosynthetic pathways have been studied extensively, using biochemistry and genomic approaches, and these efforts led to the characterization of entire terpene synthase gene families (Aubourg at al. 2002, Martin et al. 2010, Chen et al. 2011, Nieuweinhuizen at al. 2012). With this knowledge it is now possible to attempt terpene metabolic engineering in order to approach new environmentally friendly crop protection strategies. Considering the costs of many VOCs due to difficulties in their synthesis or isolation from natural sources, and considering especially that they often have a short half-life in nature because of the tendency to form isomers and/or get oxidized (Blande et al. 2014), a genetic engineering approach seems the most suitable one to study the effect of kairomone manipulation on insects behavior (Birkett and Pickett 2014).

The feasibility of this approach has been shown the first time in *A. thaliana*, where overexpression of terpene synthases has been carried out. In one case, the overexpression of a strawberry single terpene synthase (FaNES1) in the plastids of *A. thaliana* made the leaves of this plant releasing the

monoterpene linalool, which acted as a repellent for the aphid *Myzus persicae* in lab assays (Aharoni et al., 2003). The same gene targeted to mitochondria gave plants with (*E*)-nerolidol and DMNT emission, not reported in the wild type (Kappers et al., 2005). When tested in dual-choice olfactometer, the transformed plants were preferred by the predatory mite *Phytoseiulus persimilis,* a natural enemy of the spider mite *T. urticae,* which is an extremely polyphagous pest. In another case the introduced gene was TPS10 from *Zea mais*, a multiproduct sesquiterpene synthase induced after herbivory (Schnee at al. 2006). The enzyme was targeted in the cytoplasm, and the transformed *A. thaliana* plants started to emit the sesquiterpene mixture emitted by maize plants when under attack of lepidopteran larvae. The plants were also able to attract the parasitic wasp *Cotesia marginiventris*, a natural enemy of the larvae. In *Nicotiana tabacum*, the expression of a sesquiTPS from *Pogostemon cabli* was targeted to the plastids along with an additional copy of farnesyl diphosphate synthase (FPS), leading to a 1000-fold increase of patchoulol: the authors also demonstrated that the volatiles emitted from these transgenic plants significantly deterred tobacco hornworms from feeding on leaves (Wu et al., 2006). Beale et al. (2006) expressed an E-(β)-farnesene synthase gene from peppermint ubiquitously in Arabidopsis and observed E-(β)-farnesene emission. Aphid colonization was significantly diminished in transgenic plants when compared to untransformed controls, while at the same time aphid predators were attracted by the volatile emissions. So far the examples of genetic engineering were in the direction of an increased activity of TPS, however it is interesting to report that the downregulation of endogenous TPS genes has also been used successfully to increase disease resistance in plants. An antisense strategy was indeed used to reduce the expression levels of the limonene synthase gene in orange (*Citrus sinensis*): the peel of fruits form transgenic orange trees had very reduced limonene concentrations, and when challenged with the fungus *Pennicillium digitatum* or the bacterium *Xanthomonas citri* sp. *citri*, transgenic fruits showed increased resistance compared to fruit peel of control plants. Moreover, males of the citrus pest *Ceratitis capitata* were less attracted to fruit accumulating less limonene (Rodríguez et al. 2011). As no other phenotypic differences

between transgenic plants and controls were observed, according to the authors the antisense approach could potentially be used to improve pest resistance in the field.

With the establishment of reliable protocols for plant transformation, the genetic engineering approach is now being extended also to non-model species of agricultural interest. In maize for example, a beta-caryophyllene synthase gene from *Origano vulgare* was recently overexpressed: the plants attracted nematodes that killed the western corn rootworm *Diabrotica virgifera virgifera* in field trials (Degenhardt et al., 2009). This is also an example of a below-ground interaction mediated by VOCs, a situation that is likely to occur frequently in nature but which is much less studied than the above-ground interactions. More recently, Bruce et al. (2015) reported the case of *E*-(β)-farnesene-emitting wheat plants, created with the goal to repel aphids for which *E*-(β)-farnesene is an alarm pheromone. In this case, plants were repellent under lab conditions for at least three species of cereal aphids (*Sitobion avenae*, *Ropalosiphum padi* and *Metopolophium dirhodum*), although insect parasitism was not reduced during field trials. The main reason for this failure, according to the authors themselves, was the difference between *E*-(β)-farnesene release from aphids (bursts of emission followed by non-emission periods) and from plants (slow constant emission). The main challenges for the future engineering of terpenoids in plants remain the transformation of non-model species for agricultural or industrial use, the compartimentalization of the products in order not to interfere with the plant metabolism, the use of tissue-specific or stage-specific promoters and the possibility of unpredictable byproducts due to the activity of enzymes such as cytochrome P450 and glycosyl transferase (Lange and Ahkami, 2013).

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Aim of the thesis

The aim of the thesis is to investigate the role of specific grapevine VOCs in *L. botrana* attraction, and to prove if their manipulation has the potential to disrupt it. To do this, I worked with the model plant species *A. thaliana* and with *V. vinifera*, creating stable transformed lines with modified headspace profile.

First I biochemically characterized a key grapevine enzyme for the production of the kairomone *E*-(β)-caryophyllene, checking also the differences in VOCs production when expressed in heterologous and homologous systems. Then I explored the possibility of using Arabidopsis as a fast and economic *in vivo* model to test if *E*-(β)-caryophyllene and *E*-(β)-farnesene could have a role in the oviposition preference or stimulation. I created plant lines overexpressing the two TPS enzymes responsible for the synthesis of the two kairomones both singularly and in combination.

Using the same approach in the same species then I tried to create a model for the in vivo emission of DMNT, a key kairomone for *L. botrana*. However, the gene chosen for the transformation turned out to have a different in role from what its *in vitro* characterization had suggested.

Finally, in grapevine I modified with several degree of severity the ratio between *E*-(β)-caryophyllene and *E*-(β)-farnesene, and I performed wind tunnel behavioral assays to check if this ratio modification led to a decrease in attraction.

Chapter 2

Heterologous and homologous expression of grapevine *E*-(β)-caryophyllene synthase (VvGwECar2)

Running title: VvGWECAR2 characterization

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Background to chapter 2

E-(β)-caryophyllene is the most abundant sesquiterpene emitted by grapevine and it is part of a kairomonal blend that attracts females of the grapevine moth to the plants. In grapevine there are five TPS coding for β-caryophyllene synthase but only one (VvGwECar2) accounts for most of the production of VOCs in green tissues and berries, playing thus the biggest role in *L. botrana* attraction. We decided to biochemically characterize the enzyme *in vitro* and *in vivo* in homologous and heterologous systems. In all the systems the enzyme catalyzed the formation of three products, of which *E*-(β)-caryophyllene was the dominant one. We observed anyway differences in the secondary products when the gene was expressed in the heterologous plant system. The plants overexpressing this gene were created also with the intention to use them in insect behavioral assays (treated in the following chapters of the thesis).

Abstract

E–(β)-caryophyllene is a sesquiterpene volatile emitted by plants and involved in many ecological interactions within and among trophic levels and it has a kairomonal activity for many insect species. In grapevine it is a key compound for host-plant recognition by the European grapevine moth, *Lobesia botrana*, together with other two sesquiterpenes. In grapevine *E*–(β)-caryophyllene synthase is coded by the *VvGwECar2* gene, although complete characterization of the corresponding protein has not yet been achieved. Here we performed the characterization of the enzyme after heterologous expression in *E. coli*, which resulted to produce *in vitro* also minor amounts of the isomer α-humulene and of germacrene D. The pH optimum was estimated to be 7.8, and the K_m and k_{cat} values for farnesyl pyrophosphate were 31.4 μM and 0.19 s^{-1} respectively. Then, we overexpressed the gene in the cytoplasm of two plant species, *Arabidopsis thaliana* and the native host *Vitis vinifera*. In *Arabidopsis* the enzyme changed the plant head space release, showing a higher selectivity for *E*–(β)-caryophyllene, but also the production of thujopsene instead of germacrene D. Overall plants increased the *E*–(β)-caryophyllene emission in the headspace collection by 8-fold compared to *Col-0* control plants. In grapevine *VvGwECar2* overexpression resulted in higher *E*–(β)-caryophyllene emissions, although there was no clear correlation between gene activity and sesquiterpene quantity, suggesting a key role by the plant regulation machinery.

Keywords: *Vitis vinifera* Vitaceae, grapevine, sesquiterpene(s), TPS, terpene synthase, *E*-(β)-caryophyllene, *Arabidopsis thaliana* Brassicaceae, VOC(s), genetic transformation

Introduction

Terpenoids build up the biggest class of metabolites in plants, being involved both in primary (Croteau *et al.*, 2000) and in secondary metabolism (Zwenger and Basu, 2008). Humans have traditionally used them as fragrances and flavors, since many of them are present in plant essential oils. More recently, they were investigated for their role as high-value chemicals for obtaining materials (e.g. rubbers), pharmaceuticals and even biofuels (Bohlmann and Keeling, 2008). In nature however, terpenoids are best-known for their multiple ecological roles: from direct defense against pathogens and insects (Hasegawa *et al.*, 2010; Heiling *et al.* 2010) to indirect defence attracting enemies of herbivores (Turlings and Ton, 2006; Unsicker *et al*. 2009), but also attraction of pollinators (Dudareva and Pichersky, 2000), mutualistic fungi (Ditengou *et al.*, 2015) and as signals for plant-plant communication (Arimura *et al.*, 2000). Their role as semiochemicals is also well exploited by phytophagous insects, which often use them as kairomones to recognize and locate their host plants (Bruce at al., 2005). All the classes of terpenes formally derive from the C_5 isomers isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), whose biosynthesis can take place in plants via two distinct pathways: the mevalonate pathway in the cytoplasm, and the MEP/DOXP pathway in the chloroplast (Rohmer, 1999). The condensation of C_5 isomers by the prenyl-transferase enzymes leads to the C_{10}, C_{15}, C_{20}, C_{30} and C_{40} precursors of mono-, sesqui-, di-, tri- and tetra-terpenes respectively.

In the last step, the precursors are converted to terpenes by the terpene synthase (TPS) enzymes, which in each species are usually coded by gene families of 20-150 members, as predicted in the genomes sequenced so far (Chen at al., 2011). *E-(β)*-caryophyllene is an almost ubiquitous semi-volatile sesquiterpene: in particular in grapevine (*Vitis vinifera* L., Vitaceae) it is emitted by leaves, tendrils and flowers (Matarese *et al.*, 2014) and it was shown to be biologically active to the European Grapevine Moth, *Lobesia botrana* (Den. & Schiff.) *(Lepidoptera, Tortricidae)*, eliciting electrophysiological responses on their antennae (Tasin *et al.*, 2005), as well

as being part of an attractive lure both under laboratory and field conditions (Tasin *et al.*, 2006; Anfora *et al.*, 2011). The lure included also the sesquiterpene *E*-(β)-farnesene and the homoterpene *E*-(4,8)-dimethyl-(1,3,7)-nonatriene (DMNT), which is derived from the sesquiterpene alcohol *E*-nerolidol.

L. botrana is a polyphagous insect, with a wide range of host plants, but it is considered the major pest of vineyards in Europe. It recently spread also in the Neotropical Region, raising alarm over American viticulture areas (Ioriatti *et al.*, 2011). Larvae feed on grapes, allowing various fungi, as *Botrytis cinerea* to lead the whole grape cluster to rot (Fermaud & Le Menn, 1989).

In this work, we biochemically characterized the TPS enzyme responsible for most of the production of *E*-(β)-caryophyllene in grapevine (VvGwECar2). Then we created stable transgenic lines of *Arabidopsis thaliana* (L., Brassicaceae) and *V. vinifera*, and quantified the headspace emission of the volatile produced under heterologous and homologous overexpression *in planta*.

The obtained lines could be used in the future to better investigate the mechanisms of the host-finding behavior of *L. botrana* and other model systems sharing the same plant volatile signals. The possible implications for the management of *L. botrana* disrupting its long-range host recognition and the oviposition site selection by the use of transgenic/cisgenic plants with unbalanced production of kairomones are also discussed.

Results and Discussion

Bacterial *VvGwECar2* expression and characterization

In grapevine five genes are known to code for β-caryophyllene synthases (Martin *et al.*, 2011) but only one (*VvGwECar2*) is actually expressed in all plant tissues and therefore accounts for most of the volatile production in vegetative parts and berries (Matarese et a., 2014), although in flowers other two genes (*VvGwECar1* and *VvPNECar2*) seem to play a big role. Considering the importance of terpenes production in grapevine, we decided to biochemically characterize VvGwECar2. We expressed the recombinant

enzyme fused with C-terminal His-tag into *E. coli* BL21, Rosetta and RIPL strains. RIPL gave the highest amount of protein visible on SDS-PAGE both in the soluble and in the insoluble fraction, so it was used for all the subsequent expressions. After purification with IMAC resin, each batch of the fusion protein was quantified with Bradford method (Bradford, 1976) and an aliquot was loaded on SDS-PAGE where it appeared with a band with a predicted molecular weight of about 67 kDa (fig. 1).

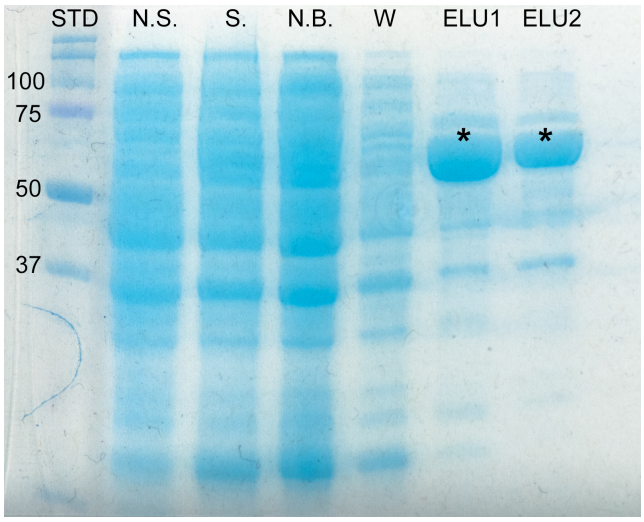

Figure 1. SDS-PAGE from *E. coli* lysis to protein purification. STD = protein standard, N.S. = non-soluble total proteins, S. = soluble total proteins, N.B. = proteins not bound to IMAC resin, W. = protein washed out from resin, ELU1 and 2 = protein elution fractions. Asterisks indicate the band corresponding to *E*-(β)-caryophyllene synthase.

To the purified recombinant VvGwECar2 protein we added farnesyl pyrophosphate (FPP) as the only substrate, since in functional assays other authors (Martin et al., 2010) reported no activity of this enzyme with geranyl pyrophosphate (GPP) or geranylgeranyl pyrophosphate (GGPP) that sometimes are accepted as alternative substrate although with a low efficiency (Cai et al., 2002). As frequently observed with other TPS enzymes, we found that more than one sesquiterpene product was formed (fig. 2).

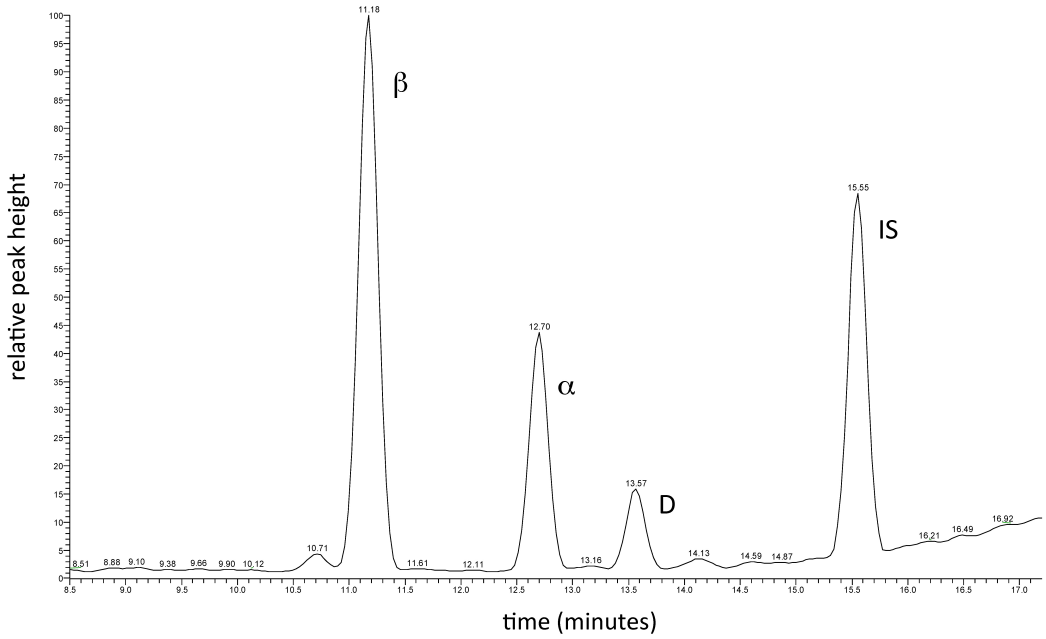

Figure 2. Chromatogram of an enzyme assay. The three products and the internal standard are visible. β = *E*-(*β*)-caryophyllene, α = humulene, D = germacrene D, IS = internal standard.

Our enzyme catalyzed the cyclization of FPP to both *b*-caryophyllene and a-humulene, plus a small amount of germacrene D. Our GC-MS analyses on the pentane layer above the enzyme buffer provided an evidence of a 70 : 23,5 : 6,5 ratio (mass of component), for E-(β)-caryophyllene, α-humulene, and germacrene D, respectively. This is in agreement with what reported in Martin *et al.* 2010. A substrate saturation and a time course were initially done to understand which concentration of the recombinant FPP was saturating under our conditions, and to estimate the length of the subsequent assays. The protein proved to be linearly stable even after overnight (15 hours) reaction, and 100mM FPP was chosen as a condition which would be saturating under any possible variation of enzyme quantity, due to variations in purification yields among the different batches (fig. 3A, B).

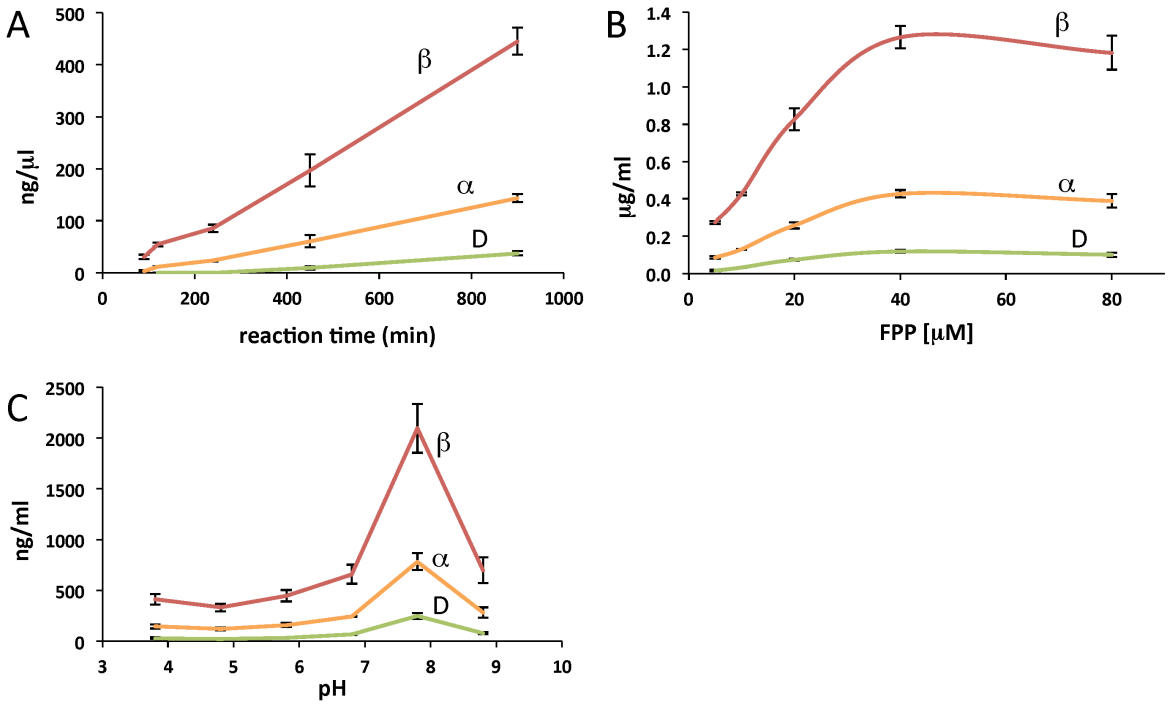

Figure 3. Products concentration in the organic phase after enzyme assays, measured after a calibration line prepared with the pure standards (α, β) or relative to internal standard (D). (A) Linearity of the VOCs production after overninght reaction. (B) Substrate (FPP) saturation. (C) pH optimum. β = *E*-(*β*)-caryophyllene, α = humulene, D = germacrene D.

The pH optimum was determined with assays from pH 4 to pH 9, with a peak of activity at pH 7.8, out of which the protein looses most of the catalytic activity (fig. 3C). This result is in accordance with the values found for other TPS enzymes and suggests that the enzyme is cytosolic, which is not surprising considering that this sesquiterpene pathway derives from the mevalonate pathway, which typically occurs in the cytoplasm. The values for K_m (31,4 mM), k_{cat} (0,19 s^{-1}) and efficiency (k_{cat} / K_m 6,1 mM^{-1} s^{-1}) differ significantly from the ones reported for sesquiterpene synthases characterized so far. Further experiments are needed to clarify if this is a general property of grapevine TPS.

Plant *VvGwECar2* overexpression

It is generally known that *in vitro* conditions can be very different from *in vivo* conditions, in terms of cofactors presence/absence, substrate concentration,

pH and post-translational modifications. For this reason, we decided to carry out *VvGwECar2* over-expressions in plants, and check for headspace sesquiterpene production *in vivo*. *A. thaliana* has been proven to emit sesquiterpenes under genetic transformation when enzymes were targeted in the plastids (Aharoni *et al.*, 2003) and mitochondria (Kappers et a., 2005), demonstrating that a pool of FPP exists in both cell compartments. However, in grapevine the main localization of FPP biosynthesis is the cytoplasm, with C_5 monomers isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) being supplied both from the cytosolic mevalonic acid (MVA) pathway and the plastidial methylerythritol phosphte (MEP) pathway (Schwab and Wüst, 2015). For this reason, sesquiterpene synthases are believed to be cytosolic enzymes, although recently in grape berries a bifunctional nerolidol/linalool synthase was found to be located only in the plastid and acting as a linalool synthase *in vivo* (Zhu et al., 2014). Given that *VvGwECar2* protein sequence apparently lacks any import signal, and that its subcellular localization prediction is cytoplasmic (SherLoc, Shatkay et al., 2007), we considered our protein expression very likely to be cytosolic.

The coding sequence of the gene was moved without his-tag into pB2WG7 plant overexpression vector (Karimi *et al.* 2007) under the control of cauliflower mosaic virus (CaMV) 35S promoter, and used to transform *Col-0* wild type plants. Screening of transformed lines were done with glyphosate selection on seeds, followed by PCR on genomic DNA to check for the presence of the insertion and also RT-PCR using total RNA from 7-day-old seedlings, to confirm the presence of the gene transcript (data not shown).

