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Developing a Sustainable Insect Pest Management System by Manipulating Plant Volatile Emissions

Scott R. Hughes
The University of Western Ontario

Supervisor
Hannoufa, Abdelali
The University of Western Ontario Co-Supervisor
Bernards, Mark
The University of Western Ontario

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Abstract

Herbivorous insects are a constant problem for farmers and growers globally, who rely on insecticides to control pest populations. Alternative measures are required due to insects developing resistance to chemical insecticides, along with increasing pressure from consumers. Integrated pest management (IPM) programs are a popular alternative. IPM uses a multifaceted approach to reduce pest populations below economic injury thresholds. Many plant-insect interactions are modulated by plant volatile organic compound (VOC) emissions. Manipulating this communication channel by producing transgenic plants with modified VOC emissions could provide another tool for use in IPM programs. I developed tomato *Solanum lycopersicum* (L.) plants expressing one of two chosen transgenes involved in VOC biosynthesis, which resulted in some changes to VOC emissions. When given the choice between transgenic and non-transgenic plants, greenhouse whitefly *Trialeurodes vaporariorum* (Westwood) preferred non-transgenic plants for oviposition. This suggests that VOC manipulation should be explored further as a potential tool for pest management.

Key Words: volatile organic compound (VOC),GC-MS, tomato, *Solanum lycopersicum*, *Arabidopsis thaliana*, CYP82G1, carotenoid cleavage dioxygenase 4, greenhouse whitefly, *Trialeurodes vaporariorum*, integrated pest management (IPM)

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List of Abbreviations

%M	Percent Match
AAFC	Agriculture and Agri-Food Canada
ABA	Abscisic Acid
ACN	Acetonitrile
AMDIS	Automated Mass Spectral Deconvolution and Identification System
ANOVA	Analysis of Variance
At	*Arabidopsis thaliana*
BHT	2,6-Di-tert-butyl-4-methylphenol
Bt	*Bacillus thuringiensis*
CCD	Carotenoid Cleavage Dioxygenase
cDNA	Complementary DNA
CI	Chemical Ionization
DAD	Diode Array Detector
DCM	Dichloromethane
DMAPP	Dimethylallyl diphosphate
DMNT	4,8-Dimethylnona-1,3,7-triene
EI	Electron Ionization
EV	Empty Vector
FAOSTAT	Food and Agriculture Organization Corporate Statistical Database

GC-MS	Gas Chromatography-Mass Spectrometery
GES	Geranyllinalool Synthase
GLV	Green Leaf Volatile
Ha	Hectares
HIPV	Herbivore Induced Plant Volatile
HPLC	High Performance Liquid Chromatography
IPP	Isopentyl diphosphate
IPM	Integrated Pest Management
JA	Jasmonic acid
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LOX	Lipoxygenase
MeJA	Methyl jasmonate
MeOH	Methanol
MEP	Methylerythritol phosphate
MeSA	Methyl salicylate
MS	Murashige and Skoog
MSD	Mass Selective Detector
MTBE	Methyl-tert-butyl-ether
MVA	Mevalonic acid
m/z	Mass to Charge Ratio

NCED	9-Cis-Epoxycarotenoid Dioxygenase
NES	Nerolidol Synthase
NIST	National Institute of Standards and Technology
OMAFRA	Ontario Ministry of Agriculture, Food, and Rural Affairs
PARADISe	PARAFAC2 Based Deconvolution and Identification System
PARAFAC2	Parallel Factor Analysis 2
P450	Cytochrome P450 Monooxygenase
PC	Principle Component
PCA	Principle Component Analysis
PCR	Polymerase Chain Reaction
PDMS/DVB	Polydimethylsiloxane/Divinylbenzene
PPM	Parts Per Million
PSI	Pounds per Square Inch
PSY	Phytoene Synthase
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RNAi	RNA Interference
ROS	Reactive Oxygen Species
RT	Retention Time
RT-PCR	Reverse Transcription Polymerase Chain Reaction
SA	Salicylic acid

SEM	Standard Error of the Mean
Sl	*Solanum lycopersicum*
SL	Strigolactone
SPME	Solid-Phase Microextraction
TMTT	4,8,12,-Trimethyltrideca-1,3,7,11-tetraene
VOC	Volatile Organic Compound

Chapter 1. General Introduction

1.1 Plant-Insect Interactions and the Role of Plant Volatiles

Plants are static organisms that rely on their ability to adapt to environmental changes on the fly in order to survive and proliferate. Thus they rely heavily on being able to perceive subtle changes in their surroundings and respond appropriately. From an abiotic standpoint, these changes can include sudden heatwaves or floods, as well as slower seasonal changes or droughts. On the biotic side, plants must deal with competition from other plants, herbivory from animals of all sizes, and pathogens.

As an added layer of difficulty, plants must be able to distinguish between these negative environmental interactions and the positive ones they may rely on for nutrients and defense. These include interactions with the soil microbiome as well as above ground interactions with pollinators and ever-present pest insects. These interactions are of increasing interest to plant scientists as well as the general public as humanity searches for new ways to feed an ever-increasing population without causing further harm to our planet through the overuse of chemical fertilizers and pesticides.

Increasingly, plant volatile organic compound (VOC) emissions have been implicated in modulating the complex interactions between plants and their surroundings. Below ground, VOC emissions can promote the growth of mycorrhizal fungi (Bécard and Piché, 1989). As well as inhibit the growth of various pathogenic fungi and bacteria (Almenar et al., 2007). Above ground, VOC emissions help guide pollinators to unpollinated flowers (Rodriguez-Saona et al., 2011). VOC emissions from aerial plant parts are also utilized by herbivores to locate optimal plants for feeding and oviposition. One of many examples of this can be seen in the interactions between cotton (*Gossypium hirsutum* (L.)) and the cotton aphid (*Aphis gossypii* (Glover)). Given the choice between infested and uninfested cotton, the cotton aphid prefers to avoid competition with other herbivores and chooses the uninfested plant (Hegde et al., 2011).

This preference is driven largely by VOC emissions, as VOCs from uninfested plants are perceived as more attractive than VOCs from infested plants, which are repellent (Hegde et al., 2011). The defense response undertaken by plants faced with herbivory generally includes an

alteration in the VOCs emitted by the plant. These herbivore-induced plant volatiles (HIPVs) can influence herbivore choices directly, as in the case of the cotton aphid. Perhaps more importantly, HIPVs can act as a form of indirect defense by attracting parasitoids and predators such as the generalist aphid parasitoid *Aphidius ervi* (Haliday) and the specialist mite predator *Phytoseiulus persimilis* (Athias-Henriot) (Bruce et al., 2008; De Boer et al., 2004).

1.2 Classes of Plant Volatiles

Across all plant species, over 1700 VOCs have been identified, the majority of which fit into three main compound classes: fatty acid derivatives, benzenoids/phenylpropanoids, and terpenoids (Dudareva et al., 2013; Knudsen et al., 2006; Mumm and Dicke, 2010). This wide array of compounds is derived from only a few precursors, modified in various ways depending on their location within the plant as well as within the cell. Fatty acid-derived volatiles are produced via the lipoxygenase (LOX) pathway, which relies on both linolenic and linoleic acids as a starting point (Dudareva et al., 2013; Feussner and Wasternack, 2002). One branch of the LOX pathway produces a wide range of short chain volatiles referred to as green leaf volatiles (GLV) (Feussner and Wasternack, 2002). These are a group of six and nine-carbon alcohols and aldehydes that, contrary to the name, may be synthesized in both reproductive and vegetative tissues (Homatidou et al., 1992; Knudsen et al., 1993). Some GLVs are emitted rapidly in response to tissue damage, and many reportedly inhibit microbial growth (Scala et al., 2013). GLVs may also be released as a signal to other plants to prime their own defenses for an impending attack (Farag and Paré, 2002; Scala et al., 2013) . This early warning function is also carried out by the major product of the second branch of the LOX pathway, methyl jasmonate (MeJA). MeJA is a volatile derivative of jasmonic acid (JA), a signalling molecule that accumulates within wounded plant tissue and begins a signaling cascade triggering inducible plant defenses (Lortzing and Steppuhn, 2016). Upon wounding, MeJA is released into the atmosphere and can diffuse to nearby undamaged plants as a warning of impending damage (Karban et al., 2013; Pierik et al., 2014).