Several independent transgenic lines were obtained and grown in the greenhouse for four generations, until all the T-DNA insertions were at homozygosity. The plants were then analyzed for volatile organic compounds (VOCs) production with the closed loop stripping analysis (CLSA, Boland *et al.*, 1984) and their emission was compared with that of control plants of the same age. In contrast with the behaviour of the enzyme *in vitro*, we did not notice any trace of germacrene D production. On the other hand, a minor peak has been identified as thujopsene, which was never observed in the in vitro assays under all the conditions tested. We also observed a higher

selectivity for *E*-(β)-caryophyllene production: the headspace of the modified plants was indeed dominated by α-humulene, thujopsene and *E*-(β)-caryophyllene, in a 1 : 0.75 : 5 mass ratio on average. However, we observed a great variation in sesquiterpenes quantity among the different lines, probably because of the difference in T-DNA copy number and expression. This variation seemed to affect mainly *E*-(β)-caryophyllene emission, while a-humulene and thujopsene were much less influenced (fig. 4).

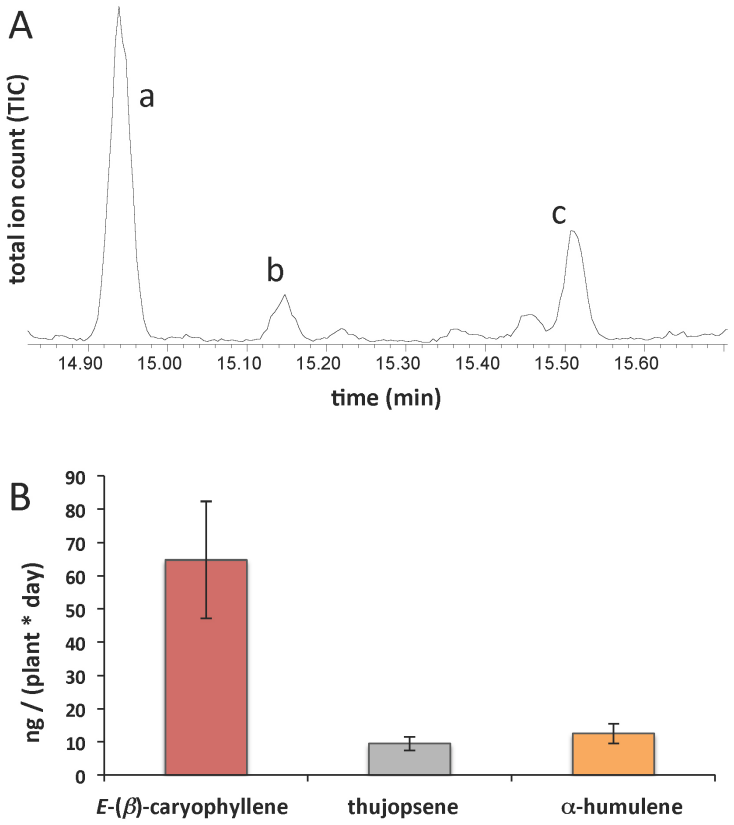

Figure 4. GC-MS analysis of the headspace of transgenic Arabidopsis plants. (A) Chromatogram (m/z =93) of the VOCs produced by *VvGwECar2* overexpression. The main peaks are *E*-(β)-caryophyllene (a), thujopsene (b) and α-humulene (c). The minor peaks are compounds released in low amounts from the plastic bag used to enclose the plants. (B) Differences of VOCs production among independent lines.

In particular *E*-(β)-caryophyllene emission was estimated to range from 39 ng plant^{-1} day^{-1} to almost 100 ng plant^{-1} day^{-1}, determining a mass ratio variation from 1 : 0.75 : 3 to 1 : 0.75 : 8. Compared to *Col-0* plants, whose emission

range was much narrower, we observed an average of 8-fold increase in VOCs.

Finally, we inserted *VvGwECar2* into grapevine to obtain homologous expression, using the transformation protocol as described in Dalla Costa *et al.* (2014). Starting from 12 Petri dishes of embryogenic *calli* (var. "Brachetto Grappolo Lungo") we obtained after one year 20 independent transgenic lines growing on the selection marker, 10 of which were acclimatized in the greenhouse and analyzed for gene expression and sesquiterpene production. As controls, to exclude effects caused by the regeneration protocol, two independent lines obtained after transformation with just *nptII* gene were also tested. Overall, no differences in plant phenotypes or growth speed were observed. At the molecular level, expression level of the *VvGwECar2,* in both controls was very similar (fig. 5), and kept as a reference for the overexpressing lines. In the over-expressing lines the expression variability was much higher than expected; indeed increase in expression levels ranged from 15-fold to 370-fold (fig. 5). Such remarkable variability is unlikely caused by position effect of T-DNA on genome, while it is probably due to very different levels of chimerism in the obtained plants, a phenomenon that has been already observed and quantified in grapevine transformations previously (Dalla Costa *et al.*, 2014).

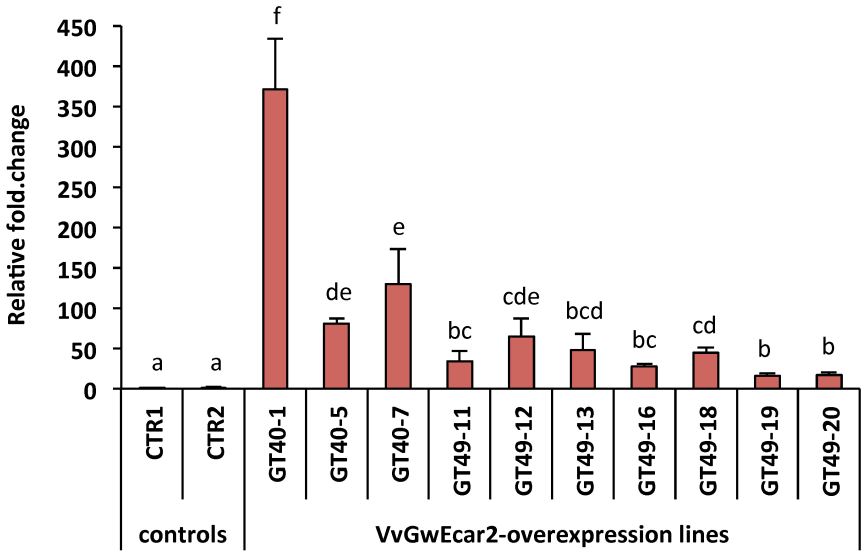

Figure 5. *VvGwEcar2* relative expression obtained by RT-qPCR. Means of controls were set to 1. At least three biological replicates were used for each line. Letters on

bars indicate different groups according to one-way ANOVA followed by Fisher LSD post hoc test ($P < 0.05$).

Since a higher level of transcript does not necessarily lead to a higher level of active protein (Keurentjes et al., 2008), we decided to screen the plants with solid-phase microextraction coupled with gas-chromatography/mass spectrometry (SPME-GC-MS) to check for changes in sesquiterpenes production compared to control plants (fig. 6).

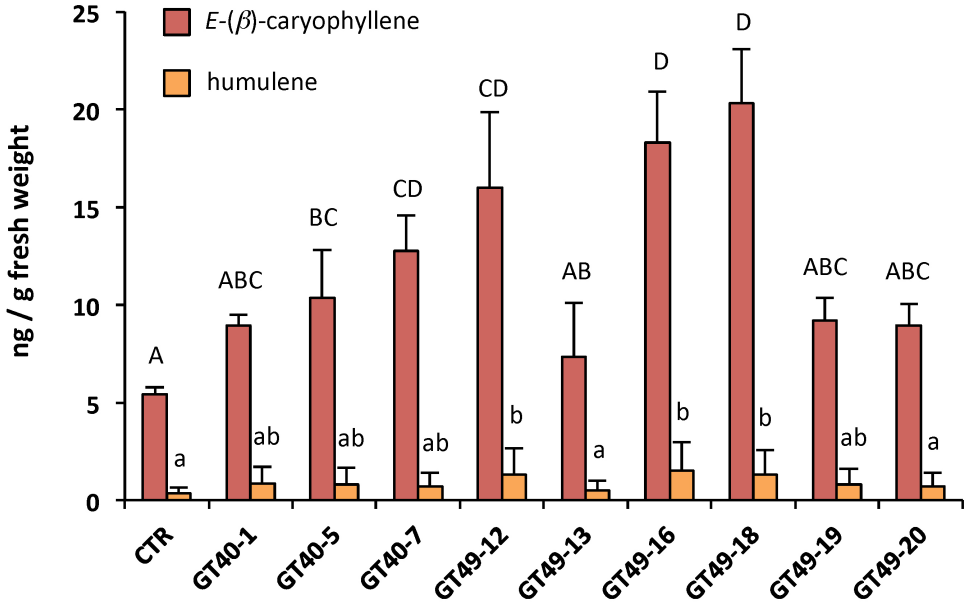

Figure 6. Volatile collection and analysis by SPME-GC-MS from young grapevine leaves taken from acclimatized plants in the greenhouse. Leaves were grounded in liquid nitrogen and the obtained powder (at least 0.5g) was weighted. Letters on bars indicate different groups according to one-way ANOVA followed by Fisher LSD post hoc test ($P < 0.05$).

SPME was chosen because it allows a very low detection threshold, considering that sesquiterpenes are usually not covering more than 1% of total amount of VOCs emitted by grapevine (Matarese *et al.*, 2014). As expected, young leaves of control plants emitted *E*-(β)-caryophyllene in the range of 5 ng/g of fresh weight, while the transgenic lines showed a strong increase, ranging from an average of 7 up to 20 ng/g, 4-fold more. Also in this

case we observed both a higher selectivity for *E*-(β)-caryophyllene production rather than a-humulene, and a high variability between lines. A similar variability was observed on the very small a-humulene content, which ranged from 0.5 ng/g to 1.5 ng/g (fresh weight) among the different lines, at most 3-fold more the wild-type plants. In contrast with Arabidopsis, we could not detect any trace of thujopsene: this sesquiterpene seems to be a byproduct of the enzyme outside the homologous expression system, and it was indeed never found in earlier grapevine headspace collections that we performed. Linearity of SPME fiber's response within this range of concentrations was tested with a calibration line from pure standards, which was also used for the quantifications. Surprisingly, there is almost no correlation between gene expression data and volatiles quantification in the transgenic lines, suggesting a deeper regulation of either the enzyme activity or the substrate availability in the cytoplasm. For future works a co-expression of a FPP-synthase may lead to a more efficient flux of substrate in the pathway and thus a higher production of sesquiterpene, as suggested recently by the work of Bruce *et al*. (2015).

Conclusions

Despite being the plant with the largest TPS gene family known so far, very few TPS from *Vitis vinifera* have been extensively characterized so far (Lücker et al., 2004; Martin et al., 2010; Zhu et al., 2014; Drew at al., 2016). K_m and k_{cat} values for grapevine *E*-(β)-caryophyllene synthase appear indeed to be quite different from the ones reported for other sesquiTPS already characterized: further studies will clarify if this is a general propriety of grapevine TPSs or not. We noticed also differences in the enzyme behaviour between three different conditions: *in vitro*, in plant in a heterologous system and in plant in the homologous system; this differences were both in different secondary products formation and in their relative abundance. Furthermore, we confirmed that a crucial role in defining the quantity of volatiles is played by the regulation of the flux in the pathway, since a great increase in one gene activity is not leading to an equivalent increase of the final product.

The availability of the genetic and metabolic tools presented in this work would accelerate also the creation of basic knowledge on the mechanisms underlying either production of volatiles in grapevine or host plant selection by phytophagous insects. This knowledge is the prerequisite to interfere with chemical, physiological and behavioural mechanisms involved in the *Vitis*-phytophagous insects, such as *L. botrana*, interactions and to develop novel sustainable control techniques (Ioriatti *et al.*, 2011; Cattaneo *et al.*, 2014). As a consequence, our study will pave the way to new possibilities for the control of the grapevine insect pests based on the manipulation of the semiochemicals scenario used by the insects during grapevine location.

Experimental

Cloning of the *E*-(β)-caryophyllene synthase into plant over-expression vectors

The coding sequence of the isoform VvGwECar2 was kindly provided by Prof. Bohlmann's laboratory into the bacterial expression vector pET28b+ with C-terminal His-tag. The vector was used as a template to amplify the coding sequence of the enzyme with Phusion High-Fidelity DNA-Polymerase (New England Biolabs) using the forward primer "attB-bCar for" (5' – GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACAATGTCTGTTCAGTCTT CAGTGG – 3') and the reverse primer "attB-bCar rev" (5' – GGGGACCAC TTTGTACAAGAAAGCTGGGTTCATATTGGCACAGAATCTATAAGC – 3'). PCR product was cloned directly into pDONR221 via BP-clonase reaction (Invitrogen), and transformed into E. Coli DH5a chemically competent cells. Plasmid minipreps from single colonies grown in 5ml cultures were sequenced to check for their quality using M13(-20) for (5' – GTAAAACGACGGCCAG – 3') and M13 rev (5' – CAGGAAACAGCTATGAC – 3') primer pair. The coding sequenced was then moved into the gateway destination vectors pK7WG2D and pB2WG2 (Karimi *et al.* 2007) via LR reaction (Invitrogen). 1ml of the reaction was used to transform chemically competent *E. Coli* TOP10 cells (Invitrogen), then plasmid minipreps from

single colonies grown in 5ml cultures were sequenced to check for their quality using my35Sprom for (5' – CCACTA TCCTTCGCAAGACCC – 3') and my35Sterm rev (5' – GAAGTATTTTACAAATACAAA TACATACTAAGG – 3') primer pair.

Heterologous expression and purification of *E*-(β)-caryophyllene synthase in *E. coli*

The plasmid pET28b+ containing the coding sequence of VvGwECar2 was transformed into chemically competent BL21-CodonPlus(DE3)-RIPL *E. coli* cells (Agilent Technologies) with heat shock method. A single colony was used to inoculate 5 ml of LB medium containing kanamycin (50 mg/L) and chloramphenicol (25 mg/L) at 37 °C overnight. 200 ml of this culture was added to 100 ml of fresh LB containing kanamycin and chloramphenicol at proper concentrations. Bacteria were grown at 28 °C until OD_{600} = 0.4, then they were grown at 16°C until OD_{600} reached 0.7. Induction was achieved by adding isopropyl-b-D-1-thiogalactopyranoside (IPTG) 0.5mM to the ice-cooled culture. Cells were maintained for 20 h at 16 °C and then harvested by centrifugation (2000g at 4°C, 15 min).

Cell pellet was suspended in 2ml in lysis buffer (50mM Tris-HCl, 200mM NaCl, 1mM EDTA, 1mM dithiothreitol (DTT), 10% v/v glycerol, pH 7.75), then 3ml of a 0.1M phenylmethanesulfonyl fluoride (PMSF (Sigma) solution were added to prevent proteolysis during the next steps. Bacteria cells were treated with three freeze-thaw cycles of 5 min in liquid nitrogen and 5 min at 42°C, 1h of incubation at 4°C with lysozyme (Sigma) 1mg/ml, and shacked with TissueLyserII (Qiagen) using 0.5g of glass beads (Sigma) and four cycles of 30sec at 30Hz.

The cell lysate was treated with DNaseI (1mg/ml) for 30 min at 37°C to reduce its viscosity and centrifuged at 4°C at 13000g for 5 min. The pellet was trashed while the supernatant, containing the soluble proteins, was used for purification, SDS-PAGE and protein quantification.

The soluble fraction of cell-lysate was combined with 80ml of Profinity IMAC resin (Bio-Rad) and tilt-shaked at 4°C for 1h to bind the his-tagged protein.

This was followed by two washing steps with 500ml of washing solution (5mM imidazole, pH 6.3) and three elution steps with 50 ml elution solution (0.3M NaCl, 50mM NaH_2PO_4, 0.4M imidazole, pH 8.0). All the fractions were analyzed on glycine-SDS-PAGE and stained with Coomassie Brilliant-Blue (Bio-Rad). The three elution steps were mixed into one, and protein concentration was determined with the Bradford method: enzymes was used directly into assays, without being stored.

Enzyme assay

Each enzyme assay was done with the same batch of freshly-purified enzyme, 3μl (about 0,5-1mg, depending on the batch) per reaction in 2ml glass vials (Agilent) in a total volume of 200ml. Standard reaction buffer was 50mM HEPES, 10mM $MgCl_2$, 20mM $MnCl_2$, 2mM DTT, 10% v/v glycerol, pH 7.0, and reaction were carried out at 30°C. To trap the volatiles produced, 200ml of a pentane layer with 10% isooctane was put above the reaction. Cuparene was used as internal standard (IS), at a final concentration of 1mg/L. Reactions were stop removing the pentane layer with a glass Pasteur pipette and transferring it into a new 2ml glass vial. Time course was done at 0min, 90 min, 120 min, 240 min, 450min and 900min using 100mM farnesyl pyrophosphate (FPP, Sigma) as substrate. pH optimum was determined from 4.0 to 9.0 after overnight reaction. K_m and K_{cat} values were determined after 11h of reaction with FPP at 2.5mM, 5mM, 10mM, 15mM using Hanes plot. The values of enzyme concentration was derived from Bradford assay done on the elution batch before starting each enzyme assay, and the size of protein was estimated to be 67 kDa (including his-tag). Three technical replicates were conducted for each reaction.

Analysis of the products

A CTC CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) equipped with a 10 mL syringe was used to inject the volatiles from the vials. 1 mL of sample was injected in splitless mode into a Trace GC Ultra Gas

Ghromatograph coupled to a Quantum XLS Mass Spectrometer (Thermo Scientific, Electron Corporation, Waltham, MA). Compounds were separated using a VF-Wax ® column (100% polyethylene glycol; 30 m ×0.25 mm ×0.25 µm, Agilent J&W Scientific Inc., Folsom, CA). The GC oven parameters were as follows: initial temperature was 70°C, maintained for 1 min, followed by an increase to 130°C at a rate of 5°C min^{-1}, the oven was then maintained at 130°C for 1min, then a rate of 20°C min^{-1} until 230°C for 1 min; splitless time of 0.8 min and GC inlet temperature of 250 °C. Helium was used as carrier gas in constant flow mode at 1.2 mL min^{-1}. The total cycle time was 20 min. The MS detector was operated in SIM mode (m/z 45, 69, 93, 119, 132, 133, 145, 161) and in scan mode (mass range 40–450 m/z) with a 0.2 sec scan time and the transfer line to the MS system was maintained at 250°C. Products were identified by comparing the mass spectra with those found in the NIST database, and by injection of standards when available. Quantifications were performed after calibration lines were prepared from the same standards.

Grapevine Transformation

The vector pK7WG2D containing *VvGwECar2* coding was use to transform via *Agrobacterium tumefaciens* embryogenic *calli* of *V. Vinifera* (variety “Brachetto Grappolo Lungo”) as described in Dalla Costa *et al.*, (2014). Transgenic plants were propagated and maintained *in vitro* until acclimatization in the greenhouse.

***Arabidopsis thaliana* transformation**

The vector pB2WG7 with constitutive CaMV 35S promoter driving *VvGwECar2* expression was used to transform *A. thaliana* Columbia 0 plants via the floral dip method (Clough and Bent, 1998). Transformed plants were screened on solid MS media (Duchefa) with sucrose (15 g/L) and 2,2 g/L Basal Salts including vitamins and supplemented with glyphosate (Sigma) 25 mg/L. After one week the resistant lines, bigger than wild-type and with visible

first root, were transferred into soil and let grow in the greenhouse for four generations, until nearly 100% of the seeds were homozygous for T-DNA insertion.

RNA extraction and RT-qPCR

RNA was extracted with Spectrum^{TM} Plant Total RNA kit (Sigma) from 7-day-old Arabidopsis seedlings and from young grapevine leaves in the greenhouse (second and third internode below the apex) following the manufacturer's guidelines. RNA quality and quantity was checked on spectrophotometer and 1% agarose electrophoresis before cDNA was retro-transcribed using SuperScript® III Reverse Transcriptase (Invitrogen) with Random Primers. A specific primer pair able to discriminate *E*-(β)-caryophyllene synthase from other TPS genes in the grapevine family (bCarRT for 5'- GAAGTGAGGAAGATGCTAATGG – 3' and bCarRT rev 5' – AACGGAACCATCGAACATG – 3') was created using the online tool Primique (Freedslund and Lange, 2007; http://cgi-www.daimi.au.dk/cgi-chili/primique/front.py), the pair had an efficiency of 91%. The qPCR was performed on an CFX96 thermocycler (Bio-Rad) using GAPDH and Actin (Reid *et al.*, 2006) as reference genes. Real-time PCR was carried out with the following cycle: 95°C 10'; 40 x (95°C 30" , 60°C 30"); The manufacturer's software (CFX Manager, Bio-Rad) was used to calculate primer pair efficiency (E) and the threshold cycle (Ct) mean and standard deviation for each sample. The analysis of relative quantification was performed using the workflow reported in Hellemans et al, 2007. For each primer pair used, Ct from control lines were used as reference to calculate the DCt. The geometric mean between the value E^DCt from Actin and GAPDH were used to calculate a normalization factor (NF), which was finally used to calculate the fold difference according to the formula: Fold Difference = E^DCt / NF.

Headspace Collection and analysis from Arabidopsis plants

Headspace volatiles of *A. thaliana* plants were collected using the close-loop stripping analysis (CLSA) method (Boland *et al.*, 1984; Abraham et al, 2014). Eight plants in five weeks old were put into a closed plastic cooking bag (45 x 55 cm, Toppits, Melitta, Sweden) where an air pump created a flow of activated-charcoal-cleaned air at 0.4 L/min. The air flow hit an adsorbent filter (ORBO 1103, Porapak Q (50/80) 150/75 mg, Supelco) for 6 hours, before trapped compounds were eluted with 1 mL of dichloromethane, and concentrated under the hood 5 times. Three bags replicates were set up for each line tested. A Gerstel MPS autosampler was used to perform liquid injections of the extracts. A 7890A GC gas chromatograph (Agilent) coupled to a 5975D MSD mass spectrometer (Agilent) was used: compounds were separated using a HP-5MS column (5% phenyl methyl siloxane; 30 m ×0.25 mm ×0.25 µm, Agilent). The GC oven parameters were as follows: initial temperature was 50°C, maintained for 1.5 min, then a rate of 7.5°C min^{-1} until 250°C maintained for 10 min; injection volume was 2µl in splitless mode and with a GC inlet temperature of 280 °C. Helium was used as carrier gas in constant flow mode at 1.2 mL min^{-1}. The total cycle time was 38.17 min. The MS detector was operated in scan mode (mass range 20–400 m/z) and the transfer line to the MS system was maintained at 230°C. Identification of the volatile compounds was made by injecting pure reference standards (Figure 7) and comparing retention index and mass spectra with the use of NIST database.

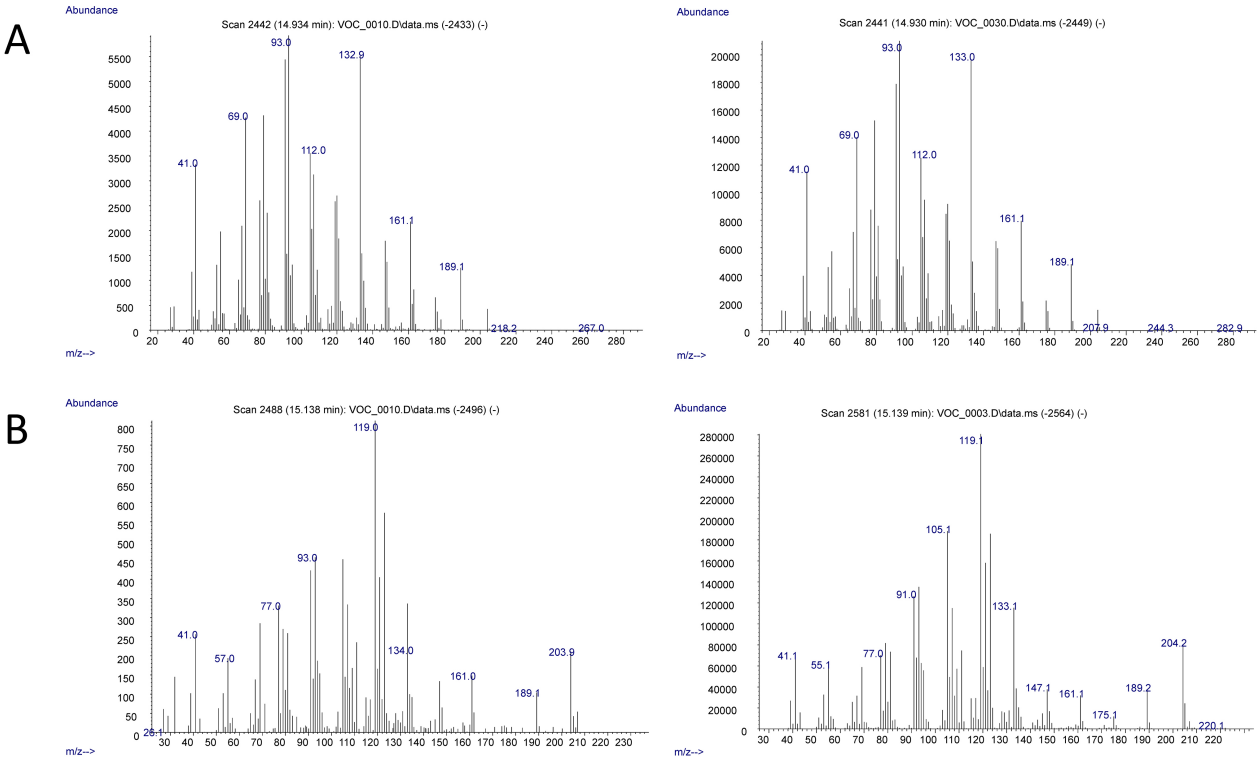

Figure 7. Mass spectra of sesquiterpenes found in Arabidopsis headspace (left) and of the injected standard (right). (A) *E*-(β)-caryophyllene (B) thujopsene (C) α-humulene.

Volatiles collection and analysis from grapevine plants

Grapevine sesquiterpene collection was performed with a method adapted from Matarese *et al.* 2014. A CTC CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) with a single magnetic mixer (SMM Chromtech) and SPME fibre conditioning station was used to extract the volatiles from the sample vial headspace. A Trace GC Ultra gas chromatograph coupled to a Quantum XLS mass spectrometer (Thermo Scientific, Electron Corporation, Waltham, MA) was used: compounds were separated using a VF-Wax ® (100% polyethylene glycol; 30 m ×0.25 mm ×0.25 µm, Agilent J&W Scientific Inc., Folsom, CA). The GC oven parameters were as follows: initial temperature was 40°C, maintained for 4 min, followed by an increase to 60°C at a rate of 2°C min^{-1}, the oven was then maintained at 60°C for 1min, then a rate of 5°C min^{-1} until 190°C for 1 min and a rate of 10°C min^{-1} until 230°C maintained for 4 min; splitless time of 5 min and a GC inlet temperature of 250

°C. Helium was used as carrier gas in constant flow mode at 1.2 mL min^{-1}. The total cycle time was 50 min. The MS detector was operated in scan mode (mass range 40–450 m/z) with a 0.2 sec scan time and the transfer line to the MS system was maintained at 250°C. SPME extraction was carried out with slight modification from the one described in Matarese et al, 2014. Half gram of leaf powder was added with 0.3 g of NaCl and 3 mL of freshly prepared citrate-phosphate buffer (0.1 M Na_2HPO_4, 50mM citric acid, pH 5.0) and put into 20 mL glass headspace vials. Each sample was spiked with 50 µL of 2-octanol at 2.13 mg L^{-1} alcoholic solution as internal standard. Samples were kept at 60°C for 20 min and then extracted for 35 min at 60°C. The headspace was sampled using 2-cm DVB/CAR/PDMS 50/30 µm fiber from Supelco (Bellefonte, PA). The volatile and semi-volatile compounds were desorbed in the GC inlet at 250°C for 4 min in splitless mode and the fibre was reconditioned for 4 min at 270°C.

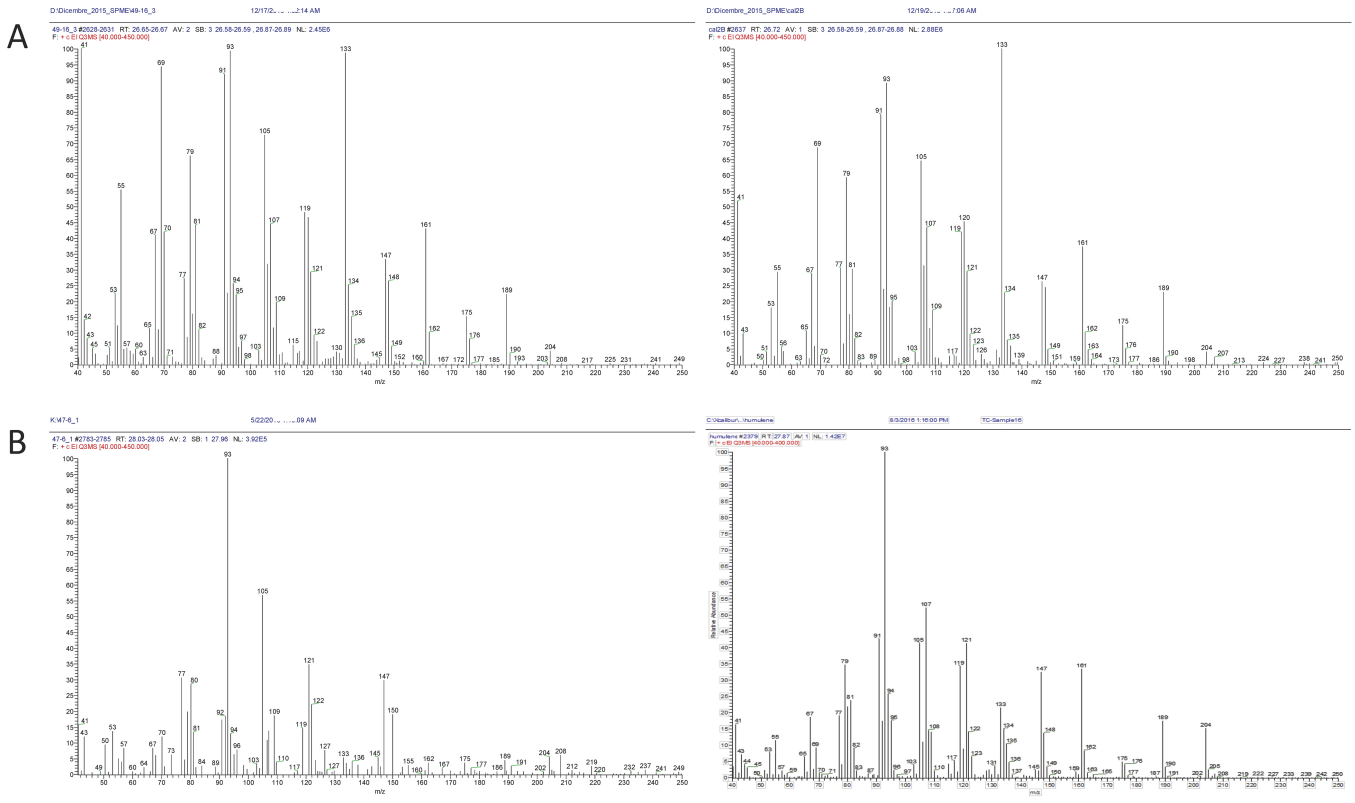

Figure 8. Mass spectra of sesquiterpenes quantified in grapevine (left) and of the injected standard (right). (A) *E*-(β)-caryophyllene (B) α-humulene.

Data processing

For GC-MS data form the enzyme assay and SPME extractions, processing was carried out with XCALIBUR™ 2.2 software provided by the vendor. Identification of the volatile compounds was made by injecting pure reference standards (Figure 8) and comparing retention index and mass spectra with the use of databases NIST MS Search 2.0.

Acknowledgments

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Chapter 3

Use of the model plant *Arabidopsis thaliana* to study the role of specific terpenoids in the oviposition preference of *L. botrana*

Running title: Use of Arabidopsis to study the role of VOCs in oviposition

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This manuscript needs the results of further experiments to be added before being submitted to a peer-reviewed journal yet to decide.

Background to chapter 3

In the last session I reported the creation of *A. thaliana* plants over-expressing a *E*-(β)-caryophyllene synthase. Here I report the creation of *A. thaliana* plants over-expressing a *E*-(β)-farnesene synthase and the cross between the two, creating lines which emit both volatile compounds in their headspace. These plants were used in dual-choice oviposition assays, to determine if the *in vivo* emission of these compounds, singularly or in couple, was enough to determine an oviposition preference or an oviposition stimulation in *Lobesia botrana* females. These VOCs indeed are part of a blend known to attract the moth from the distance, and one of them (*E*-(β)-caryophyllene) is also part of a lure that was shown to enhance oviposition, but their contribution to oviposition was never studied in detail with a model of *in vivo* emission like Arabidopsis.

Overall, it seems that these two compounds are not sufficient to stimulate oviposition or determine a substrate preference, at least in the quantity and ratio provided by Arabidopsis plants. It remains to be determined if a higher dose could reach the purpose, or if the insects need the full information provided by the presence of all the other VOCs at a close range to choose the substrate where to lay eggs.

The experiments of this session will therefore need further results to be published.

Keywords: sesquiterpene(s), TPS, terpene synthase, *E*-(β)-caryophyllene, *E*-(β)-farnesene, *Arabidopsis thaliana*, VOC(s), genetic transformation, *Lobesia botrana*, oviposition

Introduction

Adult mated females of phytophagous insects use many sensory cues to look for suitable host plants where to lay eggs. In moths, olfactory cues released from host plants attract the females from the distance (Bruce *et al.* 2005), but once the insect has landed on the plant, they might play a role also in the subsequent behavioral steps leading to oviposition (Renwick and Chew 1994).

Female of the grapevine moth, *Lobesia botrana*, are attracted by grapevine odors to lay eggs on flower buds and grapes (Masante-Roca *et al.* 2007), and a big role in this process has been attributed to contact chemosensory stimuli. Indeed contact chemoreceptors from the tarsae and ovipositor were proved to respond to different oviposition stimulants: non-volatile polar compounds extracted from grape berries stimulated the oviposition in females in choice assays (Maher & Thiéry 2004a; Maher & Thiéry 2004b), while fatty acids and their derivatives acted as deterrents (Gabel & Thiéry 1996). However, in the same species, the release of long-distance attracting VOCs has also been shown to enhance oviposition when presented combined in a six-component lure (Anfora *et al*. 2009). In this work we tested if two sesquiterpenes, namely *E*-(β)-caryophyllene and *E*-(β)-farnesene, which were shown to be the most crucial in long-distance attraction (Tasin *et al*. 2006) have also a close-range effect as oviposition stimulants when presented alone or in combination. Considering the compact size and the fast life cycle of *A. thaliana*, we reasoned that it would be a good model for an *in vivo* emission of volatiles to be used in oviposition assays. Arabidopsis indeed has a gene family of TPS (Aubourg *et al*. 2002), and it has already been proven to be a good model to study plant-insect interaction after genetic transformation with TPS gene (Aharoni et al. 2003; Kappers *et al*. 2005). Thus we produced *A thaliana* plants that emit only *E*-(β)-caryophyllene, only *E*-(β)-farnesene and both compounds contemporary, and we used them as a biological material in dual-choice oviposition assays with *L. botrana* females.

Results and Discussion

Plant VvGwECar2 and AaβFS overexpression

The coding sequences of AaβFS was moved into the plant overexpression vectors pH2WG7 (Karimi *et al.* 2007) under the control of cauliflower mosaic virus (CaMV) 35S promoter. These vectors were used to transform *Col-0* wild type Arabidopsis plants. Screening of transformed lines were done with hygromicin B on seeds in agar plates, followed by PCR on genomic DNA to check for the presence of the insertion (data not shown). The creation of lines overexpressing VvGwECar2 was reported in Salvagnin *et al*. 2016. RT-PCR using total RNA from 7-day-old seedlings was used to confirm the presence of the gene transcript (fig. 1).

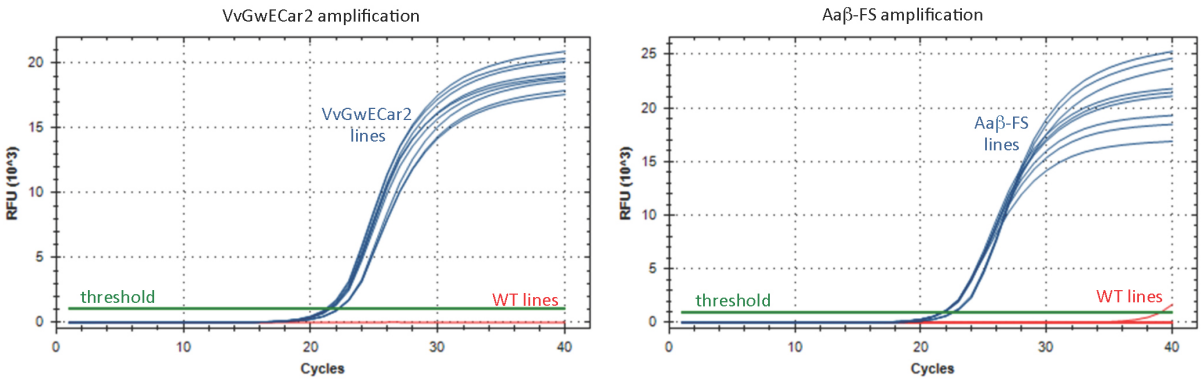

Figure 1. RT-PCR on cDNA from A. thaliana seedlings from VvGwECar2 (left) and Aaβ-FS (right) overexpressing lines. Blue lines represents amplification curves from transgenic plants, red lines from WT Col0 plants and green line is the fluorescence threshold.

The transgenic lines obtained were crossed to create a double overexpressing line, called “aaβ x β”, in which transgene expression was also confirmed (data not shown).

Plant headspace collection and analysis

All the plants were then analysed for (VOCs production with the closed loop stripping analysis (CLSA, Abraham *et al.*, 2014) and their emission was compared with that of control plants of the same age (fig. 2).

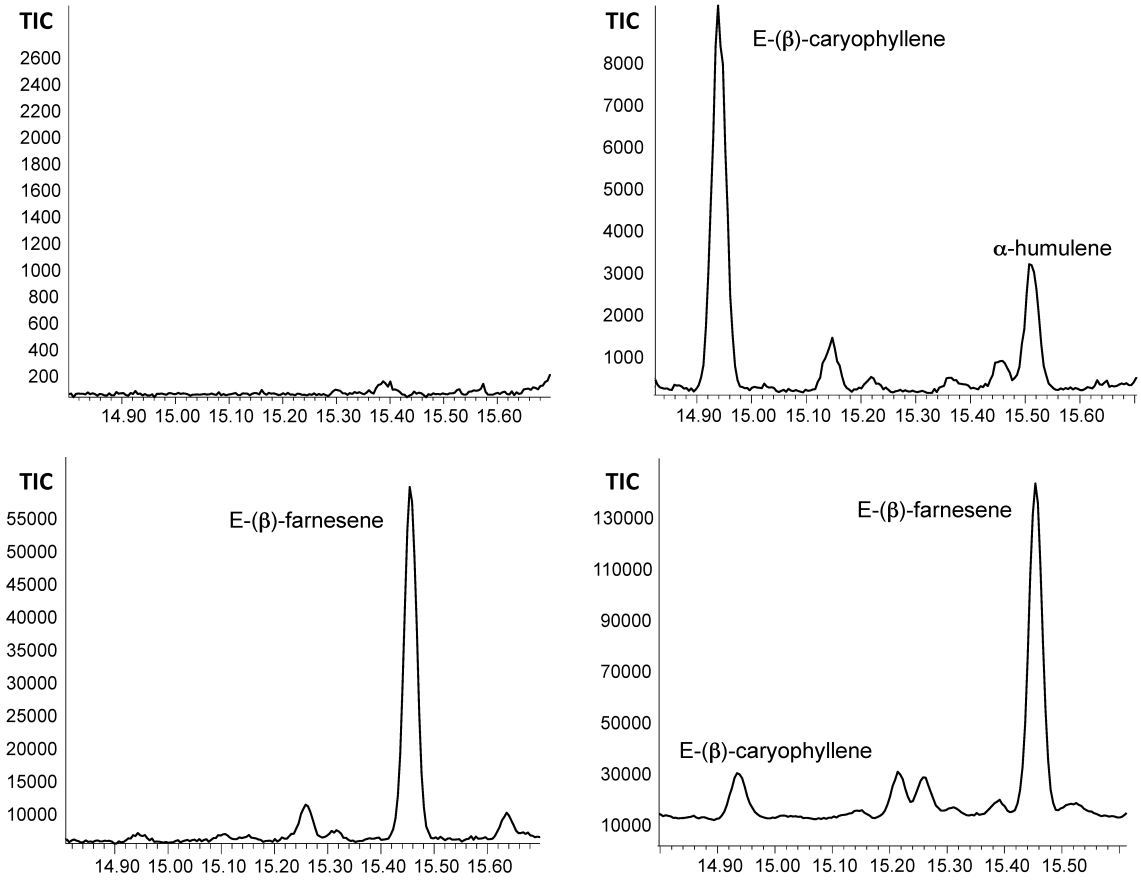

Figure 2. Chromatograms (TIC) of the VOCs produced by transgenic *Arabidopsis* plants. Top left: Wild type Col0. Top right: *VvGwECar2* overexpression. Bottom left: AaβFS overexpression. Bottom right: double aaβ x β overexpression.

Wild type Col0 plants were characterized by a very low emission, with no VOCs visible except traces of *E*-(β)-caryophyllene (8 ng plant^{-1} day^{-1}), while VvGwEcar2 overexpressing lines presented two evident peaks corresponding to *E*-(β)-caryophyllene (from 39 to 98 ng plant^{-1} day^{-1}) and its isomer α-humulene (from 7 to 17 ng plant^{-1} day^{-1}). The headspace of Aaβ-FS overexpressing lines was dominated only by *E*-(β)-farnesene (from 180 to 390 ng plant^{-1} day^{-1}) and the headspace of the double aaβ x β lines presented both *E*-(β)-caryophyllene and *E*-(β)-farnesene (19 ng plant^{-1} day^{-1} and 154 ng plant^{-1} day^{-1} respectively on average), although the latter remained the

prevalent one in quantity (figure 3). Quantifications were performed after calibration lines prepared from the standard compounds used also for VOCs identification.

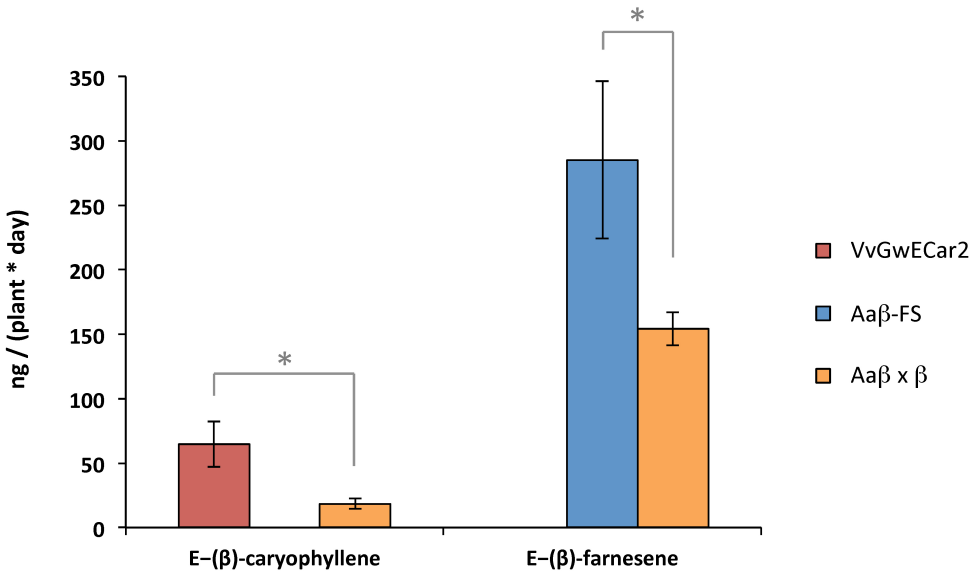

Figure 3. Quantifications of VOCs emitted by the different transgenic lines of *A. thaliana* plants. Standard error on mean (SEM) is visible for each sample. Asterisks indicate a statistical difference in the emission quantity (Student t-test, $p < 0.05$).

Aaβ x β plants emitted both volatiles, although each one was present in a smaller quantity compared to the parental lines: this is very likely to be due to a competition for the same substrate FPP, that can be limiting in the cytoplasm (Hohn and Ohlrogge, 1991; Wallaart *et al.*, 2001).

Oviposition assays

The preference for a plant genotype, and thus the VOCs emitted, was scored using an oviposition discrimination index (ODI) as described in Maher and Thiery 2006. In particular ODI is calculated with the following formula:

$$ODI = \frac{no.\ eggs_{plant\ A} - no.\ eggs_{plant\ B}}{no.\ eggs_{plant\ A} + no.\ eggs_{plant\ B}} \times 100$$

Extreme ODI values (plus and minus 100) mean that all the eggs are laid on one substrate, while a value of 0 means that there is no substrate preference, and the eggs are laid in the same quantity on the two substrates. In this case, whenever we gave a choice between a Col0 plant and one with transformed genotype, we did not observed any significant preference (paired 2-tailed t-test), regardless the VOCs emitted (fig. 4).

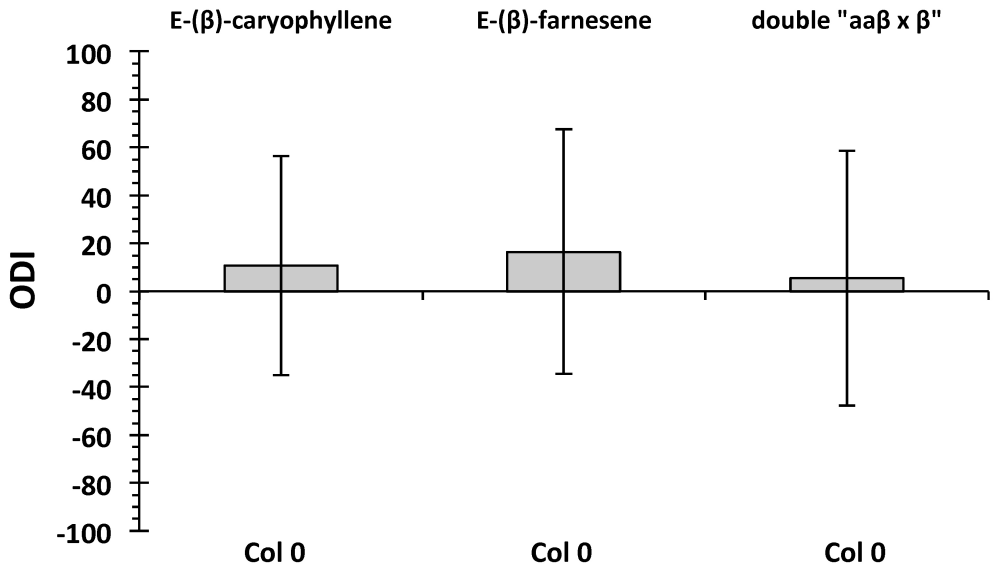

Figure 4. Results after dual-choice oviposition assays. *E*-(β)-caryophyllene test: N = 14; *E*-(β)-farnesene test: N = 17; aaβ x β test: N = 14. N = number of independent couples of females used.