Another VOC that acts as an early warning signal is methyl salicylate (MeSA), a volatile derivative of salicylic acid (SA). MeSA was the first VOC proven to be involved in signalling between healthy and diseased plants, with MeSA production from tobacco (*Nicotiana tabacum*

(L.)) plants infected with tobacco mosaic virus influencing the expression of defense genes in neighboring uninfected plants (Shulaev et al., 1997). Unlike MeJA, MeSA is synthesized via the benzenoid pathway, which, along with the phenylpropanoid pathway, generates a wide array of volatile and non-volatile secondary plant metabolites from phenylalanine (Dudareva et al., 2013; Widhalm and Dudareva, 2015). MeSA is the most widely studied VOC within the benzenoid/phenylpropanoid class, with SA having been implicated as a key regulator of many systemic acquired resistance genes (Vlot et al., 2009). Many other benzenoid/phenylpropanoid volatiles are major constituents of flower volatiles and may be involved in the attraction of pollinating insects. A good example of this is the diurnally regulated emission of methyl benzoate from snapdragon (*Antirrhinum majus* (L.)) flowers, which rely on bees for pollination and only emit methyl benzoate during the day, when bees are most active (Kolosova et al., 2001).

The third and largest group of plant VOCs are terpenoids, a group comprised of over 20,000 compounds derived from isopentenyl diphosphate (IPP) and dimethyallyl diphosphate (DMAPP) (Bernards, 2010; Dudareva et al., 2013). IPP and DMAPP are five-carbon isomers that act as the building blocks for all terpenoids (Bernards, 2010; Dudareva et al., 2013). Synthesis of IPP and DMAPP occurs via the plastid-localized methylerythritol phosphate (MEP) pathway, as well as additional, mainly cytosolic synthesis of IPP resulting from the mevalonic acid (MVA) pathway (Hsieh et al., 2008; Pulido et al., 2012; Simkin et al., 2011). Volatile terpenoids are limited to the hemiterpenes and monoterpenes which are synthesized within the plastid, as well as sesquiterpenes synthesized in the cytosol (Mumm and Dicke, 2010). Like the previous two VOC classes, terpenoid VOCs are commonly produced by many plants under non-stress conditions, but are constantly changing in response to biotic and abiotic stresses depending on the requirements of the plant including pollination and defense. For example, the sesquiterpenes β-caryophyllene, α-copaene, and germacrene D are the main VOC components emitted from tropical figs (*Ficus* spp. (L)) to attract pollenating wasps (Grison-Pigé et al., 2002).

Conversely, β-caryophyllene is also emitted as a defensive measure by maize (*Zea mays* (L.)) roots in response to feeding by Western corn rootworm (*Diabrotica virgifera* (LeConte)), which is attractive to entomopathogenic nematodes (*Heterorhabditis megidis* (Poinar, Jackson & Klein)) (Rasmann et al., 2005). A similar effect is found above ground with the emission of the

two homoterpenes 4,8-dimethylnona-1,3,7-triene (DMNT) and 4,8,12,-trimethyltrideca-1,3,7,11-tetraene (TMTT) by maize plants facing herbivory (Turlings et al., 1998). In addition to maize, these two irregular terpenes have been detected after herbivory in a number of species, and have long been reported to be involved in attracting predators and parasitoids to plants under attack from herbivores (De Boer et al., 2004; Hoballah et al., 2002; Kappers et al., 2005).

Volatile terpenoid compounds can also be produced in more indirect ways, such as from the cleavage of larger, non-volatile carotenoids. Carotenoids are a group of 40-carbon tetraterpenoid pigment compounds synthesized throughout the plant kingdom (Hannoufa and Hossain, 2012). While whole carotenoids are essential for sustained photosynthesis in plants, acting to protect the plant photosystem from photooxidative damage, they also provide colouration to plant tissues (Howitt and Pogson, 2006). In addition, carotenoid cleavage products, or apocarotenoids, influence a number of other plant functions. These include the plant hormones strigolactone (SL), which is involved in regulating shoot branching and mycorrhizal communication, as well as abscisic acid (ABA), which plays a large role in responding to a wide range of abiotic stresses (Hou et al., 2016; Schwartz et al., 2004; Seo and Koshiba, 2002) .

Volatile apocarotenoids also play a significant role in the interactions between plants and animals. Along with a few other VOCs, the apocarotenoids β-cyclocitral, geranylacetone, 6-methyl-5-hepten-2-one, and β-ionone were identified as key components in the tomato fruit flavour profile (Vogel et al., 2010). β-ionone has also been characterized as a HIPV, with emission resulting in the repellence of herbivorous insects, such as the crucifer flea beetle (*Phyllotreta cruciferae* (Goeze)) (Gruber et al., 2009; Wei et al., 2011). Pure β-ionone also repelled two-spotted spider mites (*Tetranychus urticae* (Koch)) and silverleaf whiteflies (*Bemisia tabaci* (Gennadius)) (Caceres et al., 2016).

1.3 Integrated Pest Management and the Emerging Role of Plant Volatiles

Traditionally, the management of pest insects in commercial cropping systems has focused on the use of chemical insecticides due to their ease of use, widespread availability and efficacy (Pimentel et al., 1993). Many of the defensive traits found in wild ancestors of today's modern agricultural crops have been lost over generations of domestication and breeding (Chen et al., 2015). As breeding efforts focused on more obviously beneficial traits such as aesthetically

pleasing crops with higher yield and nutritional value, traits improving resistance to insects and pathogens were not prioritized (Chen et al., 2015). Herbivorous insects have thus become increasingly problematic, as their populations rapidly expand by taking advantage of modern monoculture cropping systems. The loss of defensive capability and trend towards industrial agriculture was the driving force behind the dependence on chemical insecticides as the main mechanism of insect pest population control. As a result of heavy and prolonged insecticide exposure, many pest populations have developed some level of resistance to insecticides.

A classic example of pesticide abuse giving rise to high resistance levels is described by Dittrich et al. (1990), where the use of DDT in the 1970s and pyrethroids in the 1980s to control primary lepidopteran pests of cotton in Sudan led to the development of highly resistant silverleaf whitefly populations. Originally considered a secondary cotton pest, the silverleaf whitefly has overtaken the originally targeted lepidopterans to become a highly important pest in its own right (Dittrich et al., 1990; Oliveira et al., 2001). In addition to increased incidences of resistance, continual exposure of pests to low, non-lethal doses of insecticides may act to increase the rate of reproduction of pest insects through dose-dependent hormesis (Cutler, 2013; James and Price, 2002). The consistent use of chemical pesticides can also have serious unintended off target effects on herbivore predators and parasitoids, as well as other higher animals such as birds, amphibians and mammals (James and Price, 2002; Pimentel et al., 1993; Tanabe, 2002).