Also checking for the mean number of eggs laid per couple of females we could not find any significant effect for the different plant genotype (fig. 5), although the highest values were found in the presence of *E*-(β)-farnesene alone.

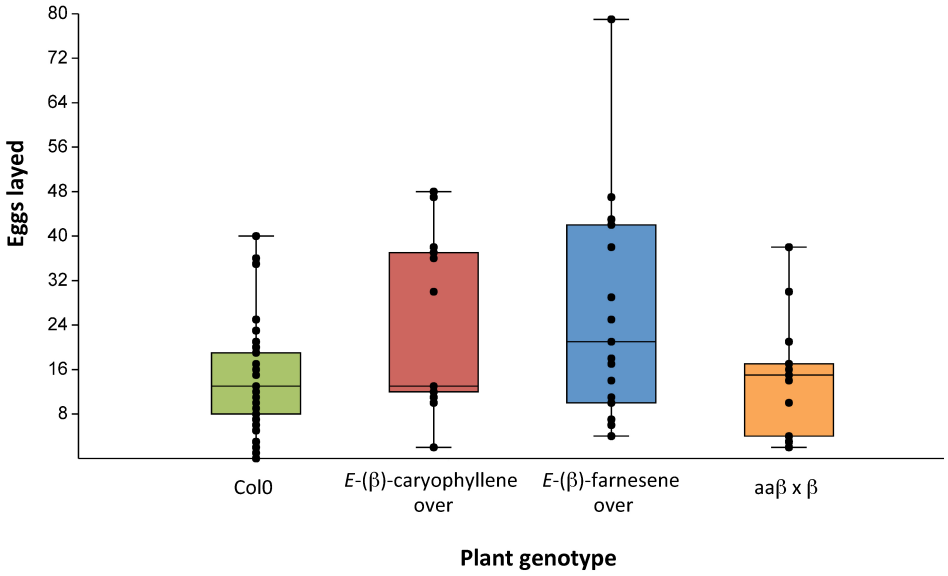

Figure 5. Boxplots of the number of eggs laid per couple of females in the oviposition assays on all the four type of plants used.

It is worth noticing that double Aaβ x β lines had a *E*-(β)-caryophyllene / *E*-(β)-farnesene emission ratio of 1:8 on average, which is very different from the ratio found in grapevine (Tasin et al. 2006). That ratio was shown to be crucial in eliciting attraction in wind tunnel assays, and we can not exclude that it might have a role also in oviposition preference. Moreover, in grapevine, a high degree of redundancy was observed (Tasin *et al*. 2007), as the lack of one compound was partially compensated by the presence of all the other background volatiles in upwind attraction tests. It could be hypothesized that during oviposition the degree of redundancy in the signal is lower, and that the complete blend is necessary to pass all the behavioural steps that lead finally to oviposition. Finally, the quantity of volatile compound should be considered as another crucial factor influencing the behaviour: the highest oviposition rate was indeed observed on *E*-(β)-farnesene emitting plant, which were those with the highest emission (more than 200 ng plant^{-1} day^{-1}) and comparable to the amount of VOCs released by grapevine plants (100-500 ng plant^{-1} day^{-1}). Future experiments with several Arabidopsis plants on the same spot will be needed to clarify this point.

Experimental

Cloning of Aaβ FS into plant over-expression vectors

Several 1-month-old *Artemisia annua* plants were obtained from seeds (www.worldseedsupply.com) germinated in the greenhouse. Mature leaves were used to extract total RNA (Spectrum^{TM} Plant Total RNA kit, Sigma) and 1.0 mg were retro-transcribed (SuperScript® III Reverse Transcriptase, Invitrogen) with the gene-specific primer "aaBfs rev" (5' – TTAGACAACCATAGGGTGAACG – 3'). cDNA was used as a template to amplify the whole coding sequence of the *E*-(*β*)-farnesene synthase (Aaβ-FS, GenBank: AY835398.1) with the forward primer "attB-β-FS for" (5' – GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACAATGTCGACTCTTCCTA TTTCTAG – 3') and the reverse primer "attB-β-FS rev" (5' – GGGGACCACTT TGTACAAGAAAGCTGGGTTTAGACAACCATAGGGTGAACG – 3'). PCR product was cloned directly into pDONR221 via BP-clonase reaction (Invitrogen), and transformed into E. coli DH5α chemically competent cells. Plasmid minipreps from single colony 5ml cultures were sequenced to check for their quality using the M13(-20) for (5' – GTAAAACGACGGCCAG – 3') and M13 rev (5' – CAGGAAACAGCTATGAC – 3') primer pair. The coding sequenced was then moved into the gateway destination vector pH2WG7 (Karimi *et al.* 2007) via LR reaction (Invitrogen). 1ml of the reaction was used to transform chemically competent *E. coli* TOP10 cells (Invitrogen), then plasmid minipreps from single colony 5ml cultures were sequenced to check for their quality using my35Sprom for the (5' – CCACTATCCTTCGCAAGA CCC – 3') and my35Sterm rev (5' – GAAGTATTTTACAAATACAAATACATAC TAAGG – 3') primer pair.

***Arabidopsis thaliana* transformation and cross**

The vector pH2WG7 with constitutive CaMV 35S promoter driving *Aaβ FS* expression was used to transform *A. thaliana* Col 0 plants with the floral dip method (Clough and Bent, 1998). Transformed plants were screened on solid MS media (Duchefa) with sucrose (15 g/L) and 2,2 g/L Basal Salts including

vitamins and supplemented with hygromycin B (Sigma) 20 mg/L. After one week the resistant lines were transferred into soil and let grow in the greenhouse for four generations, until 100% of the seeds were homozygous for T-DNA insertions. To obtain the double transformed lines, flowers from homozygous *AaβFS* overexpressing plants were emasculated with tweezers (#5, Micropoli, Milano) under a stereomicroscope and manually pollinated with anthers taken from the VvGwECar2 overexpressing lines described in Salvagnin *et al*. 20016. To select the successful crosses, seeds were screened on solid MS media (Duchefa) with sucrose (15 g/L) and 2,2 g/L Basal Salts including vitamins and supplemented with glyphosate (Sigma) 25 mg/L. After one week the resistant lines were transferred into soil and let grow in the greenhouse to reach loci homozygosity.

Headspace Collection and analysis from Arabidopsis plants

Headspace volatiles of *A. thaliana* plants were collected using the close-loop stripping analysis (CLSA) method (Boland *et al.* 1984; Abraham *et al*. 2014). Eight plants in five weeks old were put into a closed plastic cooking bag (45 x 55 cm, Toppits, Melitta, Sweden) where an air pump (DC 12/16 FK, Aersistem, Milano) created a flow of activated-charcoal-cleaned air at 0.4 L/min. The air flow hit an adsorbent filter (CLSA-Filter, LowResistance 1.8 mg, Brechbühler AG, Switzerland) for 4 hours, before trapped compounds were eluted with 140 µL of dichloromethane. Three bags replicates were set up for each line tested. A Gerstel MPS autosampler was used to perform liquid injections of the extracts. A 7890A GC gas chromatograph (Agilent) coupled to a 5975D MSD mass spectrometer (Agilent) was used: compounds were separated using a HP-5MS column (5% phenyl methyl siloxane; 30 m ×0.25 mm ×0.25 µm, Agilent). The GC oven parameters were as follows: initial temperature was 50°C, maintained for 1.5 min, then a rate of 7.5°C min^{-1} until 250°C maintained for 10 min; injection volume was 2µl in splitless mode and with a GC inlet temperature of 280 °C. Helium was used as carrier gas in constant flow mode at 1.2 mL min^{-1}. The total cycle time was 38.17 min. The MS detector was operated in scan mode (mass range 20–400 m/z) and the

transfer line to the MS system was maintained at 230°C. Identification of the volatile compounds was made by injecting pure reference standards and comparing retention index and mass spectra with the use of NIST 2011 database.

Insects

The *L. botrana* moths used in this research were reared on a semi-artificial diet in a growth chamber at 23.5°C and with 70% relative humidity. The photoperiod was 1:16:1:6 h respectively for dawn:day:sunset:night light conditions. Larvae were kept in plastic boxes (25×18×5 cm), where they could feed without limits until they pupated. All the pupae were transferred into meshed plastic boxes (30×30×30 cm), where the adults emerged. Adults were transferred into mating chambers (12 cm diameter, 22 cm long) and provided with 10% sucrose solution to obtain eggs every day, and 3-day-old mated females were used each time for all experiments. The insects never experienced any contact with plants or volatiles from the extracts before the experiments, and each female was only used once.

Oviposition assays

The dual-choice oviposition assays were done adapting a protocol from Anfora et al., 2009. Couples of mated females of the grapevine moth were put inside a ventilated cylindrical mesh cage (50 cm x 30 cm) on which the moths were not able to lay eggs. Inside each cage two *A. thaliana* plants at the same growth stage (4 weeks) were placed: a Col0 wild-type and a transformed one. The plants were placed into perforated (32 holes) transparent conical plastic glasses (bottom 61 mm, top 88 mm, height 130 mm) sealed on petri dishes (Suppl. fig. 1). Two mated females were released in the center of the cage and after 120 hrs at 70% humidity and 23.5 °C they were removed and the eggs on glasses were counted.

Supplementary figure 1. Oviposition assay set-up. (A) 4 weeks old *A. thaliana* plant inside a plastic cup with 32 holes. (B) Transformed and Col 0 plants ready to be placed inside the metal mesh cage.

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Chapter 4

The *in vitro* bi-functional grapevine linalool/nerolidol synthase acts as a mono-functional linalool synthase when overexpressed in *Arabidopsis thaliana*

Running title: Grapevine linalool/nerolidol synthase is as a linalool synthase *in vivo*

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This manuscript is meant for a short communication in a peer-reviewed journal yet to be decided.

Background to chapter 4

The grapevine gene VvCSLinNer codes for a TPS enzyme that *in vitro* was characterized as a bi-functional linalool/nerolidol synthase. Since nerolidol is the precursor of DMNT, one of the three key kairomones for *L. botrana*, our initial goal was to overexpress the gene and obtain *A. thaliana* lines emitting high levels of DMNT to be used in behavioural studies. However, after plants transformation, I could obtain plants only emitting linalool in the headspace, proving indirectly that, contrary to our expectations, the coded enzyme is imported into the plant chloroplasts. This result was contemporary confirmed by a paper (Zhu et al., 2014) where the authors definitively prove through confocal microscopy the localization of the enzyme in the plastids of *Vitis vinifera* cells. The obtained lines thus failed to produce DMNT, but their linalool content was characterized, and they could be used in the future in behavioural assays with other insects for which this compound is important in the establishment of interactions with plants.

Keywords: TPS, terpene synthase, linalool, *Arabidopsis thaliana*, VOC(s), genetic transformation

Introduction

Among the many volatile organic compounds (VOCs) used by plant to interact with the environment, terpenoids constitute the biggest group (Zwenger and Basu, 2008). Regular terpenoids such as mono (C10) and sesquiterpenes (C15) have proven their multiple ecological roles, with examples of plant defence against pathogens (Hasegawa *et al.*, 2010) and insects (Unsicker *et al*. 2009), attraction of pollinators (Dudareva and Pichersky, 2000) and priming of the defences of neighbouring plants (Arimura *et al.*, 2000). Irregular terpenoids, such as the C11 homoterpene 4,8-dimethyl-1,3,7-nonatriene (DMNT), are frequently released in response to herbivore damage on leaves (Bouwmeester *et al.*, 1999; Su *et al.*, 2008), and they are linked to the attraction of herbivore predators (Kant *et al.*, 2004) also when the emission is due to a genetic modification (Kappers *et al.*, 2005). DMNT biosynthesis starts from farnesyl pyrophosphate (FPP) which is converted to the sesquiterpene alcohol nerolidol by a nerolidol synthase, and then to DMNT through a step of oxidative degradation mediated by a P450 enzyme (fig. 1).

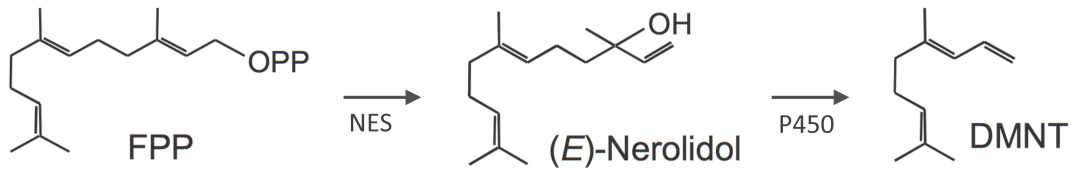


Figure 1. DMNT biosynthesis pathway in A. thaliana (adapted from Lee *et al.*, 2010). NES = nerolidol synthase; P450 = cytochrome P450 monooxygenase.

Frequently, the nerolidol synthase characterized so far *in vitro* can accept also another substrate between geranyl pyrophosphate (GPP) or geranylgeranyl pyrophosphate (GGPP), acting thus as bi-functional TPS. The enzyme responsible for the last step of the pathway was characterized so far only in Arabidopsis, and it belongs to the CYP82 family of cytochrome P450 monooxygenase (Lee at al., 2010).

In grapevine, DMNT is part of the volatile blend emitted by the canopy and flowers (Tasin *et al.*, 2005) that attracts adult females of *L. botrana*, the European grapevine moth (Tasin *et al.*, 2006). In this work we report the

creation of stable transformed *A. thaliana* plant that overexpress a previously characterized (Martin *et al*., 2011) linalool/nerolidol synthase (VvCSLinNer), with the aim of obtaining DMNT-emitting plants to use as a model in behavioral assays. Contrary to our expectations the plants emitted only linalool in their headspace, meaning that VvCSLinNer acts *in vivo* as a linalool synthase.

Results and Discussion

Plant VvCSLinNer overexpression

The coding sequences of *VvCSLinNer* was cloned into the plant overexpression vectors pK7WG2D (Karimi *et al.* 2007) respectively, under the control of cauliflower mosaic virus (CaMV) 35S promoter (fig. 2). The vector was used to transform *Col-0* wild type Arabidopsis plants with the floral dip method. Screening of transformed seeds in agar plates supplemented with kanamycine led to the identification of 10 putative lines (L1 to L10). A PCR on genomic DNA to check the presence of the insertion (data not shown) revealed three false positive line, that were discarded. Seven independent transgenic lines were thus acclimatised and grown in the greenhouse for three generations, until all the T-DNA insertions were at homozygosity. No variation on the plants size, shape or fertility was observed, although their growth was few days slower compared to control lines. This is in contrast with our findings on other Arabidopsis transformations with TPS genes, where we never saw slower growth, not even in crossings expressing two TPS contemporary. However, a similar phenotype was reported (Aharoni *et al*., 2003) in A. thaliana plants when a strawberry linalool/nerolidol synthase (*FaNES1*) was overexpressed in the plastids.

Since the copy number of T-DNA integrated into the genome can vary across the lines, as well as their site of integration, we decided to check and quantify the presence of the gene transcript in all the remaining lines.

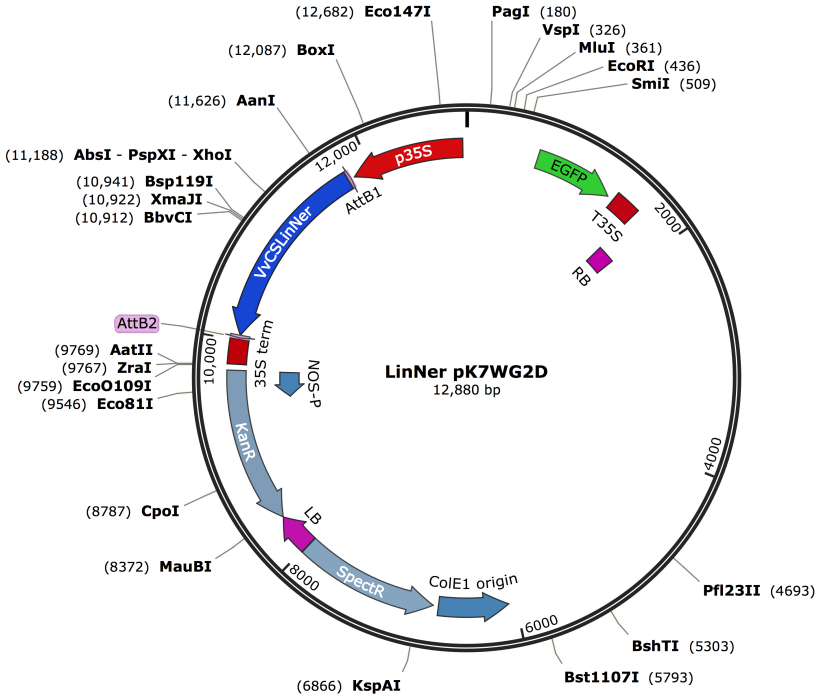


Figure 2. Map of the vector pK7WG2D harbouring VvCSLinNer under the guide of the strong CaMV 35S promoter.

Total RNA from 7-day-old seedlings was obtained and used in RT-qPCR (fig. 3).

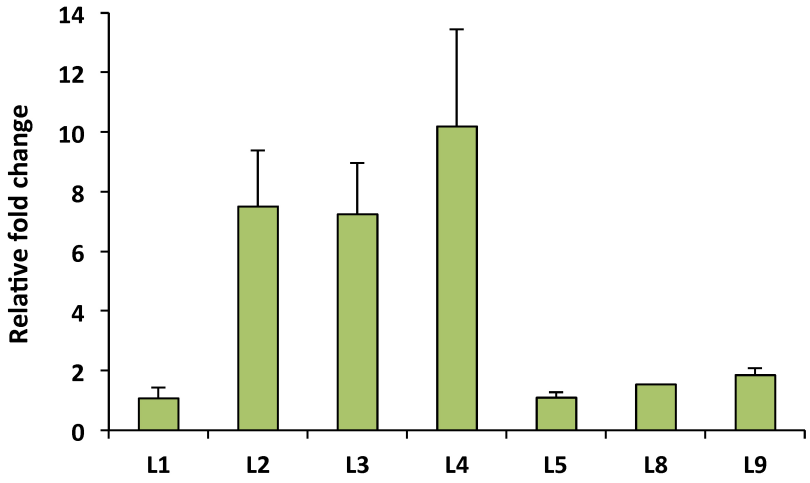


Figure 3. RT-qPCR on VvCSLinNer on different Arabidopsis lines. L1 line was used as a reference. Standard Error on Mean (SEM) is visible for each line.

Emission of linalool in transformed lines

The plants were analysed for volatile organic compounds (VOCs) production with the closed loop stripping analysis (CLSA, Boland *et al.*, 1984) and their emission was compared with that of control plants of the same age. Contrary to our expectations, the headspace was dominated by the presence of linalool (fig. 4), without any trace of nerolidol nor DMNT.

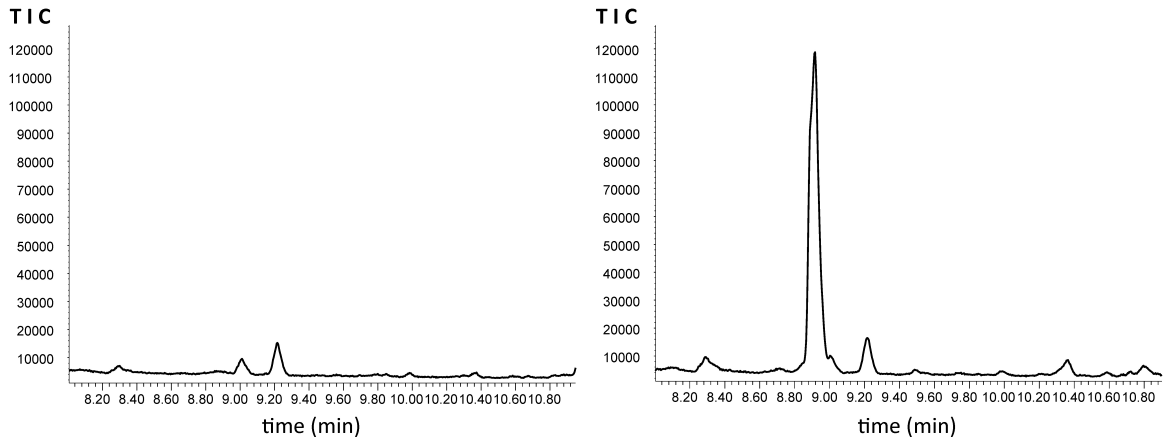


Figure 4. Gas-chromatograms (TIC) of the VOCs produced by *A. thaliana* plants wild type Col 0 (left) and overexpressing *VvCSLinNer* (right). The main peak is visible and correspond to linalool.

Overall, linalool emission ranged from 5 ng plant^{-1} day^{-1} in line L1, up to 77 ng plant^{-1} day^{-1} on average in line L2 (fig 5.), with no detection in the headspace of L5 and L8, which were two of the weakest over-expressing lines.

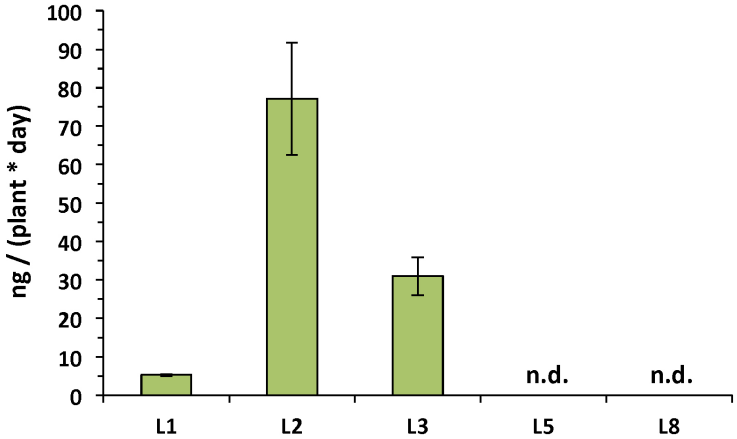


Figure 5. Quantification of linalool in *A. thaliana* plants 5 weeks old.

Considering that monoterpenes biosynthesis starts from GPP in the plastids, where there is not a pool of FPP for sesquiterpenes production, the presence of linalool suggests that VvCSLinNer protein is targeted to the chloroplast, and that although *in vitro* it is a bi-functional enzyme, *in vivo* it works only as monoterpene synthase. This indirect evidence is supported by a phylogenetic analysis on grapevine known TPS sequences, which showed that VvCSLinNer groups with monoTPS (fig. 6).

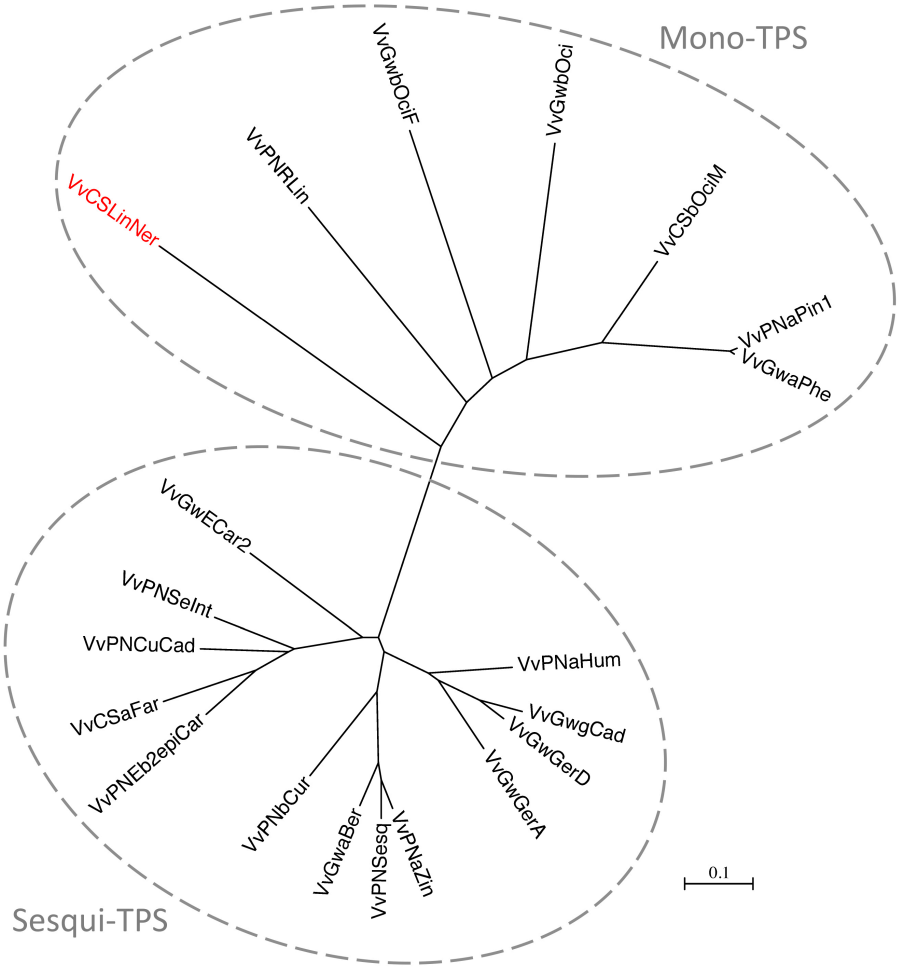


Figure 6. Phylogenetic tree illustrating VvCCLinNer (in red) as part of the mono-TPS group. The unrooted neighbor-joining tree was created with Seaview 4.6.1 after multiple alignment of the translated protein sequences. Abbreviations: VvGwECar2 = E-(β)-caryophyllene synthase (HM807374.1); VvGwGerA = germacrene A synthase (HQ326230.1); VvGwaBer = E-(α)-bergamotene synthase (HM807376.2); VvGwGerD = germacrene D synthase (HM807378.1); VvCSaFar = E,E-(α)-farnesene synthase (HM807379.1); VvGwgCad = γ-cadinene synthase

(HM807380.1); VvPNbCur = β-curcumene synthase (HM807381.1); VvPNSesq = sesquithujene synthase (HM807404.1); VvPNaZin = α-zingiberene synthase (HM807405.1); VvPNSeInt = selina-4,11-diene / intermedeol synthase (HM807406.1); VvPNCuCad = cubebol / δ-cadinene synthase (HM807407.1); VvPNaHum = α-humulene synthase (HM807408.1); vPNEb2epiCar = 2-epi-E-(β) - caryophyllene synthase (HM807409.1); VvGwaPhe = α-phellandrene synthase (HM807382.1); VvPNaPin1 = α-pinene synthase (HM807383.1); VvGwbOci = E-(β)-ocimene synthase (HM807385.1); VvCSbOciM = E-(β)-ocimene / myrcene synthase (HM807387.1); VvGwbOciF = E-(β)-ocimene / E,E-(α)-farnesene synthase (HM807388.1); VvPNRLin = linalool synthase (HM807390.1); VvCSLinNer = linalool / nerolidol synthase (HM807393.1).

The use of software for subcellular localization prediction (PSort, SherLoc2) was not considered reliable, since different tools gave different predictions for VvCSLinNer, and they also failed to guess the correct location of TPS enzymes that we already expressed in Arabidopsis. The coding sequence indeed presents no signal peptide, and share the same percentage of similarity (from 44% to 49%) with all the other grapevine TPS sequences, both mono- and sesqui-TPS (supplementary fig. 1).

A further support for a plastidic localization comes from the work of Zhu *et al*. (2014), where the researchers isolated what they consider to be an allele of VvCSLinNer in the variety *Riesling*, that was called VvRiLinNer. The two ORFs share a 99.1% of sequence similarity, and through the transient homologous expression of a VvRiLinNer-GFP fusion protein and confocal laser scanning microscopy, the authors proved the protein to be located in the chloroplasts.

In conclusion, we failed to produce *A. thaliana* plants emitting DMNT to investigate its role in *L. botrana* attraction, but we produced instead several lines emitting linalool. Considering anyway the role of linalool as semiochemical (Andersson *et al.*, 2003, Burguiere *et al.*, 2001, see also http://www.pherobase.com/database/compound/compounds-detail-linalool.php), the plants could be used in the future as a tool to investigate other plant-insect interactions.

Experimental procedures

Cloning of *VvCSLinNer* into plant over-expression vector

The coding sequence of VvCSLinNer was kindly provided by Prof. Bohlmann's laboratory into the bacterial expression vector pET28b+ with C-terminal His-tag. The vector was used as a template to amplify the coding sequence of the enzyme with Phusion High-Fidelity DNA-Polymerase (New England Biolabs) using the forward primer "attB-LinNer for" (5' – GGGGACAAGTTTGTACAAAAAAGCAGGCTTAACAATGGGATTCTCTCCG CC – 3') and the reverse primer "attB-LinNer rev" (5' – GGGGACCACTTTG TACAAGAAAGCTGGGTCTACAGGGGAAATGCTTCAAAGAGG – 3'). PCR product was cloned directly into pDONR221 via BP-clonase reaction (Invitrogen), and transformed into E. coli DH5a chemically competent cells.
Plasmid minipreps from single colony 5ml cultures were sequenced to check for their quality using the M13(-20) for (5' – GTAAAACGACGGCCAG – 3') and M13 rev (5' – CAGGAAACAGCTATGAC – 3') primer pair. The coding sequenced was then moved into the gateway destination vectors pK7WG2D (Karimi *et al.* 2007) via LR reaction (Invitrogen). 1ml of the reaction was used to transform chemically competent *E. coli* TOP10 cells (Invitrogen), then plasmid minipreps from single colony 5ml cultures were sequenced to check for their quality using my35Sprom for the (5' – CCACTATCCTTCGC AAGACCC – 3') and my35Sterm rev (5' – GAAGTATTTTACAAATACAA ATACATACTAAGG – 3') primer pair.

Arabidopsis thaliana transformation

The vector pK7WG2D with constitutive CaMV 35S promoter driving VvCSLinNer expression was used to transform *A. thaliana* Col 0 plants with the floral dip method (Clough and Bent, 1998). Transformed plants were screened on solid MS media (Duchefa) with sucrose (15 g/L) and 2,2 g/L Basal Salts including vitamins and supplemented with kanamycin (Duchefa) 45 mg/L. After one week the resistant lines were transferred into soil and let

grow in the greenhouse for two generations, until 100% of the seeds were homozygous for T-DNA insertions.

RNA extraction and RT-qPCR

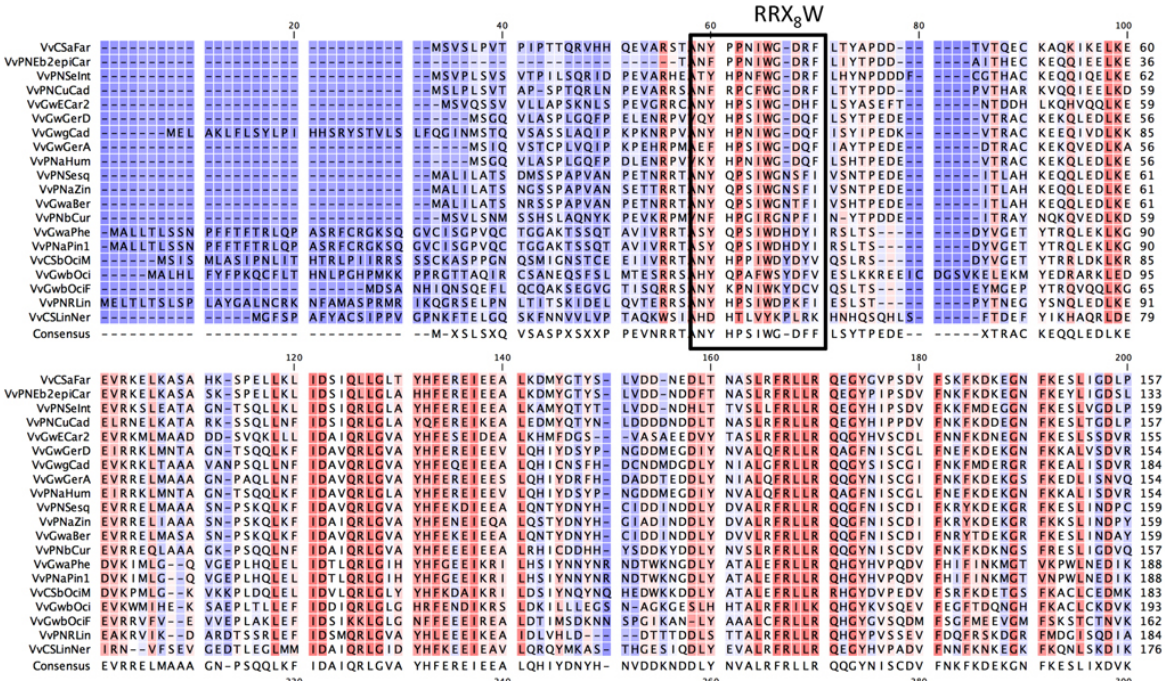
RNA was extracted with Spectrum^{TM} Plant Total RNA kit (Sigma) from 7-day-old *Arabidopsis* seedlings following the manufacturer's guidelines. RNA quality and quantity was checked on spectrophotometer and 1% agarose electrophoresis before cDNA was retro-transcribed using SuperScript® III Reverse Transcriptase (Invitrogen) with Random Primers. A specific primer pair able to discriminate linalool/nerolidol synthase (qLinNer for 5'- GGAA TGCCTCTCTCAGCATC – 3' and qLinNer rev 5' – CCTCAAGGTGTGG AAGACTC – 3') was created using the online tool Primique (Freedslund and Lange, 2007; http://cgi-www.daimi.au.dk/cgi-chili/primique/front.py), the pair had an efficiency of 95.7%. The qPCR was performed on an CFX96 thermocycler (Bio-Rad) using GAPDH and EF1a as reference genes (Czechowski *at al.*, 2005). Real-time PCR was carried out with the following cycle: 95°C 10'; 40 x (95°C 30" , 58°C 30"); The manufacturer's software (CFX Manager, Bio-Rad) was used to calculate primer pair efficiency (E) and the threshold cycle (Ct) mean and standard deviation for each sample. The analysis of relative quantification was performed using the workflow reported in Hellemans et al., 2007. For each primer pair used, Ct from a reference line were used as reference to calculate the DCt. The geometric mean between the value E^ΔCt from EF1a and GAPDH were used to calculate a normalization factor (NF), which was finally used to calculate the fold difference according to the formula: Fold Difference = E^ΔCt / NF.

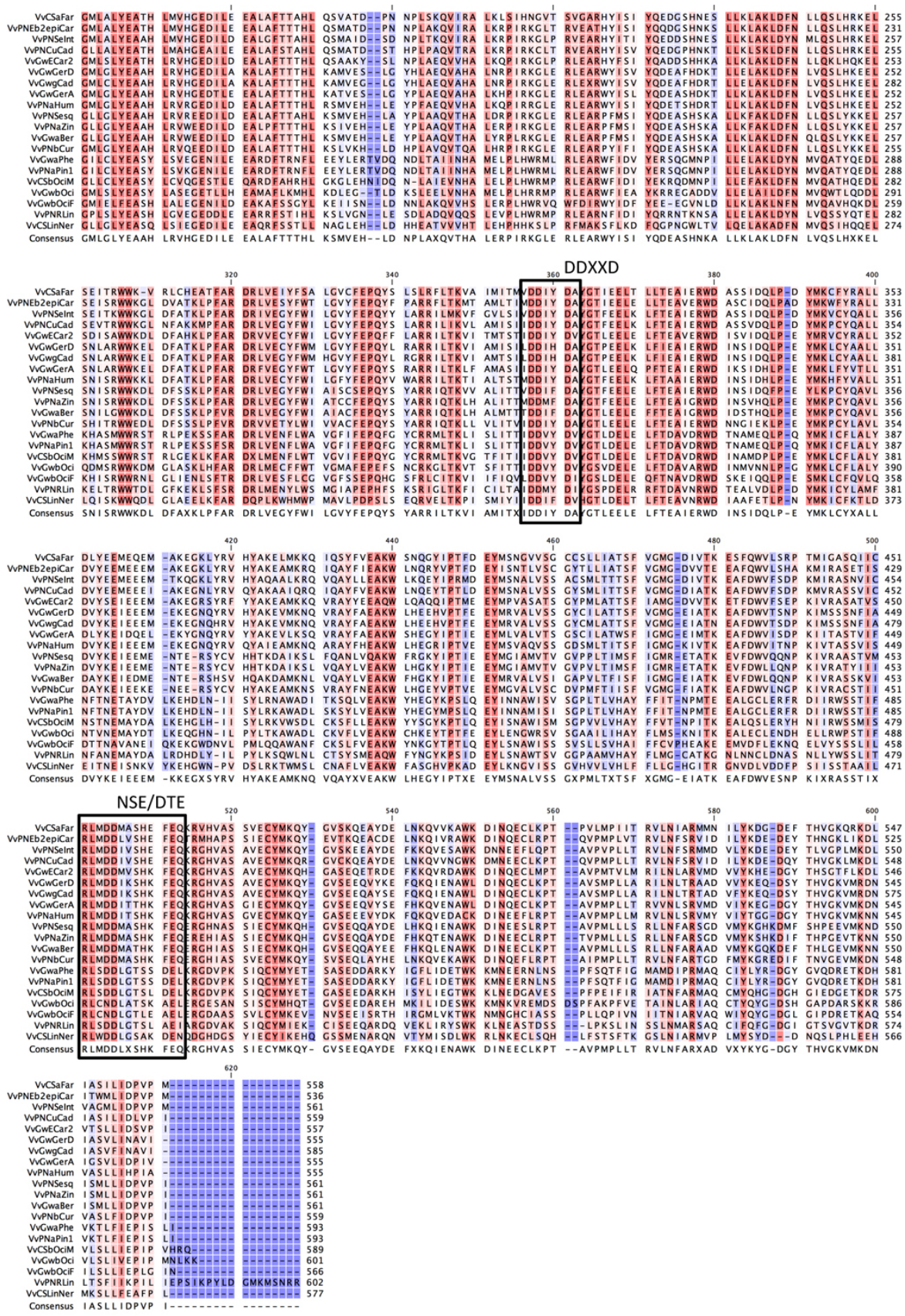
Headspace Collection and analysis from *Arabidopsis* plants

Headspace volatiles of *A. thaliana* plants were collected using the close-loop stripping analysis (CLSA) method (Boland *et al.*, 1984). Eight plants in five weeks old were put into a closed plastic cooking bag (45 x 55 cm, Toppits, Melitta, Sweden) where an air pump (DC 12/16 FK, Aersistem, Milano)

created a flow of activated-charcoal-cleaned air at 0.4 L/min. The air flow hit an adsorbent filter (CLSA-Filter, LowResistance 1.8 mg, Brechbühler AG, Switzerland) for 4 hours, before trapped compounds were eluted with 140 µL of dichloromethane. Three bags replicates were set up for each line tested. A Gerstel MPS autosampler was used to perform liquid injections of the extracts. A 7890A GC gas chromatograph (Agilent) coupled to a 5975D MSD mass spectrometer (Agilent) was used: compounds were separated using a HP-5MS column (5% phenyl methyl siloxane; 30 m ×0.25 mm ×0.25 µm, Agilent). The GC oven parameters were as follows: initial temperature was 50°C, maintained for 1.5 min, then a rate of 7.5°C min^{-1} until 250°C maintained for 10 min; injection volume was 2µl in splitless mode and with a GC inlet temperature of 280 °C. Helium was used as carrier gas in constant flow mode at 1.2 mL min^{-1}. The total cycle time was 38.17 min. The MS detector was operated in scan mode (mass range 20–400 m/z) and the transfer line to the MS system was maintained at 230°C. Identification of the volatile compounds was made by injecting pure reference standards and comparing retention index and mass spectra with the use of NIST 2011 database.

Supplementary Figure 1:




DDXXD
NSE/DTE

Supplementary Figure 1. Multiple alignment of grapevine mono- and sesqui-TPS protein sequences (CLC sequence viewer 7, Qiagen). Colors shades represent consensus (0% = blue, 50% = white, 100% = red), VvCSLinNer shares a 47% of similarity with all the other TPS. The RRX_8W, DDXXD and NSE/DTE motifs, typical of TPS, are visible inside black frames.

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Chapter 5

Exploitation of genetically modified *Vitis vinifera* plants with altered kairomone emission ratio for the control of the European Grapevine Moth *Lobesia botrana*

Running title: Genetically modified grapevine plants for the control of *L. botrana*

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The content of this section has been the subject of a paper that was submitted to Plant Biotechnology Journal while writing the thesis: Salvagnin, U., Malnoy, M., Thöming, G., Tasin, M., Carlin, S., Martens, S., Vrhovsek, U., Angeli, S., Anfora, 2016. Adjusting the scent ratio: using genetically modified *Vitis vinifera* plants to manipulate European grapevine moth behaviour *(Submitted).*

Background to chapter 5

In this chapter I describe how I used a genetic engineering approach to prove the role of the two VOCs *E*-(β)-caryophyllene and *E*-(β)-farnesene in the attraction of *L. botrana* by grapevine plants. The hypothesis is that the ratio between essential VOCs is necessary for host-plant choice, and its modification is sufficient to consistently reduce plant attractiveness. I therefore generated stable grapevine transgenic lines with altered *E*-(β)-caryophyllene and *E*-(β)-farnesene emission compared to control plants, thus modifying their kairomone ratio *in vivo*. I could do this overexpressing the TPS genes responsible for the production of these two VOCs. When I then tested headspace collections from control and modified plants, I found that those from transformed plants were less attractive than those from control plants. This result was confirmed by testing synthetic blends, imitating the ratio found on natural and transformed plants, and finally by testing the plants themselves. Based on this evidence, I suggest that modification of the kairomone ratio may also interfere with host-finding behaviour of the pest in the field, making the vineyards less susceptible to *L. botrana* herbivory, and creating the rationale for a new method in pest management.

Abstract

Herbivorous insects use olfactory cues to locate their host plant within a complex olfactory landscape. One such example is the European grapevine moth *Lobesia botrana,* a key pest of the grape in the Palearctic region, which recently expanded both its geographical and host plant range. Previous studies have showed that a synthetic blend of the three terpenoids *E*-(β)-caryophyllene, *E*-(β)-farnesene and *E*-(4,8)-dimethyl-(1,3,7)-nonatriene (DMNT) was as attractive as the complete grape odour profile in laboratory conditions. The same studies also showed that the specific ratio of these compounds in the grape bouquet was crucial, because a percentage variation in any of the three volatiles resulted in almost complete inhibition of the blend's attractiveness.

Here we report on the creation of stable grapevine transgenic lines, with modified *E*-(β)-caryophyllene and *E*-(β)-farnesene emission and thus with an altered ratio compared to the original plants. When headspace collections from these plants were tested in wind tunnel behavioural assays, they were less attractive than control extracts. This result was confirmed by testing synthetic blends imitating the ratio found on natural and transformed plants, as well as by testing the plants themselves. With this evidence we suggest that a strategy based on volatiles ratio modification may also interfere with the host-finding behaviour of *L. botrana* in the field, creating the rationale for a new pest control method.

Keywords: *Vitis vinifera*, sesquiterpene(s), *E*-(β)-caryophyllene, *E*-(β)-farnesene, VOC(s), genetic transformation, *Lobesia botrana*, wind tunnel, kairomones, grapevine, host-selection

Introduction

Terpenoids constitute the biggest class of metabolites in plants, involved both in primary (Croteau *et al.*, 2000) and secondary metabolism (Zwenger and Basu, 2008). All terpenes formally derive from the C_5 isomers isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), whose biosynthesis can take place in plants via two distinct pathways: the mevalonate pathway (MVA) in the cytoplasm and the MEP/DOXP pathway in the chloroplast (Rohmer, 1999). Condensation of C_5 isomers by prenyl-transferase enzymes leads to the C_{10}, C_{15}, C_{20}, C_{30} and C_{40} precursors of mono-, sesqui-, di-, tri- and tetra-terpenes respectively. In the last step of the pathway, precursors are converted to terpenes by terpene synthase (TPS) enzymes, which are usually coded by gene families of 20-150 members in each species (Chen at al., 2011). In nature terpenes have many ecological roles, such as direct and indirect defence against pathogens and insects (Hasegawa *et al.*, 2010; Heiling *et al.* 2010; Huang et al. 2012; Unsicker *et al*. 2009), attraction of pollinators (Dudareva and Pichersky, 2000) and mutualistic fungi (Ditengou *et al.*, 2015), as well as being used as signals for plant-to-plant communication (Arimura *et al.*, 2000). As semiochemicals, their emission is often exploited by phytophagous insects, which use them as kairomones to recognise and locate their host plants (Bruce at al., 2005). This is also the case of the European grapevine moth *Lobesia botrana* (Den. & Schiff.) (Lepidoptera, Tortricidae), a polyphagous insect which is considered the main pest of vineyards in Europe and which has the potential to become an invasive species, as recently observed in California, Argentina and Chile (Ioriatti *et al.*, 2011). Concerns about *L. botrana* management arise when considering that in worldwide fruit production, grapes are in first place in terms of crop surface area, with over 3,200,000 ha cultivated and more than 26 Mtons of fruit production in Europe (FAOSTAT, data until 2013, http://faostat.fao.org/). Control strategies against this pest still rely mainly on the use of pesticides, with environmentally friendly techniques such as mating disruption covering less than 4% of European vineyards (Ioriatti et al., 2011). With a view to using semiochemicals against insect pests (Birkett and Pickett,

2014), grapevine VOCs involved in host-plant interaction were studied for *L. botrana*, and it was found that a specific blend of the terpenoids *E*-(β)-caryophyllene and *E*-(β)-farnesene and the homoterpene *E*-(4,8)-dimethyl-(1,3,7)-nonatriene (DMNT) elicits electrophysiological responses on female antennae (Tasin *et al.*, 2005). Moreover, the blend itself was attractive in laboratory and field conditions (Anfora *et al.*, 2009), and the attractiveness was shown to be dependent on the kairomone ratio, decreasing significantly when deviating from the ratio found in the grapevine headspace collection (Tasin *et al.*, 2006).

In this work, we generated stable grapevine transgenic lines with altered *E*-(β)-caryophyllene and *E*-(β)-farnesene emission compared to control plants, thus modifying their kairomone ratio *in vivo*. We then tested headspace collections from control and modified plants, finding that those from transformed plants with unbalanced kairomonal emission were less attractive than those from control plants. This result was confirmed by testing synthetic blends, imitating the ratio found on natural and transformed plants, and finally by testing the plants themselves. Based on this evidence, we suggest that modification of the kairomone ratio may also interfere with host-finding behaviour of the pest in the field, making the plants less susceptible to *L. botrana* herbivory.

Results

Creation of grapevine lines with altered *E*-(β)-caryophyllene and *E*-(β)-farnesene emission

To modify sesquiterpene emission, both TPS gene overexpression and a TPS gene silencing approach were considered. In grapevine there are five genes known to code for *E*-(β)-caryophyllene synthase (Martin et al. 2010), but *VvGwECar2* (GenBank: HM807374) alone is responsible for most of the volatile production in the green tissues of the plant (Matarese et a., 2014), so it was chosen as the target for gene silencing using RNAi. However, after plant transformation and regeneration we could not obtain any significantly

silenced line (data not shown), probably because of the difficulty in specifically targeting only one TPS gene in the whole grapevine gene family, in which there is a high degree of sequence similarity among members. By contrast, overexpression of *VvGwECar2* was successful and resulted in the regeneration of many independent lines, as we already reported in a previous study (Salvagnin et al. 2016).