Given their important role in directly repelling herbivores and attracting predators and parasitoids, plant VOCs are of increasing interest in integrated pest management (IPM) programs. The goal of all IPM programs is to maintain pest populations below an economic threshold level using a variety of control methods simultaneously (Kogan, 1988). A major focus of IPM programs is the use of control measures that are both environmentally and economically friendly (Kogan, 1988, 1998). This normally includes some combination of biological control agents such as the natural predators and parasitoids of crop pests as well as improved cultural practices and biopesticide application, with chemical pesticides kept in reserve as a last resort (Chandler et al., 2011; Kogan, 1998). Advances in biotechnology have also allowed for the integration of transgenic crops with improved pest resistance into IPM programs, mainly through the introduction of crop plants expressing endotoxin genes from the soil microbe *Bacillus thuringiensis* (Bt) (Romeis et al., 2008). By utilizing transgenics, plant defense traits that have

previously been lost over generations of breeding could be rapidly reintroduced from wild populations, while defense traits from other species could potentially be added (Ahmad et al., 2012; Kos et al., 2009).

The implementation of Bt crops has benefited growers who focus on IPM as a first line of defense. The systemic toxins control for many lepidopteran and coleopteran pests, reducing the amount of pesticide sprays required and decreasing the negative impacts on populations of biological control insects (Romeis et al., 2008; Shelton et al., 2002). While Bt crops represent the vast majority of pest resistant transgenic crops on the market, there are a large number of other traits with the potential to improve pest control. This includes direct resistance mechanisms such as RNA interference (RNAi) and indirect resistance mechanisms such as VOC manipulation (Kos et al., 2013; Price and Gatehouse, 2008). By altering the VOC emissions from crop plants, herbivores, their predators, and their parasitoids can be influenced to achieve an overall reduction in herbivory. An excellent case study for manipulating pest behaviour with plant VOCs is described by Khan et al. (2016). This review describes a push-pull system developed in order to control lepidopteran pests on smallholder farms in sub-Saharan Africa by intercropping repellant silverleaf desmodium (*Desmodium uncinatum* (Jacq.)) with the main crop and surrounding the entire field with a border crop of attractive napier grass (*Pennisetum pupureum* (Schumach.)) (Khan et al., 2016). Users of this system have reported reduced populations of lepidopteran pests accompanied by an increase in crop yields (Khan et al., 2008). While not yet commercialized, the potential to utilize transgenic plants with altered VOC profiles to influence plant-insect interactions has shown promise in controlled greenhouse experiments (Degenhardt et al., 2003; Kos et al., 2013; Wei et al., 2011).

1.4 Project Rationale and Objectives

The tomato (*Solanum lycopersicum* (L.)) is a globally important food crop, with worldwide production reaching 177 million tonnes grown across 4.7 million hectares (ha) in 2016 (FAOSTAT, 2016). In Canada, tomatoes are cultivated on over 7000 ha with total exports valued at $372.5 million (AAFC, 2016). As of 2014 nearly 700 ha of greenhouses were devoted to tomato cultivation, with Ontario accounting for 66.3% of greenhouse tomato production across the country (AAFC, 2014). While greenhouse tomato production is steadily growing

across the country, there are many biotic and abiotic stress factors that hold back yields each year.

One of the major biotic stress factors hindering tomato crop production in Ontario greenhouses is the greenhouse whitefly (*Trialeurodes vaporariorum* (Westwood) (Hemiptera: Aleyrodidae)) (OMAFRA, 2014). Greenhouse whiteflies are small (1-2 mm) generalist phloem feeding hemipterans that have developed into a major primary pest for a wide array of greenhouse and field grown crops, including tomatoes (OMAFRA, 2014). In addition to draining plant resources directly through phloem feeding, whiteflies can transmit numerous plant viruses and the honeydew resulting from their feeding can promote the growth of molds that may further damage the crop (OMAFRA, 2014). While the damage caused by one whitefly can be nearly imperceptible, a single female can lay up to 300 eggs in a lifetime (OMAFRA, 2014). As such, whitefly populations can rapidly reach high densities with the potential to inflict major crop damage and economic losses.

The rapid regeneration rate of whitefly populations coupled with past irresponsible use of broad spectrum insecticides has resulted in the development of whitefly populations with high insecticide resistance levels (OMAFRA, 2014). This has led to the development of a multifaceted IPM approach to control whitefly populations in Ontario greenhouses that utilizes a combination of physical controls such as attractive yellow sticky traps, fine mesh air filters, and quarantine of infested plants alongside biological controls including parasitic wasps (*Encarsia formosa* (Gahan), *Eretmocerus eremicus* (Rose & Zolnerowich), and *Eretmocerus mundus* (Mercet)), ladybeetles (*Delphastus catalinae* (LeConte)), and mirid bugs (*Dicyphus Hesperus* (Knight)).

The objectives of this thesis were to generate transgenic tomatoes with altered VOC emissions and investigate the potential for their use in a push-pull based pest control program. I developed tomatoes expressing one of two genes directly involved in modulating the plant's VOC profile. The first gene of interest chosen for my study was carotenoid cleavage dioxygenase *4* (*CCD4*). *CCD4* is one of four *CCD* genes within the nine membered 9-cis-epoxycarotenoid dioxygenase (*NCED*) gene family in the model plant *Arabidopsis thaliana* (Auldridge et al., 2006). Originally, CCD4 was found to be responsible for pigment variations in the petals of mums

(*Chrysanthemum morifolium* (Ramat.)) (Ohmiya et al., 2006). More recently, expression *in vitro* showed CCD4 had the potential to form the apocarotenoid volatile β-ionone (Huang et al., 2009). Previously, members of our lab showed that when the closely related *CCD1* gene was overexpessed in *Arabidopsis thaliana,* the increased levels of β-ionone emitted from flowering plants reduced herbivory by crucifer flea beetles (Wei et al., 2011). Further *in planta* studies have shown that expression of *AtCCD4* in rice (*Oryza sativa* L.) could lead to heightened emission of β-ionone and β-cyclocitral (Song et al., 2016).

The second gene of interest chosen for my study was the cytochrome P450 monooxygenase gene *CYP82G1*. CYP82G1 is responsible for the synthesis of TMTT in *A. thaliana* (Lee et al., 2010). TMTT is synthesized *de novo* and emitted by *A. thaliana* leaves in response to herbivory (Lee et al., 2010). In addition, AtCYP82G1 can also convert (E)-nerolidol to DMNT *in vitro* (Lee Et al., 2010). Both DMNT and TMTT have been described as volatile signals of herbivory that are able to be sensed and acted upon by members of higher trophic levels (De Boer et al., 2004; Kappers et al., 2005; Turlings et al., 1998). Additionally, DMNT has been identified as a potential repellent of maize stalk borers (*Busseola fusca* (Full)) (Khan et al., 2000). Given the potential influence of their products on pest insects, CCD4 and CYP82G1 were chosen for further study in tomato.

To test if CCD4 or CYP82G1 could provide an altered volatile profile in tomato, *CCD4* and *CYP82G1* were cloned from *A. thaliana* and expressed in the model tomato cultivar MicroTom. For both *AtCYP82G1* and *AtCCD4* lines, expression levels were evaluated using reverse transcription followed by quantitative PCR (RT-qPCR). Volatile profiles of intact leaves and flowers were collected *in vivo* using an untargeted, rapid solid-phase microextraction (SPME) technique, followed by gas chromatography-mass spectrometry (GC-MS) analysis. In addition, volatile emissions from ground leaf tissue of *AtCCD4* expression lines was evaluated in a similar fashion, along with analysis of the major leaf carotenoids using high performance liquid chromatography (HPLC). Finally, choice oviposition preference bioassays were conducted to evaluate greenhouse whitefly preference between *AtCCD4* lines and empty vector (EV) controls. The experiments within shed light on the feasibility and challenges associated with working with transgenics to manipulate plant volatiles and offer some insight for future attempts to influence plant-insect interactions through VOCs.