In order to modify *E*-(β)-farnesene emission we could not silence or overexpress any grapevine gene, since there are no known *E*-(β)-farnesene synthases in the grapevine genome, but only a *E*-(α)-bergamotene and a β-curcumene synthase that also produce *E*-(β)-farnesene as a minority by-product (Martin et al. 2010). For this reason, we decided to enhance the level of the sesquiterpene by the insertion and expression of a gene from the sweet wormwood *Artemisia annua* L. which had already been characterised as a *E*-(β)-farnesene synthase (Aab-FS, GenBank: AY835398.1; Picaud et al. 2005). All the transformations were carried out as described in Dalla Costa et al. (2014), starting from “Brachetto Grappolo Lungo” grapevine cv. embryogenic calli (Martinelli et al. 2001), and obtaining a dozen independent transgenic lines after one year. A subset of the lines was extensively propagated and acclimatised in the greenhouse, and this was used for all the experiments. Overall, we did not notice any difference in plant phenotypes or growth speed in the different lines or compared to the controls.

As frequently observed with random integration of T-DNA, transgene expression levels varied among lines (One-way ANOVA, $F = 6.37$; $df = 24$; $p < 0.001$) when measured with RT-QPCR (Fig. 1), with a 30-fold difference in transcript level between the weakest and strongest overexpressing lines.

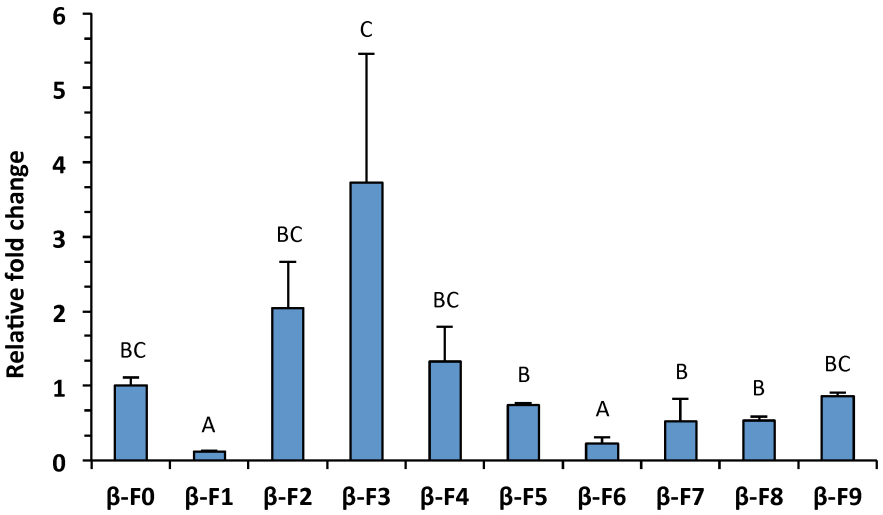

Figure1. *Aaβ-FS* relative expression obtained using RT-QPCR. Means of reference line β-F0 were set to 1. At least three biological replicates were used for each line. Letters on bars indicate different groups, according to one-way ANOVA followed by Fisher's LSD post hoc test (P < 0.05)) with standard deviations (SDs) visible for each line.

To check for changes in sesquiterpene emission, we screened the plants with solid-phase microextraction coupled with gas-chromatography/mass spectrometry (SPME-GC-MS) (Fig. 2).

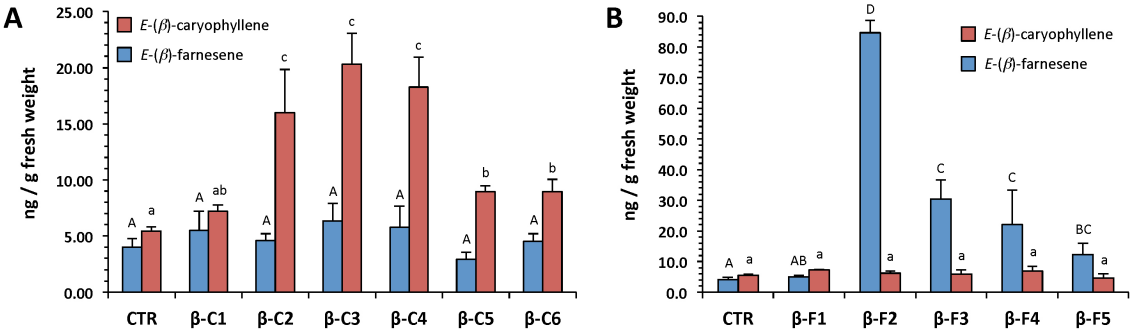

Figure 2. Volatile collection and analysis using SPME-GC-MS from young grapevine leaves taken from plants acclimatised in the greenhouse. (A) *E*-(β)-caryophyllene overexpressing lines and (B) lines with enhanced *E*-(β)-farnesene emission. Letters on bars indicate different groups according to one-way ANOVA followed by Fisher's LSD post hoc test (P < 0.05) with the standard error of the mean (SEM) visible for each line.

SPME was chosen because of its speed and sensitivity, considering that sesquiterpenes usually cover no more than 1% of the total amount of VOCs emitted by the grapevine (Matarese *et al.*, 2014). Young leaves of the control plants emitted *E*-(β)-caryophyllene in the range of 5 ng/g of fresh weight and *E*-(β)-farnesene in the range of 4 ng/g. As already reported in Salvagnin et al. 2016, *E*-(β)-caryophyllene overexpressing lines (βC1 to 6) showed an increase of up to 20 ng/g of fresh weight in terms of *E*-(β)-caryophyllene content (One-way ANOVA, $F = 11.92$; $df = 20$; $p < 0.001$), which was poorly correlated with the gene transcript level. *E*-(β)-farnesene overexpressing lines (βF1 to 5) showed a more pronounced increase (One-way ANOVA, $F = 18.54$; $df = 18$; $p < 0.001$), with up to 20 times more *E*-(β)-farnesene, and a level of 85 ng/g of fresh weight. The correlation between gene activity and metabolites was also higher than the βC lines (Pearsons's $r = 0,65$). The linearity of SPME fibre response within this concentration range was tested with calibration lines from pure standards, which were also used for quantification. It is worth noting that in βC lines the levels of *E*-(β)-farnesene were not statistically different from those of control plants (One-way ANOVA, $F = 0.66$; $df = 22$; $p = 0.68$), and that the same thing happened with βF lines and *E*-(β)-caryophyllene levels (One-way ANOVA, $F = 0.77$; $df = 21$; $p = 0.59$): in other words, a change in the abundance of one volatile did not affect the abundance of the other.

Plant headspace extraction with closed-loop stripping analysis

Although SPME allowed us to have fast confirmation of the plant phenotype, it also had the limitation of basing the sampling process only on a few young leaves from an entire plant. Indeed, terpenoid emission is known to vary significantly in the different organs of *Vitis vinifera* (Matarese at al. 2014) and in the field insects are likely to choose their host plant based on VOC emission from the whole body of the plant, rather than from just a few leaves. For this reason we decided to extract and characterise the plant headspace through closed-loop stripping analysis (CLSA, (Boland *et al.*, 1984; Abraham et al. 2014). This method also has the advantage of being non-destructive,

and is carried out at environment temperature, thus not altering plant physiology and more reliably reflecting the emission of VOCs in real ecological conditions. Sampling was done on plants of the same age and of a similar size (30-35 leaves) and was started at the same time every day to exclude the possible effects of the day/night cycle on VOC emission (Chalal et al. 2015; Giacomuzzi et al. 2016). Quantification of *E*-(β)-caryophyllene and *E*-(β)-farnesene (Fig.3A) confirmed the pattern observed with SPME quantification, although it showed greater variability between biological replicates, and in some lines (βC5, βC6, βF3) it showed that SPME sampling had underestimated VOC emission. Overall, our initial goal of obtaining grapevine plants with an *E*-(β)-caryophyllene / *E*-(β)-farnesene emission ratio divergent from the wild type was achieved (One-way ANOVA, F = 24.07; df = 45; p < 0.0001), with extracts ranging from 0.22:1 to 38:1, while the control plants were in the range of 4:1 (Fig. 3B).

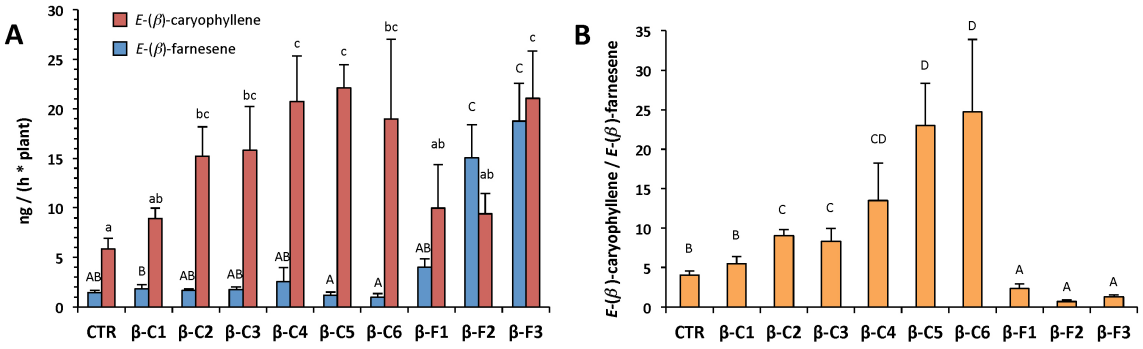

Figure 3. Volatile collection and analysis of the whole above-ground part of grapevine plants using CLSA-GC-MS. (A) Quantification of *E*-(*β*)-caryophyllene and *E*-(*β*)-farnesene emission from the different lines. (B) *E*-(*β*)-caryophyllene / *E*-(*β*)-farnesene mass ratio in the same lines. Letters on bars indicate different groups according to one-way ANOVA followed by Fisher's LSD post hoc test (P < 0.05) with the standard error of the mean (SEM) visible for each line.

Wind tunnel behavioural assays

All the extracts were sorted and grouped into 7 categories (Fig. 4A) according to their *E*-(β)-caryophyllene / *E*-(β)-farnesene ratio: six categories (A to F)

from transformed plants with increasingly different ratios (from 35:1 to 0.5:1) and one category (CTR) with extracts only from control plants (ratio = 4:1 on average). Categories A, B and C, with a higher ratio compared to the controls, were obtained from βC lines, while categories E and F, with a lower ratio compared to the controls, were obtained from βF lines. Category D extracts were obtained from βC lines whose kairomone emissions were not statistically different from the CTR category, and were thus used as a further control. It is worth noting that the ratio found on control plants of our variety (4:1) was different from the ratio (10:1) found in previous studies (Tasin et al. 2006), where Chardonnay plants were used, highlighting a dependency on variety. Extracts from all the groups were tested for attraction in wind tunnel assays (Fig. 4B) using adult *L. botrana* mated females 3 days after eclosion, reared on a semi-artificial diet.

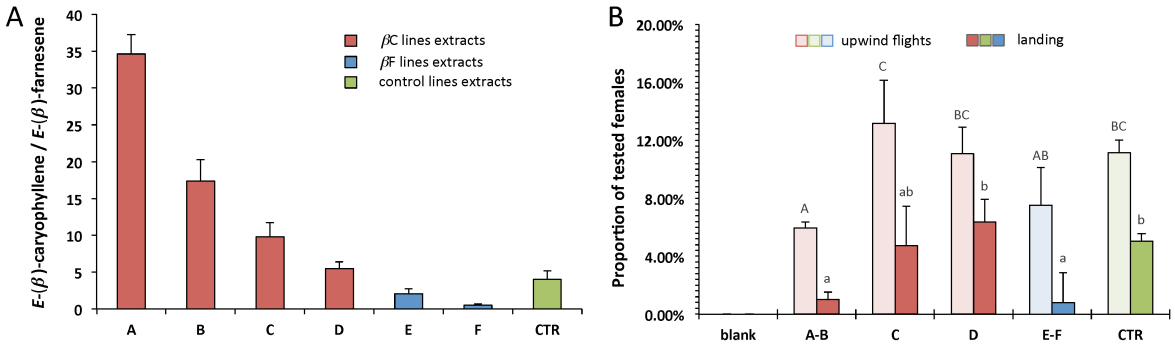

Figure 4. Wind tunnel behavioural assays. (A) Plant extracts used in the wind tunnel sorted by *E*-(*β*)-caryophyllene / *E*-(*β*)-farnesene ratio, with each group separated using Fisher's LSD post hoc test ($P < 0.05$), except for groups D and CTR, which are not statistically different. The standard deviation (SD) for each category is visible. (B) Attraction (upwind in light and landing in dark) elicited on 3-day-old mated *L. botrana* females. Blank: N = 60; A-B: N = 432; C: N = 207; D: N = 316; E-F: N = 207; CTR: N = 231. The standard error of the mean (SEM) is visible for each category and letters on bars indicate different groups according to one-way ANOVA followed by Fisher's LSD post hoc test ($P < 0.05$).

Responding insects took flight from the tubes on the downwind side of the wind tunnel and locked into the odour plume, starting to fly upwind. The maximum linear flying distance that an insect could cover before hitting the

protective cage in front of the sprayer was 180 cm: any flight between 50 cm and 170 cm from the odour source was defined as upwind flight, while arrival at any point within 10 cm of the sprayer was defined as landing (Fig. 5). The insect response rate was low, but in line with previous studies performed on this species when reared in semi-artificial conditions (Tasin et al. 2005), and it proved to be consistent over time within the same groups of extracts and throughout all the batches of insects tested.

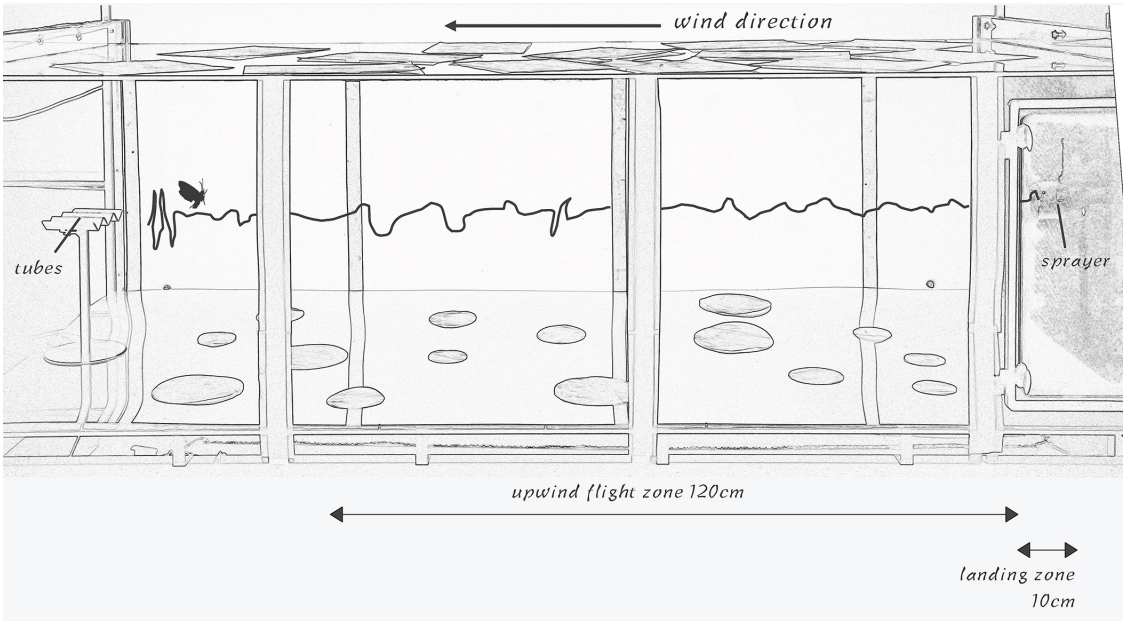

Figure 5. Sketch of the wind tunnel used in the behavioural experiments. Visual stimuli for insect orientation during flight were visible as paper disks on the floor of the tunnel and paper sheets on the roof.

Extracts from control plants elicited on average 11% of upwind flights and 5% of landings. Group D elicited the same attraction as compared to the CTR group, both in terms of upwind flights and landings, as could be expected, given that the VOC ratio was not statistically different. Group C contained extracts from plants which weakly overexpress *E*-(β)-caryophyllene, with an emission ratio close to 10:1 on average. This ratio is different from that of the "Brachetto Grappolo Lungo" variety wild type, but not from that of the "Chardonnay" variety wild type used in previous studies, and indeed all the extracts tested from group C elicited attraction very similar to group D and the CTR group (Fig. 4B). Groups A, B, E and F were the most divergent from the

CTR group, with all ratios higher than 17:1 or lower than 2:1. These groups all showed very similar responses, namely a modest decrease in the number of upwind flights (One-way ANOVA, F = 4.49; df = 15 ; p = 0.021) and a major decrease in the number of landings (One-way ANOVA, F = 4.02; df = 20 ; p = 0.019), with less than 1% of the total insects tested. Moreover, especially with groups of extracts A and F, we observed some differences in the way insects flew towards the sprayer: when leaving the tubes downwind they seemed to maintain casting behaviour longer, and they frequently lost the odour plume before giving up and resting on the walls of the tunnel.

To confirm that the different behaviour of the moths was due to the changing ratio between *E*-(β)-caryophyllene and *E*-(β)-farnesene, we created three synthetic blends based on the original three-component lure described in Tasin et al. 2006. The control (CTR) blend mimicked the ratio of control plants, which for our variety was 4:3.3:1 for *E*-(β)-caryophyllene, DMNT and *E*-(β)-farnesene respectively, while the βC blend had a ratio of 25:3.3:1 (similar to βC5 plants) and βF blend had a ratio of 0.3:3.3:1, similar to βF2 plants. The blends were sprayed into the wind tunnel at a rate of 35 ng/h of the main compound, and female attraction was scored in the same way used with plant extracts (Fig. 6A). Insect response percentages were very similar to those found with headspace collections, and both the βC and βF blends were less attractive than the CTR blend (upwind: $\chi^2 = 7.65$; $df = 2$; $p < 0.05$; landing: $\chi^2 = 6.76$; $df = 2$; $p < 0.05$). Finally, to confirm the difference in behaviour, we tested two representative plant lines, βC5 and βF2, in the wind tunnel, and compared their attractiveness to the CTR line (Fig. 6B). All the plants were of the same age, grown in the same conditions and had a similar number of leaves (about 80).

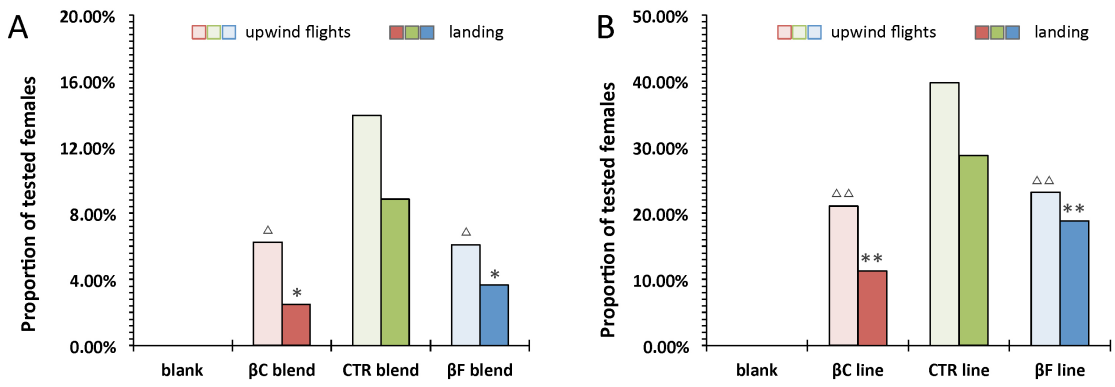

Figure 6. Wind tunnel behavioural assays. (A) Attraction (upwind in light and landing in dark) elicited on 3-day-old mated *L. botrana* females from synthetic three-component blends that mimic respectively the βC, CTR and βF profile. Blank: N = 60; βC blend: N = 80; CTR blend: N = 79; βF blend: N = 82. Symbols indicate statistical significance: $\Delta : \chi^2 = 7.65; df = 2; p < 0.05$ and $* : \chi^2 = 6.76; df = 2; p < 0.05$. (B) Attraction (upwind in light and landing in dark) elicited on 3-day-old mated *L. botrana* females from real plants. Blank: N = 39; βC line: N = 71; CTR blend: N = 73; βF line: N = 69. Symbols indicate statistical significance: $\Delta\Delta : \chi^2 = 13.92; df = 2; p < 0.001$ and $** : \chi^2 = 18.14; df = 2; p < 0.001$.

Insect response percentages here were much higher than in previous experiments, probably because the plants had a higher emitting surface compared to the sprayer, and their odour profile was not affected by any sampling steps. In any case, the trend observed remained the same, with lower attractiveness for plants with an impaired *E*-(β)-caryophyllene / *E*-(β)-farnesene ratio compared to the control, both in terms of upwind flights ($\chi^2 = 13.92$; $df = 2$; $p < 0.001$) and landing ($\chi^2 = 18.14$; $df = 2$; $p < 0.001$).

Discussion

Herbivorous insects use olfactory cues to locate their host plants in complex environments (Bruce et al. 2005). This selectivity can be achieved either by the perception of species-specific VOCs (Nottingham at al. 1991, Blight et al.

1995) or more frequently by the presence of a blend of common VOCs emitted in a specific ratio (Thöming and Knudsen 2014, Birkett at al. 2004). Approaches based on the use of synthetic blends in a wind tunnel showed that ratio modification of the components led to a decrease in attractiveness (Cha et al. 2011, Tasin et al. 2006), suggesting that VOC manipulation could be exploited to establish new pest control methods (Turlings and Ton 2006, Unsicker et al. 2009). Considering the costs of many VOCs due to difficulties in their synthesis or isolation from natural sources, and considering especially that they often have a short half-life in nature because of the tendency to form isomers and/or become oxidized (Blande et al. 2014), a genetic engineering approach seems most suitable for studying the effect of kairomone manipulation on insect behaviour (Birkett and Pickett 2014). In plants of agricultural interest, only a few cases of insect semiochemical biosynthesis have already been reported, curiously regarding the same VOCs we targeted in our study. In maize, it was shown that *E*-(β)-caryophyllene-emitting plants could attract insect-killing nematodes (Degenhardt et al. 2009), suffering less root damage in the field from *Diabrotica virgifera virgifera* compared to control lines. More recently, Bruce et al. (2015) reported the case of *E*-(β)-farnesene-emitting wheat plants, created with the scope of repelling aphids, for which *E*-(β)-farnesene is an alarm pheromone. In this case, plants were repellent under laboratory conditions for at least three species of cereal aphids, but insect parasitism was not reduced during field trials.