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Chapter 2. Evaluation of Volatile Organic Compound Emissions from Tomato Plants Expressing *AtCYP82G1*

2.1 Introduction

Terpenoids are a diverse class of plant-specific metabolites comprised of over 20,000 members, many of which are involved in modulating plant interaction with the surrounding environment (Bernards, 2010; Dudareva et al., 2013). These interactions are of increasing importance within the plant science community, as researchers look for new and innovative ways to improve crop performance in order to feed a growing human population. Traditionally, research examining plant interactions with other organisms has focused on pest insects, pathogenic bacteria, and viruses, but more recently the focus has shifted to encompass interactions with beneficial microbes and insects. This includes interactions with pollinators as well as herbivore predators and parasitoids. While many terpenoids have been implicated in the interactions that plants have with other organisms, the two homoterpenes 4,8-dimethylnona-1,3,7-triene (DMNT) and 4,8,12,-trimethyltrideca-1,3,7,11-tetraene (TMTT) have been given special attention since their identification as important components of herbivore-induced plant volatile (HIPV) profiles (Dicke et al., 1990).

Initial experiments investigating the volatile organic compound (VOC) emissions of lima bean (*Phaseolus lunatus* (L.)) plants infested with spider mites (*Tetranychus urticae* (Koch)) identified DMNT and TMTT as major components of the HIPV blend (Dicke et al., 1990). Work by Dicke et al. (1990) showed that the predatory mite *Phytoseiulus persimilis* (Athias-Henriot), a now common biological control agent for spidermites, was attracted to DMNT. Around the same time, Turlings et al. (1990) showed that *Cotesia marginiventris* (Cresson), a parasitoid of beet armyworm (*Spodopter exigua* (Hübner)) larvae, relied on a blend of HIPVs emitted by corn plants upon *S. exigua* larvae feeding that included both DMNT and TMTT. These two studies paved the way for further research into the intricacies of how plants cope with herbivory and how their VOC emissions influence their interactions with higher trophic levels.

Since then, further details of the regulation and synthesis of DMNT and TMTT have been uncovered in addition to more information pertaining to their roles in plant-insect interactions. The conserved production of DMNT and TMTT from nerolidol and geranyllinalool,

respectively, was demonstrated by utilizing isotope labeling and tracking techniques (Boland et al., 1992; Boland and Gäbler, 1989). These homoterpenes along with their precursors have been detected in the volatile blends of many agriculturally important crops, including numerous *Brassica* spp., tomato, rice, cotton, and cucumber (Boland et al., 1992; Boland and Gäbler, 1989; Tholl et al., 2011). In *Arabidopsis thaliana,* the cleavage reaction producing TMTT from geranyllinalool is carried out by a cytochrome P450 enzyme, CYP82G1, which can also cleave nerolidol to DMNT *in vitro* (Lee et al., 2010). Closely related P450 enzymes have been described that carry out the formation of TMTT and DMNT in cotton, but alternative pathways to these compounds also exist as monocots lack CYP82 family of P450 enzymes but still emit DMNT and TMTT (Liu et al., 2017; Tholl et al., 2011). In corn, synthesis is carried out by CYP92C5 and CYP92C6, two distinct enzymes that produce DMNT and TMTT, respectively (Richter et al., 2016). Indeed, the utilization of different pathways leading to the production of these two compounds is evident even just within *A. thaliana*, where DMNT production in the roots is carried out by a different enzyme that utilizes an entirely different precursor (Sohrabi et al., 2015). Upon infection with root rot *Pythium irregular*e, the triterpene arabidiol is cleaved by a CYP705A1 enzyme to produce DMNT, which acts as a partial inhibitor of *P. irregulare* germination *in vitro* (Sohrabi et al., 2015).

While DMNT was noted as an attractive compound to *P. persimilis,* TMTT appeared to have no direct effect (Dicke et al., 1990). Upon further study, it was shown that TMTT acts synergistically with other lima bean HIPVs to steer *P. persimilis* preference towards spidermite infested plants (De Boer et al., 2004). A similar synergistic effect was found to occur with another generalist biological control mite *Neoseiulus californicus* (Mcgregor), where transgenic *Lotus japonicus* (L.) plants emitting heightened levels of TMTT were more attractive than wild type controls (Brillada et al., 2013). The idea of TMTT acting in a synergistic manner to heighten the response of parasitoids is also likely, as TMTT is a noted member of the HIPV blend emitted by soybean (*Glycine max* (L.) Merr.) that attracted the stinkbug egg parasitoid *Telenomus podisi* (Ashmead) (Moraes et al., 2009).

In addition to attracting predators and parasitoids, DMNT and TMTT may have a direct influence on herbivores as both attractants and repellents. The pollen beetle *Meligethes aeneus* (Fabricius) is a common *Brassica* pest that is attracted by DMNT (Bartlet et al., 2004). DMNT is

also an important component of the volatile blend emitted by the fruit of hawthorn (*Crataegus* spp. (Gand.)) that are attractive to apple maggot (*Rhagoletis pomonella* (Walsh)) flies (Nojima et al., 2003). Similarily, grapes (*Vitis vinifera* (L.)) emit DMNT, a noted attractant of mated female grapevine moths (*Lobesia botrana* (Denis and Schiffermüller)) in search of optimal fruit for oviposition (Tasin et al., 2006). Conversely, a blend of natural cashew (*Anacardium occidentale* (L.)) containing DMNT and TMTT repelled Asian citrus psyllids (*Diaphorina citri* (Kuwayama)), which are a common vector of the economically damaging citrus greening disease (Fancelli et al., 2018). TMTT is also a component of maize VOC emissions that repelled bird cherry-oat aphids (*Rhopalosiphum padi* (L.)) and along with DMNT is utilized by boll weevils (*Anthonomus grandis* (Boh)) to discriminate between cotton plants at the vegetative and reproductive stages (Magalhaes et al., 2016; Schröder et al., 2015).

Given their wide range of potential influences on herbivores, predators and parsitoids, DMNT and TMTT have become volatiles of interest for potential use in IPM programs. Using advances in molecular breeding technologies, plants with altered volatile profiles could be rapidly generated and used to augment IPM programs, especially in the form of a push-pull system. Push-pull is an IPM strategy where pest damage is reduced by pushing herbivores away from the main crop with repellent intercrops while at the same time pulling them towards attractive border crops (Khan et al., 2016; Meats et al., 2012). While previously employed successfully in an outdoor farm setting, push-pull technology is also of interest in a greenhouse setting (Du et al., 2016; Li et al., 2014; Pickett and Khan, 2016). In this chapter, transgenic tomato (*Solanum lycopersicum* cv. MicroTom) plants expressing *AtCYP82G1* were generated. Expression analysis was carried out using reverse transcription followed by quantitative PCR (RT-qPCR), and the *in vivo* volatile profiles of *AtCYP82G1* expressing plants and empty vector controls were collected, analyzed and compared using rapid solid phase microextraction (SPME) and gas chromatography-mass spectrometry (GC-MS). The advantages, disadvantages and potential pitfalls of using transgenics as a means of VOC manipulation were also discussed.