In this work, we report for the first time on the transformation of a woody plant species of agricultural interest with a modified kairomonal biosynthetic pathway. In particular, we produced *V. vinifera* plants keeping all the kairomones used by *L. botrana* for host-location, but with several degrees of ratio modification between two key components, namely *E*-(β)-caryophyllene and *E*-(β)-farnesene. Biosynthesis of the third key component DMNT was not a goal of our genetic modification, and its emission remained unaltered in the different lines (One-way ANOVA, $F = 1.35$; $df = 47$; $p = 0.24$). The data presented show a certain degree of plasticity in terms of insect choice, considering that mild but significant kairomone ratio variations were not sufficient to lose *L. botrana* attraction. The most probable reason for this

plasticity is the natural variation in VOC emission in different plants, even when the genetic background is the same (clones) and they are in the same conditions, as was the case here. Different genetic backgrounds, as takes place within a natural population and/or in the case of different varieties, is likely to enhance ratio differences. Indeed, previous works on “Chardonnay” (Tasin et al. 2006) found a typical average ratio of 10:1, which is the same as for “Brachetto Grappolo Lungo” transformed βC plants falling into category C, whose ratio was at least double that of wild types on average. However, when the kairomone ratio is very distant from the average (more than three times higher or less than half, as in this case) there is a clear decrease in attractiveness and increased difficulty in recognising the host plant, despite the presence of all the other VOCs in the headspace. Overall, we conclude that modification of the kairomone ratio is sufficient to interfere with the host-finding behaviour of *L. botrana*, and we believe that this could be the rationale for establishment of a new environmentally friendly pest control method to be applied in the field. In particular, in *V. vinifera*, apart from keeping the genetic background of elite varieties unaltered, the use of genetically engineered plants could also have the advantage of modifying the behaviour of other grapevine phytophagous insects (e.g. *Eupoecilia ambiguella*, *Scaphoideus titanus*), which may use the same VOCs as part of their kairomonal blend, although this remains to be investigated. For the same reason, care should be taken not to underestimate possible negative effects on beneficial insects, which help to contain pest populations in vineyards naturally.

Experimental procedures

Cloning of the *E*-(β)-farnesene synthase into plant overexpression vectors

A 1-month-old *Artemisia annua* plant was obtained from seeds (www.worldseedsupply.com) germinated in the greenhouse. Mature leaves were used to extract total RNA (Spectrum™ Plant Total RNA kit, Sigma) and 1.0 mg were retro-transcribed (SuperScript® III Reverse Transcriptase,

Invitrogen) with the gene-specific primer "aaBfs rev" (5' – TTAGACAA CCATAGGGTGAACG – 3'). cDNA was used as a template to amplify the whole coding sequence of the *E*-(*β*)-farnesene synthase (Aaβ-FS, GenBank: AY835398.1) with the forward primer "attB-β-FS for" (5' – GGGGACAAGTTT GTACAAAAAAGCAGGCTTAACAATGTCGACTCTTCCTATTTCTAG – 3') and the reverse primer "attB-β-FS rev" (5' – GGGGACCACTTTGTACAAGA AAGCTGGGTTTAGACAACCATAGGGTGAACG – 3'). PCR product was cloned directly into pDONR221 via BP-clonase reaction (Invitrogen), and transformed into *E. coli* DH5a chemically competent cells. Plasmid minipreps from single colony 5ml cultures were sequenced to check for their quality using the M13(-20) for (5' – GTAAAACGACGGCCAG – 3') and M13 rev (5' – CAGGAAA CAGCTATGAC – 3') primer pair. The coding sequenced was then moved into the gateway destination vectors pK7WG2D (Karimi *et al.* 2007) via LR reaction (Invitrogen). 1ml of the reaction was used to transform chemically competent *E. coli* TOP10 cells (Invitrogen), then plasmid minipreps from single colony 5ml cultures were sequenced to check for their quality using my35Sprom for the (5' – CCACTATCCTTCGCAAGACCC – 3') and my35Sterm rev (5' – GAAGTATTTTACAAATACAAATACATACTAAGG – 3') primer pair.

Grapevine Transformation

The vector pK7WG2D containing the Aaβ-FS coding sequence was used to transform embryogenic *calli* of *V. Vinifera* ("Brachetto Grappolo Lungo" variety) via *Agrobacterium tumefaciens,* as described in Dalla Costa *et al.*, (2014). Transgenic plants were propagated and maintained *in vitro* until acclimatisation in the greenhouse.

RNA extraction and RT-qPCR

RNA was extracted with the Spectrum^{TM} Plant Total RNA kit (Sigma), from young grapevine leaves (second and third internode below the apex) that had previously been acclimatised in the greenhouse following the manufacturer's

guidelines. RNA quality and quantity was checked on a spectrophotometer and 1% agarose electrophoresis before cDNA was retro-transcribed using SuperScript® III Reverse Transcriptase (Invitrogen) with Random Primers.
The primer pair "Aaβ-FS RT for" (5' – TGAGGGTGGAAGATGAAACAATA – 3') and "Aaβ-FS RT rev" (5' – CTTAGGGAAGAGTCACAAGAAGG – 3') had an efficiency of 94.3% and was used in RT-qPCR to determine transgene expression. qPCR was performed on an CFX96 thermocycler (Bio-Rad), using GAPDH and Actin (Reid *et al.*, 2006) as reference genes. Real-time PCR was carried out with the following cycle: 95°C 10'; 40 x (95°C 30" , 60°C 30"); The manufacturer's software (CFX Manager, Bio-Rad) was used to calculate primer pair efficiency (E) and the threshold cycle (Ct) mean and standard deviation for each sample. Analysis of relative quantification was performed using the workflow reported in Hellemans et al. (2007). For each primer pair used, Cts from reference lines were used as references to calculate DCt. The geometric mean between the E^DCt value from Actin and GAPDH was used to calculate a normalisation factor (NF), which was finally used to calculate the fold difference according to the formula: Fold Difference = E^ΔCt / NF.

Volatile analysis with SPME-GC-MS

Sesquiterpene collection was performed with a method adapted from Matarese *et al.* (2014). A CTC CombiPAL autosampler (CTC Analytics, Zwingen, Switzerland) with a single magnetic mixer (SMM Chromtech) and SPME fibre conditioning station was used to extract the volatiles from the sample vial headspace. A Trace GC Ultra gas chromatograph coupled to a Quantum XLS mass spectrometer (Thermo Scientific, Electron Corporation, Waltham, MA) was used: compounds were separated using a VF-Wax® (100% polyethylene glycol; 30 m×0.25 mm×0.25 µm, Agilent J&W Scientific Inc., Folsom, CA). The GC oven parameters were as follows: initial temperature was 40°C, maintained for 4 min, followed by an increase to 60°C at a rate of 2°C min^{-1}, the oven was then maintained at 60°C for 1 min, then increased at a rate of 5°C min^{-1} up to 190°C for 1 min and at a rate of 10°C

min^{-1} up to 230°C, maintained for 4 min; splitless time of 5 min and GC inlet temperature of 250 °C. Helium was used as carrier gas in constant flow mode at 1.2 mL min^{-1}. The total cycle time was 50 min. The MS detector was operated in scan mode (mass range 40–450 m/z) with a 0.2 sec scan time and the transfer line to the MS system was maintained at 250°C.

SPME extraction was carried out with a slight modification as compared to that described in Matarese et al, 2014. Half a gram of leaf powder was added to 0.3 g of NaCl and 3 mL of freshly prepared citrate-phosphate buffer (0.1 M Na_2HPO_4, 50mM citric acid, pH 5.0) and put into 20 mL glass headspace vials. Each sample was spiked with 50 µL of 2-octanol at 2.13 mg L^{-1} alcoholic solution as internal standard. Samples were kept at 60°C for 20 min and then extracted for 35 min at 60°C. The headspace was sampled using 2-cm DVB/CAR/PDMS 50/30 µm fiber from Supelco (Bellefonte, PA). The volatile and semi-volatile compounds were desorbed in the GC inlet at 250°C for 4 min in splitless mode and the fibre was reconditioned for 4 min at 270°C.

Headspace collection and analysis through *CLSA*

Plant headspace was collected using the closed-loop stripping analysis (CLSA) method (Boland *et al.*, 1984; Abraham et al, 2014). The above-ground part of the plant (approximately 30 leaves) was put into a closed plastic cooking bag (25 x 38 cm, Cuki, Italy) where an air pump (DC 12/16 FK, Aersistem, Milano) created a flow of activated-charcoal-cleaned air at 0.8 L/min. The air flow hit an adsorbent filter (CLSA-Filter, LowResistance 1.8 mg, Brechbühler AG, Switzerland) for 3.5 hours, before trapped compounds were eluted with 160 µL of dichloromethane. Before being used again, each filter was rinsed with 6 mL of dichloromethane and left to dry. At least three acclimatised plants were used as biological replicates for each line tested.

A HP6890 autosampler was used to perform liquid injection of the extracts. An Agilent 6890N gas chromatograph coupled to an Agilent 5973 mass spectrometer was used: compounds were separated using a J&W Scientific DB-Wax column (100% PEG; 30m × 0.25mm × 0.25µm, Agilent) and a 2.5m methyl-deactivated pre-column (Varian Inc., Lake Forest, CA, USA) with the

same internal diameter that was connected to the analytical column via a press-fit connector (BGB Analytik AG, Boeekten, Switzerland). The GC oven parameters were as follows: initial temperature was 40°C, maintained for 2 min, then increased at a rate of 6.9°C min^{-1} up to 160°C, and then 21.5°C min^{-1} to 250 °C for 3.60 min; injection volume was 1µl in splitless mode with a GC inlet temperature of 250 °C. Helium was used as carrier gas in constant flow mode at 1.6 mL min^{-1}. The total cycle time was 27.18 min. The MS detector was operated in scan mode (mass range 40–550 m/z) and the transfer line to the MS system was maintained at 250°C.

Data processing

For SPME-GC-MS data, processing was carried out with XCALIBUR™ 2.2 software provided by the vendor, while for CLSA-GC-MS data, processing was carried out with MSD ChemStation E.02.02.1431 (Agilent). Identification of the volatile compounds was made by injecting pure reference standards when available, or by comparing the retention index and mass spectra with those in the NIST 2011 database. Quantification was performed after calibration lines had been prepared with the same standards.

Insects

The *L. botrana* moths used in this research were reared on a semi-artificial diet in a growth chamber at 23.5°C and with 65% relative humidity. The photoperiod was 1:16:1:6 h respectively for dawn:day:sunset:night light conditions. Dawn and sunset conditions were simulated with a single lamp put on one side of the growth chamber and switched on for 1 hour by a timer before and after the 16h full light period. Larvae were kept in plastic boxes (25×18×5 cm), where they could feed without limits until they pupated. All the pupae were transferred into meshed plastic boxes (30×30×30 cm), where the adults emerged. Adults were transferred into mating chambers (12 cm diameter, 22 cm long) and provided with 10% sucrose solution to obtain eggs every day, and 3-day-old mated females were used each time for all

experiments. The insects never experienced any contact with plants or volatiles from the extracts before the experiments, and each female was only used once.

Wind Tunnel

The wind tunnel used with plant headspace extracts and synthetic blends had a flight section of 67×88×200 cm and was kept in a room at 23°C and with 55-65% relative humidity. The air flow through the tunnel was 25 cm/s, and inside the tunnel the light intensity was approximately 10 lux. An ultrasonic sprayer equipped with a conical nozzle (Sono-Tek corporation, Milton, NY) and connected to a model 102 syringe pump (CMA Microdialysis AB, Solna, Sweden) was used to introduce the volatiles into the tunnel. The tip of the nozzle was installed through a metal grid into the centre of the upwind end of the flight section, 30 cm above the floor, and the tip was covered with a glass cylinder (diam. 10 cm, length 8 cm) and a metal mesh.
Each headspace sample was concentrated under the fume hood to get rid of most of the dichloromethane, and diluted with pure ethanol to a volume of 1.800 mL, which corresponded to 3 hours headspace collection time when released at a rate of 10 ml / min in the wind tunnel. Groups of 5 mated females were placed in glass tubes (12.5 × 2.6 cm) capped with a nylon mesh and transferred into the wind tunnel room at least 3 h before the start of the experiment. The wind tunnel tests started 0.5 h before the start of the scotophase: 3 tubes with moths were placed in the centre of the downwind end of the tunnel, 30 cm above the floor and 160 cm from the sprayer. Each batch of females was allowed to respond for 20 min, and no more than 3 batches per day were used. The nozzle of the sprayer was cleaned with pure ethanol for 10 min before and after use, as well as between different extracts when sprayed the same day. The glass cylinders, glass tubes and metal mesh were heated to 300 C for 8 h before use, to prevent any odour contamination. The flight behaviour of the moths was observed during that time, and the numbers of landings as well as upwind flights were recorded. For each of the tested extracts, from 70 to 90 females were used, on at least

two different days, and the order of the stimuli was randomised. The percentage of females responding was transformed to $\log_{10}$ values and submitted to one-way analysis of variance (ANOVA), followed by Fisher's LSD test, with a significance level of 0.05 between groups for multiple comparisons.

Real plants were instead tested in a wind tunnel close to the containment greenhouse, which had a flight section of 63 × 90 × 200 cm and was kept in a room with the same conditions as described above. The plant was put behind a metal grid in the upwind end of the flight section, with the pot wrapped in aluminium foil. The preparation of the experiment was the same as described for the sprayed extracts. Each batch of females was allowed to respond for 10 min, and no more than 4 batches per day were used. The flight behaviour of the moths was observed during that time, and the numbers of landings as well as upwind flights were recorded. Again in this case, from 70 to 90 females were used for each of the tested plants, on at least three different days, and their order was randomised. The percentage of females responding in the different groups was tested for statistical significance using a chi-squared test.

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Chapter 6

Concluding remarks and future perspectives

Despite being the major insect pest of the fruit crop with the largest harvested surface worldwide, the management of *L. botrana* still relies mostly on classical pest-control strategies based on pesticides, and environmentally friendly techniques like Mating Disruption are still poorly applied (less than 4% of European vineyards).

In my thesis I used a genetic engineering approach to investigate the role of known grapevine kairomones in host-finding and egg-laying behavior, with the perspective to prove, at least theoretically, the feasibility of a new pest-control method based on the modification of their emission.

First I biochemically characterized the enzyme responsible for the synthesis of the majority of *E*-(β)-caryophyllene, which is the most abundant grapevine kairomone. After that, I performed its expression in two plant systems, *A. thaliana* and *V. vinifera*, to demonstrate that VOCs engineering is possible within these species which I need to study changes in *L. botrana* behavior.

The study of GM Arabidopsis plants showed that the emission of *E*-(β)-caryophyllene and *E*-(β)-farnesene, both singularly and contemporary, did not affect neither oviposition preference nor oviposition stimulation in the moth, suggesting that maybe these kairomones are involved only in long-distance interaction resulting in proper host plant choice, and not in close-range interactions. However, further experiments are needed, since it remains to be determined whether the addition of few other key VOCs would trigger oviposition, or if this role is mainly the result of the perception of less volatile compounds present on the surface of grape berries.

The attempt to express the grapevine gene VvCSLinNer in Arabidopsis was done with the purpose of adding the key compounds DMNT in the plants headspace: the coded enzyme indeed is an *in vitro* bi-functional linalool / nerolidol synthase, and nerolidol is known to be the precursor of DMNT *in vivo*. However, in the plants headspace after transformation I could detect just linalool, proving indirectly that the enzyme acts in plant as a mono-functional linalool synthase, and it is probably imported into the chloroplasts, where monoterpenes are synthetized.

V. vinifera GM plants instead confirmed the essential role of *E*-(β)-caryophyllene and *E*-(β)-farnesene in host selection. In fact, in this system, I managed to prove that the ratio between these two essential VOCs is necessary for host-plant choice, and its modification is enough to consistently reduce plant attractiveness. The results obtained with headspace extracts from GM plants were confirmed with synthetic blends and with the plants themselves in wind tunnel experiments. Based on this pieces of evidence, I suggest that modification of the *E*-(β)-caryophyllene / *E*-(β)-farnesene ratio may also interfere with host-finding behaviour of *L. botrana* in the field, making the vineyards less susceptible to herbivory, and thus creating the rationale for a new pest-control method. For the future, this possibility should be explored in field or semi-field conditions, either by using GM grapevine genotypes with modified *E*-(β)-caryophyllene or *E*-(β)-farnesene emission, or by using standard genotypes along other plant species that modify the global headspace of the field.

Appendices

5th European PhD Network in Inset Science, June 7-8, 2014, Orosei, Sardegna (Italy)

Control of the Grapevine Moth *Lobesia botrana* through the genetic engineering manipulation of the host plant's volatiles

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Abstract

The European grapevine moth *Lobesia botrana* is one of the key pests of grape. The caterpillar feeding activity leads to a direct damage on reproductive plant tissues (flower buds and berries) but also to an indirect damage by promoting secondary infections of microorganisms. Current control systems are based on the use of insecticides or on mating disruption: while the first is not environmentally friendly, the second is not particularly suitable for non-delimited areas, or areas where pest population is high.

Here we explore the possibility to develop a new control strategy of *L. botrana* by interfering with the female host-finding and egg-laying behaviors, which are mostly mediated by the host-plant-volatiles (kairomones).

Recent wind-tunnel studies have shown that a blend of 3 specific grapevine plant terpenoids, namely (*E*)-β-caryophyllene, (*E*)-β-farnesene and (*E*)-4,8-dimethyl-1,3,7-nonatriene, elicited attraction comparable to that of the complete plant odor profile. Moreover, the specific ratio among these compounds showed to be crucial, since the subtraction or the percentage variation of any of the three chemicals resulted into an almost complete inhibition of the attractive blend. The same chemicals gave also promising result when tested in field conditions, but their direct application is not a strategy economically feasible due to their extremely high cost. A promising

alternative approach may consist in modifying the releasing ratio of these compounds by modifying the metabolic pathways of the plant. To achieve this goal we are at present working on cv. Chardonnay, by creating new genetically stable lines. Preliminary data will be presented at the workshop.

Keywords: VOCs, L. botrana, genetic engineering, kairomones

Accompanying poster and short oral talk were presented by the PhD candidate on the 7th of June.

ICE15: Graduate Course in Insect Chemical Ecology, Ethology and Evolution, June 8-19, 2015, Alnarp, (Sweden)

Control of the Grapevine Moth *Lobesia botrana* through Genetic Manipulation of Plant VOCs

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Abstract

The European grapevine moth *Lobesia botrana* is one of the key pests of grape: current control systems are based either on the use of insecticides or on mating disruption, but while the first is not environmentally friendly, the second is not particularly suitable for small and isolated areas, or regions where pest population is high.

Here we explore the possibility to develop a new control strategy of *L. botrana* by interfering with the female host-finding and egg-laying behaviors, which are mostly mediated by the volatile organic compounds (VOCs) emitted by the host plant (kairomones).

Wind-tunnel studies have shown that a blend of 3 specific grapevine plant terpenoids, namely (*E*)-β-caryophyllene, (*E*)-β-farnesene and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), elicited attraction comparable to that of the complete plant odor profile. Moreover, the specific ratio among these compounds has been shown to be crucial, since the subtraction or the percentage variation of any of the three chemicals resulted into an almost complete inhibition of the blend attractiveness.

To prove the role of these VOCs as kairomones, we chose a genetic engineering approach: we produced grapevine (host) and *Arabidopsis* (non-host) plants with altered Terpene Synthases gene expression according to RT-PCR analysis. The same plants presented also changes in the quantity of the released terpenoids when analyzed with CLSA-GC-MS and SPME-GC-MS. Next step in the project will be the selection of few lines of plants to use in insect behavioral assays.

Keywords: VOCs, L. botrana, genetic engineering, kairomones

Accompanying poster and short oral talk were presented by the PhD candidate on the 9th of June.

Research article, 13th July 2016

TRPA5, an Ankyrin Subfamily Insect TRP Channel, is Expressed in Antennae of *Cydia pomonella* (Lepidoptera: Tortricidae) in Multiple Splice Variants

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Abstract

Transient receptor potential (TRP) channels are an ancient family of cation channels, working as metabotropic triggers, which respond to physical and chemical environmental cues. Perception of chemical signals mediate reproductive behaviors and is therefore an important target for sustainable management tactics against the codling moth *Cydia pomonella* L. (Lepidoptera: Tortricidae). However, olfactory behavior strongly depends on

diel periodicity and correlation of chemical with physical cues, like temperature, and physical cues thus essentially contribute to the generation of behavioral response. From an antennal transcriptome generated by next generation sequencing, we characterized five candidate TRPs in the codling moth. The coding DNA sequence of one of these was extended to full length, and phylogenetic investigation revealed it to be orthologous of the TRPA5 genes, reported in several insect genomes as members of the insect TRPA group with unknown function but closely related to the thermal sensor pyrexia. Reverse transcription PCR revealed the existence of five alternate splice forms of CpTRPA5. Identification of a novel TRPA and its splice forms in codling moth antennae open for investigation of their possible sensory roles and implications in behavioral responses related to olfaction.

Key words: transient receptor potential cation channel, TRPA subfamily, TRPA5, splice form, *Cydia pomonella*

My contribution to this paper consisted in the molecular cloning of the full-length coding sequence of TRPA5 gene from antennal cDNA. The paper was sent to the Journal of Insect Science the 17th of February 2017, and was accepted on July 13th.

The paper can be referred to as:

Cattaneo, A.M., Bengtsson, J.M., Montagné, N., Jacquin-Joly, E., Rota-Stabelli, O., Salvagnin, U., Bassoli, A., Witzgall, P., Anfora, G., 2016. TRPA5, an Ankyrin Subfamily Insect TRP Channel, is Expressed in Antennae of *Cydia pomonella* (Lepidoptera: Tortricidae) in Multiple Splice Variants. J. Insect Sci. 16, 83. doi:10.1093/jisesa/iew072

Research article, 24th January 2017

Candidate pheromone receptors of codling moth *Cydia pomonella* respond to pheromones and kairomones

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Abstract

Olfaction plays a dominant role in the mate-finding and host selection behaviours of the codling moth (*Cydia pomonella*), an important pest of apple,

pear and walnut orchards worldwide. Antennal transcriptome analysis revealed a number of abundantly expressed genes related to the moth olfactory system, including those encoding the olfactory receptors (ORs) CpomOR1, CpomOR3 and CpomOR6a, which belong to the pheromone receptor (PR) lineage, and the co-receptor (CpomOrco). Using heterologous expression, in both *Drosophila* olfactory sensory neurons and in human embryonic kidney cells, together with electrophysiological recordings and calcium imaging, we characterize the basic physiological and pharmacological properties of these receptors and demonstrate that they form functional ionotropic receptor channels. Both the homomeric CpomOrco and heteromeric CpomOrco + OR complexes can be activated by the common Orco agonists VUAA1 and VUAA3, as well as inhibited by the common Orco antagonists amiloride derivatives. CpomOR3 responds to the plant volatile compound pear ester ethyl-(E,Z)-2,4-decadienoate, while CpomOR6a responds to the strong pheromone antagonist codlemone acetate (E,E)-8,10-dodecadien-1-yl acetate. These findings represent important breakthroughs in the deorphanization of codling moth pheromone receptors, as well as more broadly into insect ecology and evolution and, consequently, for the development of sustainable pest control strategies based on manipulating chemosensory communication.

My contribution to this paper consisted in the molecular cloning of the full-length coding sequence of OR3 gene from antennal cDNA. The paper was sent to the Journal of Insect Science the 24th of August 2016, and was accepted on December 15th.