2.2 Materials and Methods

2.2.1 Plant Material

Solanum lycopersicum (tomato) cv. MicroTom seeds were sterilized for 20 minutes in 100% commercial bleach using a three dimensional rotating mixer. The bleach solution was then pipetted off in a sterile flow hood and seeds were washed three times with 1 mL autoclaved milli-Q water and plated on Germination media (Appendix A). Seeds were stratified in the dark at 4°C for 3 days then transferred to a growth cabinet set to 25°C, with a light intensity of 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 16 hrs followed by 8 hrs of darkness. Approximately two weeks after germination, seedlings were transplanted to plastic plug trays containing Pro-mix MX soil (Pro-Mix, Rivière-du-Loup, Québec) in a growth chamber and covered with a plastic dome. The growth chamber was set to 22°C and 70% relative humidity, with a light intensity level of 130-150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ over the same photoperiod as the aforementioned growth cabinet. Seedlings were monitored for a week with water being added as needed, then subsequently transplanted to 15 cm plastic pots in the same growth chamber. Potted plants were watered every other day, with bi-weekly supplementation of 20-20-20 all-purpose fertilizer (Plant Products, Leamington, ON).

2.2.2 Cloning of *CYP82G1* and Tomato Leaf Transformation

To clone *A. thaliana CYP82G1* (AT3G25180), genomic DNA was extracted from 50 mg of rosette leaves using a DNeasy Plant Mini Kit (QIAGEN,Toronto, Ontario). *AtCYP82G1* was PCR amplified with gene-specific primers (AtCYP82G1-F and AtCYP82G1-R, Table 2.1) designed to permit gateway cloning using Phusion® High-Fidelity DNA Polymerase (New England Biolabs, Whitby, Ontario). An aliquot of PCR product was run on a 1% agarose gel in to confirm the presence of a single PCR product of the correct size. Once confirmed, the remaining PCR product was purified with a GeneJET PCR purification kit (ThermoFisher Scientific, Burlington, Ontario). The purified PCR product was subsequently cloned into the entry vector pENTR™/D-TOPO® (ThermoFisher Scientific) and transferred by heat shock into chemically competent *Escherichia coli* One Shot™ TOP10 (ThermoFisher Scientific). Kanamycin (50 mg/L) was added to all LB media used for subsequent transformed *E. coli* growth as both the entry vector and the expression vector pMDC-32 contain kanamycin resistance genes (Curtis and Grossniklaus, 2003). The plasmid was isolated from an overnight

culture of transformed *E. coli* with a QIAprep Spin Miniprep Kit (QIAGEN) and sequenced to confirm successful cloning. Once the correct sequence was obtained, *AtCYP82G1* was sub-cloned into the expression vector pMDC-32 using a Gateway™ LR Clonase™ II Enzyme Mix (ThermoFisher Scientific) and again transformed into *E. coli.* Colonies were screened for transformation with pMDC-32 containing a single copy of *AtCYP82G1* by extracting plasmid DNA for digestion with BamHI restriction enzyme (New England Biolabs). Banding patterns differ between colonies containing the entry vector and pMDC-32, allowing for differentiation between colonies transformed with either plasmid. The plasmids that showed the proper banding pattern were then sent for sequencing to confirm *AtCYP82G1* insertion. Finally, the *AtCYP82G1* expression vector was transformed into the *Agrobacterium tumefaciens* strain EHA105 by electroporation.

Tomato transformation was carried out using a modified protocol provided by Cruz-Mendivil et al. (2011). A schematic of the transgene insertion is depicted in figure 2.1. Ingredients for the media used in each step of the tissue culture process are detailed in Appendix A. *A. tumefaciens* EHA105 containing the pMDC-32 expression vector alone (empty vector, EV) or with *AtCYP82G1* were grown in LB media containing 50 mg/L kanamycin and 25 mg/L rifampicin at 28°C with shaking for 48 hours. Four hours prior to infection, the cells were centrifuged at 1700g for 30 min and resuspended in infection media to an OD_{600} of 0.3, then allowed to grow for approximately 3 hrs to an OD_{600} of 0.5-0.6. Leaves of four- week-old tomato plants were cut into pieces approximately $1cm^2$ and placed adaxial side down on preculture media for 24 hrs. Explants were then transferred to *A. tumefaciens* culture in infection media for a minimum of 20 min. Explants were removed from the suspension, blotted dry and transferred to co-culture media, adaxial side down. Co-culture media and explants were incubated in the dark at 25°C for five days. After co-culture incubation, explants were washed in sterile milli-Q water containing 300 mg/L timentin, blotted dry and transferred to shoot induction media I (SIM-I), abaxial side down, and further incubated at 25°C under lights kept at 80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ on a 16:8 light:dark schedule. Explants were incubated on SIM-I for seven days before transfer to SIM-II, and another seven days before transfer to SIM-III. After transfer to SIM-III, explants were monitored closely for calli and eventual shoot production, as well as contamination, and calli were moved to fresh SIM-III media approximately every 2-3 weeks until shoot production. Once shoot formation occurred, shooting calli were transferred to shoot elongation media. Once shoots

elongated to approximately 2 cm in height, shoots were excised and placed on root induction media until a root system established. Finally, putatively transgenic plants were transferred from sterile root induction media to 15 cm pots and PCR screened for the transgene (At3g25180Fq and At3g25180Rq, Table 2.1) Plants were kept under a plastic dome and monitored closely for two weeks to allow for proper soil acclimation. Plants were then grown until fruit maturation, after which seeds were collected from putative transgenic plants and sown on germination media supplemented with 40 mg/L hygromycin followed by transplanting to soil for further analysis.

Primer Name	Primer Sequence	Purpose	Amplicon	GenBank Accession
AtCYP82G1-F AtCYP82G1-R	CACCATGACTTTTCTCTTTAGTACTCTCCAGT TCACAAGAAGCTCCCAATAATTATCGAG	Gateway Cloning	1871	NM_113423
35S-F3 (pMDC) NOS-R2 (pMDC)	CAATCCCACTATCCTTCGCAAGACCC ATAATCATCGCAAGACCGGCAAC	PCR confirmations, sequencing	Variable	-
M13 F (pentr) M13 R (pentr)	GTAAAACGACGGCCAG CAGGAAACAGCTATGAC	PCR confirmations, sequencing	Variable	-
AtCYP82G1Mid-F AtCYP82G1Mid-R	GTTATTCCATGGTTGGGATGGTTGG GCTTGTAGGTACTTGAGGTTTTGTATATCGG	PCR confirmations, sequencing	577	NM_113423
AtCYP82G1sq-R1 AtCYP82G1sq-F1	GGTGTCCGAATAGAGGCAAAGCTC CTGCTACTCAACAATCCAGCTGCTTTAG	PCR confirmations, sequencing	1052	NM_113423
At3g25180Fq At3g25180Rq	CAATGCAAGTCTGACTCTGGC CTTCTGAAGAACGAATGTGACC	RT-qPCR	117	NM_113423
Sl-ExpSq-Fq Sl-ExpSq-Rq	GCTAAGAACGCTGGACCTAATG TGGGTGTGCCTTTCTGAATG	RT-qPCR Reference Gene	183	XM_004242916
Sl-CAC-Fq Sl-CAC-Rq	CCTCCGTTGTGATGTAACTGG ATTGGTGGAAAGTAACATCATCG	RT-qPCR Reference Gene	173	NM_001324017
SlACTINF163 SlACTINR347	CATGCCATTCTTCGTTTGGA GAGCTGCTCCTGGCAGTTTC	RT-qPCR Reference Gene	184	NM_001321306

Table 2.1. Primers used for cloning, sequencing, and expression analysis of plants expressing *AtCYP82G1*. Primers with variable amplicons are primers designed for the plasmid backbone listed in brackets that would amplify any insert.

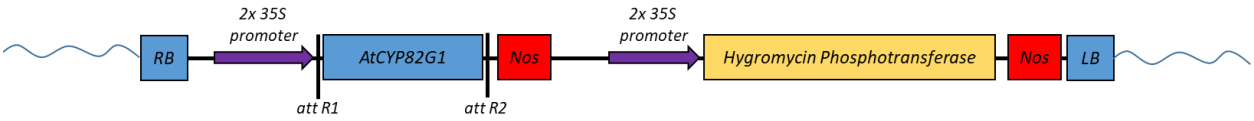

Figure 2.1. Schematic of transgene insertion cassette for *AtCYP82G1* expression. The pMDC-32 vector contains *attR1* and *attR2* sites to allow for gateway cloning of *AtCYP82G1* between a 2x35S promoter and *Nos* terminator (Curtis and Grossniklaus, 2003). The expression of the selectable *hygromycin phosphotransferase* gene is controlled in a similar fashion. Upon infection with *Agrobacterium tumefaciens*, the entire region between the flanking border sequences (*RB*, *LB*) is randomly inserted into the plant genome.

2.2.3 *CYP82G1* Expression Analysis

In order to screen for *AtCYP82G1* expression levels, RT-qPCR experiments were conducted on the transgenic lines. Leaf tissue from a young, expanding leaf near the shoot apex of T0 plants was used for expression profiling, with the expanding leaf nearest the stem apex collected for RNA extraction. Leaves were collected and snap frozen in liquid nitrogen before storage at -80°C. RNA was extracted using the TRIzol™ Plus RNA Purification Kit (ThermoFisher Scientific) supplemented with the Plant RNA Isolation Aid (ThermoFisher Scientific). Isolated RNA was treated with TURBO DNA-free™ Kit (ThermoFisher Scientific) and quality was confirmed by running an aliquot of extracted RNA on an agarose gel. The presence of intact ribosomal RNA bands was used as an indicator of overall RNA quality. The concentration of RNA extracted from each sample was assessed using a Nanodrop (ThermoFisher Scientific).

A total of 300 ng of RNA was used as a template for reverse transcription with iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Mississauga, ON). Quantitative PCR (qPCR) experiments were run using SsoFast™ EvaGreen® Supermix (Bio-Rad) on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad) and results were analyzed using the accompanying CFX Manager Software (Version 3.1, Bio-Rad). The conditions for qPCR were as follows: an initial 30 seconds at 95°C, followed by 45 cycles of 95°C for 5 seconds and 15 seconds at 60°C. Melt curve analysis was conducted after the 45th PCR cycle with an incremental temperature increase of 0.5°C every 5 seconds, from 65°C to 95°C. Three reference genes (*SlActin*, *SlCAC*, *SlEXP*) were chosen based on previous work in the Hannoufa lab as well as on previous expression studies in tomato. The primers for *SlActin* were designed based on the gene sequence retrieved from the Sol Genomics Network (www.solgenomics.net), while the primers *SlCAC* and *SlEXP* were designed by González-Aguilera et al. (2016). The qPCR primers for *AtCYP82G1* were designed based on gene sequences from the Arabidopsis information resource (TAIR, www.arabidopsis.com). All primers were analyzed for secondary structure formation and self-complementarity using the DNASTAR Lasergene Core Suite (Version 12). All primers were also checked for potential off target amplification targets within the tomato genome using NCBI Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). Sequences for primers and target genes can be found in table 2.1.

2.2.3 Collection and Analysis of Plant Volatiles

VOCs were collected from leaves and flowers *in vivo* to determine the effect of *AtCYP82G1* expression on undamaged plant VOC profiles. The static headspace collection method used was modified from Caceres et al. (2015). The first expanded leaf of 4-week-old plants was carefully placed within a 27 mL glass adaptor open at both ends and sealed using parafilm to generate a static headspace. The same procedure was conducted on the same plants at approximately six weeks for flower VOC collection. Leaves or flowers were held within the headspace for 40 min to allow VOCs to accumulate. After 40 min, a 65 µm polydimethylsiloxane/divinylbenzene (DVB) solid phase microextraction (SPME) fibre was exposed to the headspace for 20 min to extract VOCs. After the 60 min extraction protocol, the loaded fibres were manually injected into an Agilent 7890A GC system equipped with a 5975 inert XL EI/CI MSD quadrupole mass spectrometer. VOCs were separated using helium carrier gas on an Agilent J&W DuraGuard DB-5ms, 30m x 0.25 mm x 0.25 µm column with 10 m of guard column at a constant pressure of 12.445 psi. The GC was run in pulsed splitless mode in order to concentrate the trace VOCs onto the column. The inlet was maintained at 250°C for fibre desorption, while the oven temperature was held at 30°C for 1 min before ramping at a rate of 5°C/min to 200°C, where it was held again for 1 min. The total run time was 36 min, with the fibre used for collection held in the inlet for the duration of the run in order to reduce carryover of extracted VOCs. The mass spectrometer ionization energy was set to 70 eV and mass spectra were collected at a scan speed of 3.75 scans/sec in the range of 20-400 m/z. All VOCs were collected over a period of five days between the hours of 8 AM and 10 AM in order to avoid variations in VOC emissions due to circadian rhythms. . In order to putatively identify compounds, a combination of different GC-MS analysis software was employed. Agilent GC-MS files were batch deconvoluted using the Automated Mass Spectral Deconvolution and Identification System (AMDIS, version 2.71, https://chemdata.nist.gov/mass-spc/amdis/downloads/). The settings for deconvolution resolution, sensitivity and shape were high, high, and medium, respectively. The resulting files were uploaded to SpectConnect (http://spectconnect.gatech.edu/) for alignment (Styczynski et al., 2007). Along with the deconvoluted files, a library file consisting of 615 compounds was uploaded for putative identification based on mass spectra.

This library file was constructed using the PARAFAC2 based Deconvolution and Identification System (PARADISe version 2.6, http://www.models.life.ku.dk/paradise) (Johnsen et al., 2017). As the name suggests, PARADISe utilizes a modeling system known as Parallel Factor Analysis 2 (PARAFAC) in order to deconvolute and align raw GC-MS files. PARADISe also interfaces with the National Institute of Standards and Technology (NIST) mass spectral library for putative identification of unknown peaks. PARADISe intervals were assigned visually and the top 5 matches in the NIST mass spectra database were returned. NIST was then used to construct a library file consisting of the putatively identified compounds for subsequent upload to SpectConnect. Aligned SpectConnect data was processed by subtracting peaks present in blank samples from each test sample. In addition, features that were present in less than 50% of biological replicates of any line were removed. Finally, the processed peak areas from SpectConnect were submitted to MetaboAnalyst (Version 4.0, www.metaboanalyst.ca) for statistical analysis using principle component analysis (PCA) in order to visualize differences between EV control and transgenic lines (Xia et al., 2015). Data was log transformed and pareto scaled prior to PCA plot construction.