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Curriculum Vitae

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EDUCATION

2014 PhD Student at the Free University of Bolzano with the project "Control of the grapevine moth *Lobesia botrana* through the genetic manipulation of the host plant volatiles"

2012 Research Fellowship at Fondazione Edmund Mach on the project "Genetic engineering manipulation of terpene biosynthesis in *V. vinifera* for the control of the grapevine moth *Lobesia botrana"*

2011 Master degree (110/110 with honors) in Industrial Biotechnology at the University of Padova with the thesis "Initial functional characterization of a peach gene coding for an apoplastic peptide hormone involved in fruit ripening"

2009 Bachelor degree (110/110 with honors) in Biotechnology at the University of Padova with the short thesis "Homologous expression of recombinant forms of the [FeFe]-hydrogenase of the green unicellular alga *Chlamydomonas reinhardtii*"

2006 Scientific High School diploma (100/100) at Istituto Superiore Statale "A. Einstein – G. Cardano" in Piove di Sacco (PD)

FORMATION, CONGRESS AND SUMMER SCHOOLS

2016 6 months of research activities at the Norwegian Institute for Bioeconomy Research (NIBIO) in Ås (Norway)

2015 Attendance of the Graduate Course in Insect Chemical Ecology (ICE15) in Alnarp (Sweden), with the talk "Control of the grapevine moth *Lobesia botrana* through the genetic manipulation of the host plant VOCs*"*

2015 Tutorship in the master thesis "Use of the model plant Arabidopsis thaliana to study the role of specific terpenoids in plant-insect interaction", master student Carlotta Pirrello, graduate course "Plant and Microbial Biotechnologies", University of Pisa

2014 Attendance of the 5th European PhD Meeting in Insect Science in Orosei (Sardinia), with the poster "Control of the grapevine moth *Lobesia botrana* through the genetic manipulation of the host plant volatiles"

2013 Attendance of the international TERPNET2013 congress in Kolimvari (Crete), with the poster "Control of the grapevine moth *Lobesia botrana* through the genetic manipulation of the plant terpenoid profile"

PUBLICATIONS

2017	Salvagnin, U., Malnoy, M., Thöming, G., Tasin, M., Carlin, S., Martens, S., Vrhovsek, U., Angeli, S., Anfora, G. Exploitation of genetically modified *Vitis vinifera* plants with altered kairomone emission ratio for the control of the European Grapevine Moth *Lobesia botrana. Submitted.*
2016	Salvagnin, U., Carlin, S., Angeli, S., Vrhovsek, U., Anfora, G., Malnoy, M., Martens, S., 2016. Homologous and heterologous expression of grapevine E-(β)-caryophyllene synthase (VvGwECar2). Phytochemistry 131, 1–8. doi:10.1016/j.phytochem.2016.08.002

PERSONAL SKILLS AND COMPETENCES

Languages	Good knowledge and understanding of the English language (written and oral)
Computer skills	Use of both Windows and Apple operating systems. Use of electronic sheets, common text editing softwares, and slides creation. Good knowledge of image-editing softwares (Adobe Photoshop and similar). Use of statistics software (STATISTICA, Past), GC-MS data analysis (Agilent ChemStation) and RT-QPCR data analysis(CFX Manager, BioRad). Use of common bioinformatics tools (Blast, Pubmed, Snapgene, SerialCloner, Amplify, Primer3, Primique).
Photographic skills	Use of DSLR and film cameras. Macrophotography and Scientific photographty. Digital negative file development (Adobe Photoshop Lightroom, CaptureOne).
Scientific skills	Nucleic acids extraction, purification and quantification; agarose-gel electrophoresis; DNA cloning (restriction/ligation, topo-cloning, gateway cloning); PCR and RT-qPCR; *E. Coli* and *A. tumefaciens* cell cultures and transformation; Protein expression in *E. Coli*; recombinant protein purification via his-tag; SDS-PAGE; enzymatic assay on TPS and enzymes that produce volatile compounds; GUS enzymatic and hystochemical assay; SPME and CLSA-GC-MS; plant cell culture (*N. tabacum, A. thaliana, S. lycopersicum, V. vinifera*) and *A. tumefaciens* mediated plant transformation (transient and stable); *in vitro* and *ex vitro* plant acclimatization; greenhouse management; basic optical microscopy.

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Summary

The European grapevine moth *Lobesia botrana* (Lepidoptera: Tortricidae) is a known pest of economic significance that originated in Europe and has spread to portions of Africa, the Middle East as well as some wine-growing regions in North and South America.

Two main pest control strategies are currently in use to limit the infestation of *L. botrana* in the vineyards: chemical insecticides and pheromone-based strategies. The first is not considered environmentally friendly, raising issues like side effects on beneficial insects or the presence of residual chemicals for the consumers, for which they are gradually being phased out.

Pheromone-based strategies on the other hand are not based on the toxicity of the released compounds, but they take advantage of the behavioral effect that pheromones have targeting the olfactory communication between sexes in the moth. However, when a population density above the critical threshold is reached, it is necessary to apply pesticides, and reduce the population to a level at which pheromone-based mating disruption is effective again.

The intrinsic limitation of this strategy is that it can affect only males behavior: to have a stronger control it would be desirable to affect also the female host-finding and egg-laying behaviors. These in many insects are mediated by the release of volatile compounds from the host plant which act as kairomones.

Wind-tunnel studies have shown that a blend of 3 specific grapevine plant terpenoids, namely (*E*)-β-caryophyllene, (*E*)-β-farnesene and (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), elicited attraction comparable to that of the complete plant odor profile. Moreover, the specific ratio among these compounds has been shown to be crucial, since the subtraction or the percentage variation of any of the three chemicals resulted into an almost complete inhibition of the blend attractiveness.

In particular, *E*-(β)-caryophyllene is the most abundant sesquiterpene emitted by grapevine: in its genome there are five TPS genes coding for E-(β)-caryophyllene synthase but only one (VvGwECar2) accounts for most of the production of VOCs in green tissues and berries, playing thus the biggest role in *L. botrana* attraction. I decided to biochemically characterize the enzyme *in*

vitro and *in vivo* in homologous and heterologous systems. In all the systems the enzyme catalyzed the formation of three products, of which *E*-(β)-caryophyllene was the dominant one. I observed anyway differences in the secondary products when the gene was expressed in the heterologous plant system (*A. thaliana*) compared to the homologous one (*V. vinifera*). The Arabidopsis plants overexpressing this gene were created also with the intention to use them in insect behavioral assays, to determine if the volatile had an effect on a close-range plant interaction like oviposition. For the same purpose, I created Arabidopsis plants overexpressing also a *E*-(β)-farnesene synthase from *Artemisia annua*, plus plants derived by the cross with the *E*-(β)-caryophyllene emitting ones. All these plants were used in dual-choice oviposition assays, to determine if the *in vivo* emission of these VOCs, singularly or in couple, was enough to determine an oviposition preference or an oviposition stimulation in *Lobesia botrana* females. Overall, it seems that the two compounds, even when presented combined, are not sufficient to stimulate oviposition or to determine a substrate preference, at least in the quantities and ratio provided by Arabidopsis plants. It remains to be determined if a higher dose could reach the purpose, or if the insects need the full information provided by the presence of all the other host VOCs at a close range to choose the substrate where to lay eggs.

Using the same approach, Arabidopsis plants were modified also with the insertion of the grapevine gene VvCSLinNer, that codes for a TPS enzyme that *in vitro* was characterized as a bi-functional linalool/nerolidol synthase. Since nerolidol is the precursor of DMNT, one of the three key kairomones for *L. botrana*, the initial goal was to overexpress the gene and to obtain DMNT-emitting plants to be used in behavioural studies. However, after plants transformation, I could obtain plants only emitting linalool in their headspace, proving indirectly that, contrary to my expectations, the coded enzyme is imported into the plant chloroplasts. This result was contemporary confirmed by a paper where the authors definitively prove through confocal microscopy the localization of the enzyme in the plastids of *Vitis vinifera* cells. The obtained lines thus failed to produce DMNT, but their linalool content was

quantified, and they could be used in the future in behavioural assays with other insects for which this compound is an important semiochemical.

In the last part, I used a genetic engineering approach to prove the role of the two VOCs *E*-(β)-caryophyllene and *E*-(β)-farnesene in the long-distance attraction of *L. botrana* by grapevine plants. Considering the initial observation about the ratio between essential VOCs being necessary the attraction to the host plant, I generated stable grapevine transgenic lines with altered *E*-(β)-caryophyllene and *E*-(β)-farnesene emission compared to control plants. In other words, these plants had a modified kairomone ratio, and I used them to check if this change could impact on plant attractiveness. When I tested headspace collections from control and modified plants in the wind tunnel, I found that those from transformed plants were less attractive than those from control plants. This result was confirmed by testing synthetic blends, imitating the ratio found on natural and transformed plants, and finally by testing the plants themselves. Based on this evidence, I suggest that modification of the kairomone ratio may also interfere with host-finding behaviour of the pest in the field, making the vineyards less susceptible to *L. botrana* herbivory, and creating the rationale for a new method in pest management.

Zusammenfassung

Der Europäische oder Bekreuzte Traubenwickler (*Lobesia botrana*, Lepidoptera; Tortricidae) ist einer der bekanntesten und bedeutesten Schädlinge im Weinbau mit grosser wirtschaftlicher Bedeutung. Das Insekt hat seinen Ursprung in Europa, hat sich aber mittlerweile auch auf Teile Afrikas, den Nahen Osten sowie einige Weinregionen in Nord- und Südamerika ausgebreitet.

Bei der Bekämpfung kommen momentan im wesentlichen zwei Strategien zum Einsatz - Insektizide und Pheromone. Beide Anwendungen haben zum Ziel den Befall von *L. botrana* in den Weinbergen zu begrenzen, wobei Insektizide als nicht umweltfreundlich betrachtet werden. Mögliche Nebenwirkungen, die zu Problemen mit Nützlingen führen können, und das kritische Bewusstsein der Verbaucher gegenüber dem Vorhandensein von Restchemikalien im Produkt führen momentan zu einem schrittweise Auslaufen entsprechender Produkte.

Pheromonbasierte Strategien beruhen nicht auf der Toxizität der freigesetzten Verbindungen (Terpenoide), sondern sie nutzen deren Wirkung auf das Verhalten der Insekten, dass die Pheromone mittels der olfaktorischen Kommunikation zwischen den Geschlechtern des Wicklers besitzen. Wenn jedoch die Populationsdichte der Insekten oberhalb der kritischen Schwelle angewachsen ist, ist es notwendig Insektizide anzuwenden und die Population auf ein Niveau zu senken, bei dem die pheromonbasierte Paarungsbeeinflussung (Verwirrmethode) wieder wirksam ist.

Die wesentliche Einschränkung dieser Strategie liegt darin, dass sie nur das Verhalten der männlichen Insekten beeinflussen kann; um jedoch eine stärkere Kontrolle zu erreichen, wäre es wünschenswert, auch das weibliche Wirtsfindungs- und Eiablageverhalten direkt zu beeinflussen. Dieses artspezifische Verhaltensmuster wird bei vielen Insekten durch die Freisetzung flüchtiger Verbindungen aus der Wirtspflanze, die als sogenannte Kairomone wirken, beeinflusst.

Untersuchungen im Windtunnel haben gezeigt, dass eine Mischung von drei spezifischen Terpenoiden der Weinrebe (*Vitis vinifera*), (E)-β-Caryophyllen,

(E)-β-Farnesen und (E)-4,8-Dimethyl-1,3,7-nonatrien (DMNT), eine vergleichbare Wirkung wie die des gesamten Profils an flüchtigen Inhaltsstoffen besitzen. Darüber hinaus hat sich das spezifische Verhältnis dieser Verbindungen als entscheidend erwiesen, da das Fehlen einer oder eine Veränderung im Verhältnis der drei Substanzen zu einem nahezu vollständigen Ausbleiben der anlockenden Wirkung führt.

Das (E)-β-Caryophyllen ist das am häufigsten von Weinreben gebildete und abgegebene Sesquiterpenoid. Im Genom konnten fünf putative Terpensynthase Gene (TPS), die für eine (E)-β-Caryophyllensynthase kodieren, identifiziert werden. Nur eines der kodierten Proteine (VvGwECar2) ist aber für den Großteil der Synthese in Blättern und Beeren der Pflanze verantwortlich und spielt damit eine besondere Rolle in der Kommunikation zwischen Insekt und Pflanze. Aus diesem Grund wurde beschlossen, dieses Protein *in vitro* und *in vivo* sowohl in homologen als auch in heterologen Systemen biochemisch zu charakterisieren. In allen Systemen katalysierte das aktive Enzym die Synthese von drei Produkten, von denen das E-(β)-Caryophyllen deutlich überwiegte. Dem gegenüber konnten wesentliche Unterschiede bei der Bildung der Nebenprodukte beobachtet werden, wenn das Gen im heterologen Pflanzensystem (*Arabidopsis thaliana*) oder im Vergleich dazu im homologen System (*V. vinifera*) exprimiert wurde. Arabidopsis Pflanzen, die dieses Gen überexprimieren, wurden mit der Absicht entwickelt, sie in Verhaltenstest mit den Insekten zu verwenden, und um zu bestimmen, ob die neuen flüchtigen Verbindungen einen Einfluss oder eine Wirkung auf die Pflanzen-Insekten Interaktion sowie die Eiablage haben. Für den gleichen Zweck wurden Arabidopsis Pflanzen, die eine (E)-β-Farnesensynthase aus *Artemisia annua* überexpremieren generiert. Pflanzen, die in der Lage sind beide Verbindungen zu bilden, wurden durch ensprechende Kreuzung mit den (E)-β-Caryophyllen Pflanzen erhalten. Um zu bestimmen, ob die *in-vivo* Emission dieser Terpenoide, einzeln oder beide, ausreicht, um eine Präferenz oder Stimulation der Eiablage durch die *L. botrana* Weibchen zu beeinflussen, wurden sogenannte “Dual-Choice” Tests durchgeführt. Insgesamt scheint es, dass die beiden Verbindungen jedoch nicht ausreichen, um die Eiablage zu stimulieren oder eine Wirtspräferenz zu

beeinflussen; zumindest nicht in den Mengen und dem Verhältnis, die von den Arabidopsis Pflanzen gebildet und abgegeben wurden. Es bleibt jedoch zu klären, ob mit einer höheren Dosis dieses Ziel erreicht werden kann oder ob die Insekten die vollständigen Informationen benötigen bzw. auf das Vorhandensein aller anderen Terpenoide in einem engen Bereich angewiesen sind, um den Wirt für die Eiablage zu waehlen.

Mit dem gleichen obenbeschriebenen Ansatz wurden Arabidopsis Pflanzen durch Einfügen des Wein Gens VvCSLinNer modifiziert, das für ein TPS Protein kodiert, das *in vitro* als bi-funktionelle Linalool/Nerolidolsynthase charakterisiert wurde. Da Nerolidol der Vorläufer von DMNT, einem der drei Schlüsselkairomone für *L. botrana*, ist, war das ursprüngliche Ziel, das Gen überzuexprimieren und DMNT-emittierende Pflanzen zu erhalten, die dann in Verhaltensstudien verwendet werden sollten. Nach der entsprechenden Transformation wurden jedoch keine Pflanzen erhalten, die nur Linalool emittieren, was indirekt zeigt, dass das kodierte Protein im Gegensatz zu den Erwartungen in die Chloroplasten eingeführt wurde. Dieses Ergebnis konnte in einer neueren Veröffentlichung bestätigt werden, in der die Autoren durch konfokale Mikroskopie die Lokalisation des Proteins in den Plastiden der *V. vinifera* Zellen eindeutig nachweisen konnten. Die erhaltenen Linien waren daher nicht in der Lage DMNT zu produzieren, aber ihr Linaloolgehalt wurde quantifiziert, und sie könnten in nachfolgenden Arbeiten in Verhaltenstests mit anderen Insekten verwendet werden, für die diese Verbindung eine wichtige semiochemische Bedeutung hat.

Im letzten Teil dieser Arbeit wurde ein gentechnischer Ansatz verwendet, um die Rolle der beiden Terpenoide, (E)-β-Caryophyllen und (E)-β-Farnesen, in der Erkennung der Wirtspflanze durch *L. botrana* über eine grössere Entfernung zu zeigen. Unter Berücksichtigung der früheren Beobachtung zur Bedeutung des Verhältnisses der essentiellen Terpenoide auf die Attraktivität der Wirtspflanze, wurden stabile transgene Weinpflanzen generiert, die im Vergleich zu Kontrollpflanzen ein verändertes (E)-β-Caryophyllen und (E)-β-Farnesen Emissionenprofil besitzen. Mit anderen Worten, diese Pflanzen besitzen ein modifiziertes Kairomon-Verhältnis, und wurden in weiteren Untersuchungen genutzt, um zu überprüfen, ob diese Veränderung die

Attraktivität der Pflanzen beeinflusst. Die Untersuchung von Headspace-Sammlungen aus Wildtyp und modifizierten Pflanzen im Windkanal, ergab, dass jene die von transformierten Pflanzen gesammelt wurden weniger attraktiv waren als von Kontrollpflanzen. Dieses Ergebnis wurde durch das Testen synthetischer Mischungen, durch Nachahmung des Verhältnisses, das auf natürlichen und transformierten Pflanzen gefunden wurde, und durch Testen der Pflanzen selbst bestätigt. Auf der Grundlage dieser Ergebnisse kann postuliert warden, dass eine Modifizierung des Kairomon-Verhältnisses die Wirtfindung und das Verhalten des Schädlings auf dem Feld in der Art und Weise beeinflussen kann, das in den Weinbergen die Anfälligkeit und der Befall mit *L. botrana* als Pflanzenschädling reduziert werden kann und damit ein wichtiger und neuer Ausgangspunkt für die Entwicklung neuer Methoden in der biologischen Schädlingsbekämpfung sein kann.

Riassunto

La tignoletta della vite *Lobesia botrana* (Lepidoptera: Tortricidae) è un noto insetto nocivo di rilevanza economica che, originario dell'Europa, si è diffuso in parti dell'Africa, del Medio Oriente e in regioni vitivinicole nel Nord e Sud America.

Due principali strategie di controllo sono usate correntemente per limitare l'infestazione di *L. botrana* nei vigneti: insetticidi tradizionali e lotta basata sui feromoni. I primi non sono considerati ambientalmente sostenibili, sollevando preoccupazioni su effetti collaterali verso insetti benefici o sulla presenza di residui per il consumatore, motive per cui il loro uso sta venendo sempre più dismesso.

La lotta basata sull'impiego di feromoni d'altro canto non si basa sulla tossicità dei composti rilasciati, ma si avvantaggia dell'effetto sul comportamento che i feromoni hanno bersagliando la comunicazione olfattiva tra i diversi sessi della falena. Tuttavia quando la densità di popolazione supera una soglia critica, è necessario applicare insetticidi e ridurre il numero di insetti a un livello in cui la confusione sessuale è nuovamente efficace.

Il limite intrinseco di questa strategia è che ha influenza solo sul comportamento del maschio: per avere un controllo più forte sarebbe desiderabile influire anche sui comportamenti di ricerca della pianta ospite e ovideposizione della femmina. Questi comportamenti in molti insetti sono mediati dal rilascio di composti volatili dalla pianta ospite che agiscono quindi da cairomoni.

Studi in tunnel del vento hanno mostrato che una miscela di 3 specifici terpenoidi della vite, nello specifico (*E*)-β-cariofillene, (*E*)-β-farnesene e (*E*)-4,8-dimetill-1,3,7-nonatriene (DMNT), elicitano una attrazione comparabile a quella che si ha con il completo profilo odoroso della pianta. Inoltre, il rapporto specifico tra questi composti si è rivelato cruciale, dato che sia la sottrazione sia il cambiamento di questo rapporto ha condotto a una quasi totale inibizione dell'attrattività della miscela.

In particolare, l'*E*-(β)-cariofillene è il sesquiterpene più abbondante emesso dalla vite: nel suo genoma esistono cinque geni per TPS che codificano per

E-(β)-cariofillene sintasi, ma solo uno (VvGwECar2) è responsabile della maggior parte della produzione di volatile nei tessuti verdi e negli acini, giocando così il ruolo più importante nell'attrazione di *L. botrana*. Ho deciso di caratterizzare biochimicamente l'enzima *in vitro* e *in vivo,* in sistema sia omologo che eterologo. In tutti i sistemi l'enzima ha catalizzato la formazione di tre prodotti, di cui l' *E*-(β)-cariofillene era il principale. Ho notato tuttavia differenze nei prodotti secondari quando il gene era espresso in pianta nel sistema eterologo (*A. thaliana*) rispetto a quello omologo (*V. vinifera*). Le piante di Arabidopsis sovraesprimenti questo gene sono state create anche con l'intenzione di usarle in saggi comportamentali, per determinare se il voltile avesse effetti in interazioni a corta distanza come l'ovideposizione. Allo stesso scopo ho creato piante di Arabidopsis sovraesprimenti anche l'*E*-(β)-farnesene sintasi di *Artemisia annua*, e anche altre piante derivate dall'incrocio con quelle emettenti *E*-(β)-cariofillene. Tutte queste piante sono state usate in saggi di ovideposizione a doppia scelta per determinare se l'emissione in vivo di questi VOCs, singolarmente o in coppia, fosse sufficiente a innescare una preferenza o una stimolazione dell'ovideposizione da parte di femmine di *Lobesia botrana*. Complessivamente, sembra che i due composti, anche quando presenti in combinazione, non bastino a stimolare la deposizione delle uova, né a far preferire un substrato piuttosto che un altro, per lo meno non nelle quantità e nei rapporti forniti dalle piante di Arabidopsis. Resta da determinare se una dose emessa più alta possa avere questo effetto, o se l'insetto abbia bisogno di tutta l'informazione data dalla presenza degli altri composti di vite a breve distanza per sceglier il substrato di ovideposizione.

Usando lo stesso approccio, piante di Arabidopsis sono state modificate anche con l'inserzione del gene di vite VvCSLinNer, che codifica per una TPS che *in vitro* è stata caratterizzata come una bifunzionale linalolo/nerolidolo sintasi. Dato che il nerolidolo è il precursore del DMNT, uno dei tre cairomoni chiave per *L. botrana*, lo scopo inziale era quello di sovraesprimere il gene per ottenere delle piante emettenti DMNT da usare in saggi comportamentali. Tuttavia, dopo la trasformazione, ho potuto ottenere solo piante emettenti linalolo nel loro spazio di testa, provando indirettamente che, contrariamente

alle mie aspettative, l'enzima codificato è importato nei cloroplasti. Questo è stato confermato contemporaneamente da una articolo in cui gli autori dimostrano definitivamente, tramite microscopia confocale, la localizzazione dell'enzima nei plastidi di cellule di *Vitis vinifera*. Le linee di piante ottenute non producono quindi DMNT, ma il loro contenuto in linalolo è stato quantificato, e potrebbero esser usate in futuro in saggi comportamentali su altri insetti per cui il linalolo è un importante semiochimico. Nell'ultima parte, ho usato un approccio di ingegneria genetica per provare il ruolo dei due volatili *E*-(β)-cariofillene e *E*-(β)-farnesene nell'attrazione a lunga distanza di *L. botrana* da parte della vite. Considerando l'osservazione iniziale circa il fatto che il rapporto tra i volatili è essenziale per l'attrazione verso la pianta, ho generato line di vite transgeniche stabilmente trasformate con emissione di *E*-(β)-cariofillene e *E*-(β)-farnesene alterata rispetto a piante di cotrollo. In altre parole, queste piante hanno un rapporto tra cairomoni alterato, e sono state usate per verificare se questo cambiamento impatta sull'attrattività della pianta stessa. Quando ho testato estratti degli spazi di testa da piante trasformate e modificate nel tunnel del vento, ho trovato che quelli da piante modificate erano meno attrattivi di quelli delle piante di controllo. Questo risultato è stato confermato testando delle miscele di composti sintetici che imitano i rapporti trovati nelle piante modificate e non, e alla fine anche testando le piante stesse. Basandomi su queste prove, propongo che la modifica del rapporto tra cairomoni possa interferire con il comportamento di ricerca della pianta ospite anche in campo, rendendo i vigneti meno suscettibili all'attacco di *L. botrana*, e creando la base per un nuovo metodo di controllo.