2.3 Results

2.3.1 *AtCYP82G1* Expression Analysis

Overall, 10 transgenic plants from nine calli were transferred to soil and grown for seed. One of the calli generated two viable shoots that produced seed, which were cut separately and subsequently labeled 4.1 and 4.2. Given the imprecise nature of *Agrobacterium* transformation, it is possible that 4.1 and 4.2 were generated from the same transgene insertion and so they were named to reflect that. Due to the tissue culture process, the T0 plants tested were generally smaller and had abnormal growth habits compared to plants grown from seed (Figure 2.2). To determine the suitability of the reference genes used for expression analysis, the M-value and coefficient of variance (CV) calculated by the CFX manager software were compared. These values indicate the stability of the reference genes and those that have the lower of these two values are considered the most stable (Bustin et al., 2009; Taylor et al., 2010). *SlActin* (CV=0.6321, M-value=1.3296) was found to be the least stable, so *SlCAC* (CV=0.4371, M-Value=1.1792) and *SlEXP* (CV=0.4901, M-value=1.1491) were chosen as reference genes for

AtCYP82G1 expression analysis. Analysis of primers using NCBI Primer-BLAST found no potential for non-specific amplification, and melt curve analysis following each qPCR assay confirmed that each primers amplicon was of a single sequence. The qPCR product was also run on a gel in order to further confirm that a single product was produced and only from the cDNA of transgenic plants (Figure 2.3A). *AtCYP82G1* was expressed in each line tested, with line 2 having the highest level of expression and line 7 having the lowest (Figure 2.3B). Of the 10 lines generated, five (2, 6, 7, 8, 9) produced little to no seeds and as such were not suitable for line advancement and subsequent volatile analysis. Based on their seed production levels, lines 1, 5, 4.1, 4.2 and 3 were selected for further analysis.

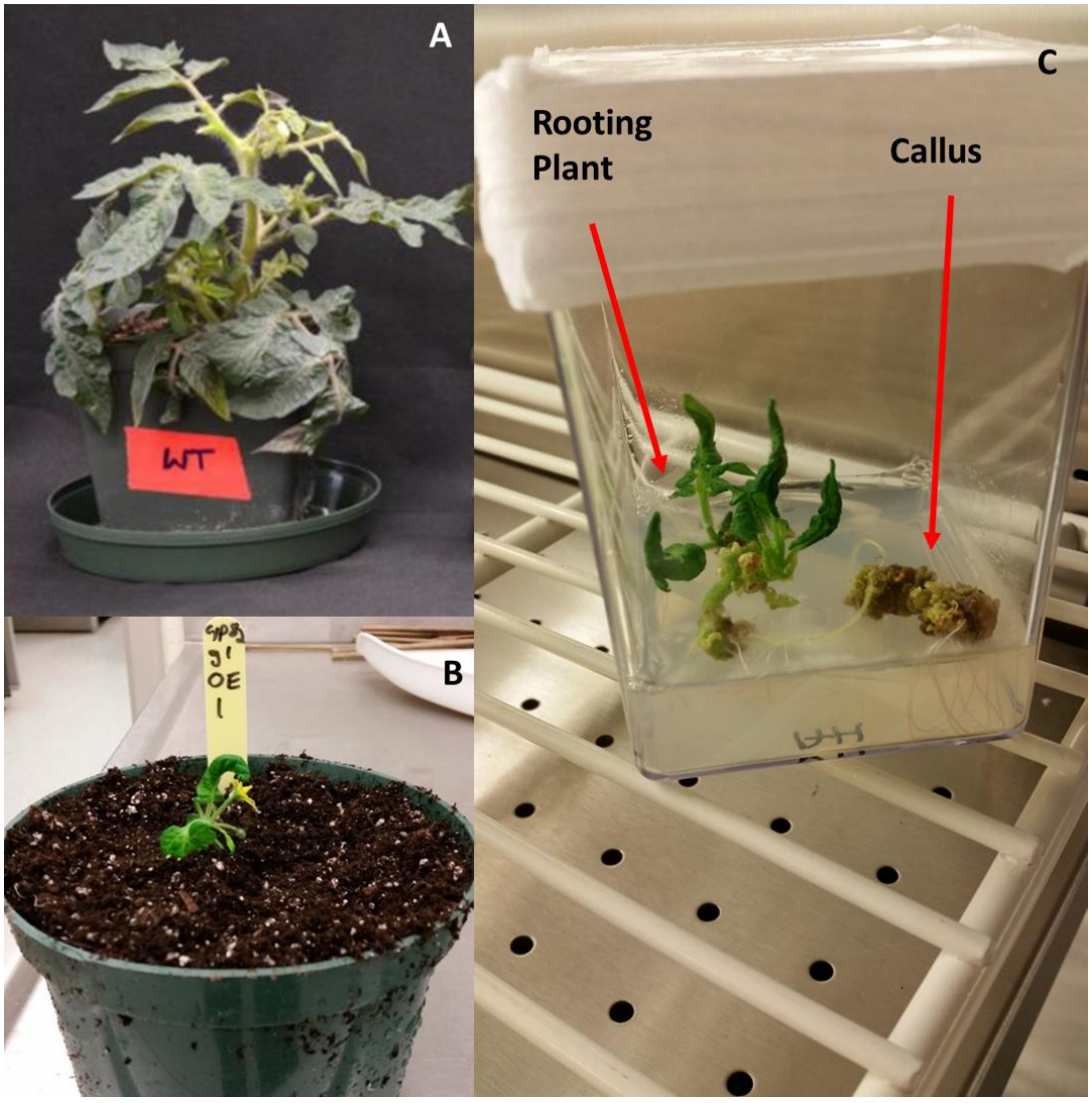

Figure 2.2. Representative photos of wild type and *AtCYP82G1* T0 MicroTom Plants. Compared to wildtype (A), the transgenic plants generated through tissue culture initially showed abnormal growth habits such as curled leaves and early flowering (B,C).

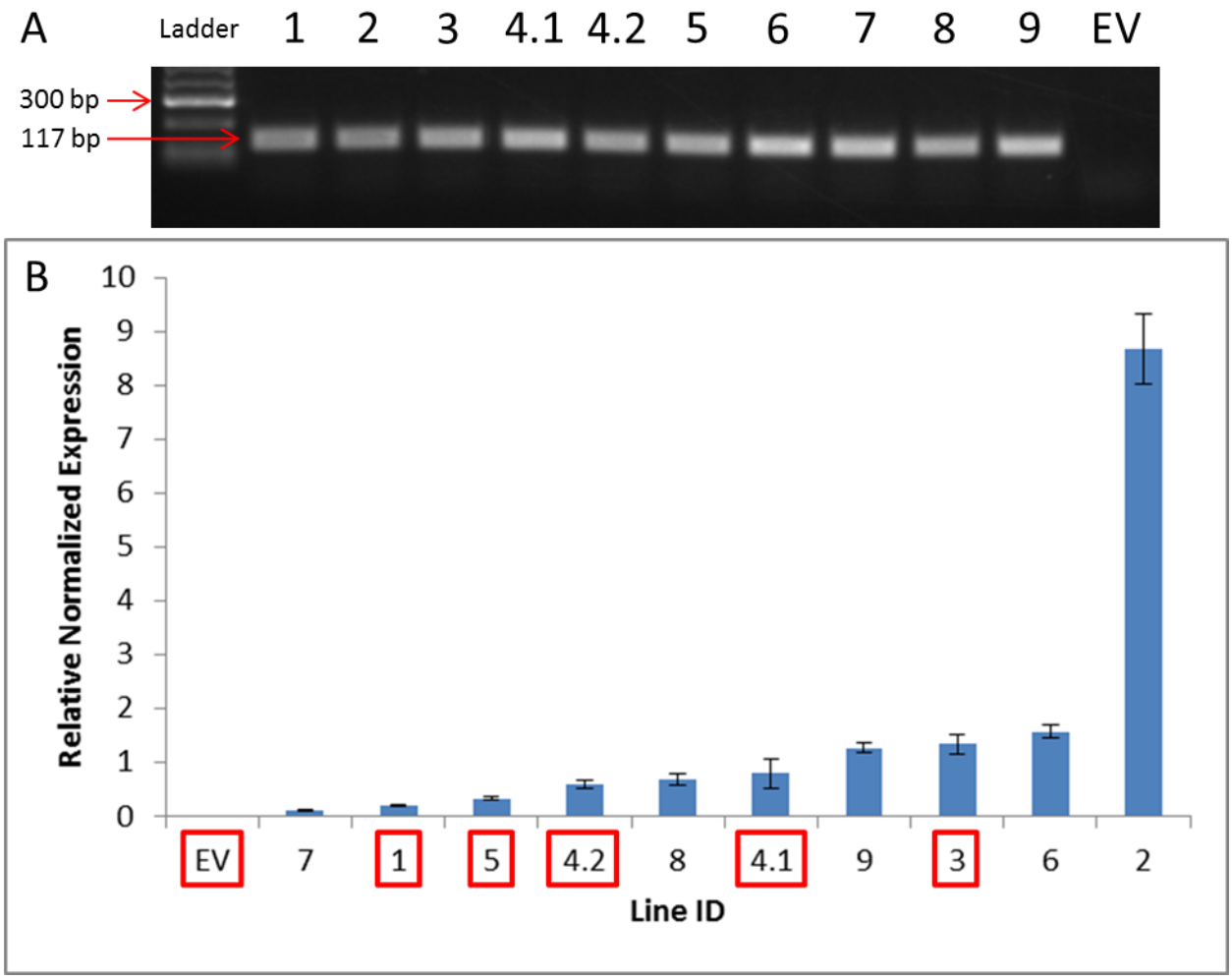

Figure 2.3. Expression analysis of *AtCYP82G1* in transgenic tomato leaves. Primers used for RT-qPCR amplified a single target of 117 bp from each transgenic line (A). RT-qPCR revealed a range of expression levels in the plants tested (B). Each line is an individual plant, with three technical replicates indicated by the error bars. Lines selected for volatile analysis are shown in red boxes.

2.3.2 Volatile Analysis of *CYP82G1* T1 Plants

VOC emissions were collected and analyzed via SPME and GC-MS for five transgenic lines and EV controls. VOCs were collected from T1 plants that were grown from seeds of the T0 plants that had previously been subject to expression analysis. While the T0 plants exhibited some abnormal morphology as side effects of the tissue culture process, the T1 plants were visually indistinguishable from the EV controls. The leaf VOC profile consisted mainly of α-pinene, which accounted for between 68.82% (line 3) and 79.09% (line 1) of leaf emissions (table 2.2). Other compounds consistently detected from leaves included hexanal, β-cymene, and caryophyllene. In total, 31 compounds were tentatively identified from the leaf VOC collections. Many compounds identified were not detected in all lines, and many of these were present in trace amounts. DMNT and TMTT, the two compounds that are produced by CYP82G1 *in vitro*, were not detected in the leaf VOC emissions of any plants tested.

Much like the leaf VOC profile the flower VOC profile also consisted mainly of α-pinene, however to a greater extent (table 2.3). In flowers, α-pinene made up between 82.51% (line 1) and 91.05% (line 4.2) of flower VOC emissions. Other trace compounds in the flower emission profile were hexanal, β-cymene, propylcyclopropane, caryophyllene and methyl salicylate (MeSA). The VOC profile of the flower was more complex than that of the leaf, as 48 compounds were detected. Like the leaf VOC profile though, a number of compounds were not detected in every line. In addition, DMNT and TMTT were not detected in any flower VOC emissions.

Principal Component Analysis (PCA) was used to visualize differences in the entire volatile emission profiles of each line. Each point on the plot represents an individual plant and those points that are grouped closest together have the most similarities in their volatile emission profiles. Analysis of whole leaf VOC emissions consisting of 235 features using PCA revealed some separation between the transgenic lines and EV control profiles, with the most pronounced separation found between EV and line 3 (Figure 2.4). Line 3 was separated along both principle component (PC) 1 (11.8%) and PC2 (10.2%), while lines 1, 4.2, and 5 were mostly separated along PC1. In contrast, when PCA was conducted on the 31 features where tentative identifications had been made the differences were much less pronounced (Figure 2.5). In the

PCA plot constructed with only identified compounds, each line had individuals spread along PC1 (19.2%), while lines 3 and 1were somewhat separated from the EV controls along PC2 (15%).

Similar patterns were revealed in PCA plots constructed from flower VOC emission data. The entire flower VOC emission profile consisted of 347 features. The PCA plot constructed from these data showed good separation of all transgenic lines along PC1 (9.7%) with lines 4.1 and 3 the most distant from EV controls (figure 2.6). Lines 3, 4.2 and 4.5 were also separated from EV along PC2 (8.1%). This separation nearly disappears in the PCA plot of identified features, with only slight separation of line 4.1 from EV along PC1 (15.7%) and little notable separation of any lines along PC2 (11%) (Figure 2.7). Taken together, the PCA analysis suggests that the majority of differences between EV control plants and the transgenic plants tested lies in the features whose identities remain unknown.

Table 2.2. Tentatively identified VOCs emitted from leaves of *AtCYP82G1* and EV control plants. Chromatographic features are listed by retention time (RT). Averages (Avg) of the percentage peak area along with standard error of the mean (SEM) are shown, with each calculated from a minimum of six biological replicates. P-Val is indicative of the p-value returned from a one-way ANOVA test conducted on each feature, with values less than .05 considered significant. A minimum of 60% mass spectral match (%M) was set for tentative identification of the compounds listed. Compounds that were not detected in the profiles of a specific line are marked as n.d.

Table 2.2				**EV**			**1**			**5**			**4.2**			**4.1**			**3**			
Tentative Compound ID	**m/z**	**%M**	**RT**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**p-val**
Toluene	91	83	5.25	0.36	±	0.15	0.18	±	0.09	0.14	±	0.02	0.39	±	0.15	0.82	±	0.43	0.11	±	0.05	0.27
Hexanal	44	87	6.20	2.29	±	1.27	1.72	±	0.80	3.85	±	1.03	2.57	±	0.60	4.75	±	1.40	1.34	±	0.32	0.81
p-Xylene	91	64	7.95	n.d.	±	-	0.18	±	0.08	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.11	±	0.03	0.15
Styrene	104	80	8.58	n.d.	±	-	n.d.	±	-	1.44	±	0.42	0.69	±	0.22	n.d.	±	-	0.58	±	0.19	0.45
α-Pinene	93	98	9.96	72.45	±	5.28	79.09	±	6.25	73.68	±	3.27	74.40	±	5.68	76.86	±	7.37	68.82	±	5.61	0.47
α-Thujene	93	71	10.22	1.90	±	1.04	0.55	±	0.22	1.29	±	0.24	4.88	±	3.22	3.59	±	1.33	7.12	±	5.67	0.61
Benzaldehyde	77	84	10.81	n.d.	±	-	0.44	±	0.12	n.d.	±	-	0.45	±	0.11	n.d.	±	-	0.24	±	0.07	0.43
Cyclofenchene	93	61	11.06	9.15	±	5.44	16.24	±	6.01	n.d.	±	-	5.33	±	1.85	n.d.	±	-	6.71	±	3.39	0.34
2-Propenylidene-cyclobutene	91	63	11.11	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	3.15	±	0.90	0.16
β-Cymene	119	87	13.00	0.62	±	0.19	0.82	±	0.15	0.89	±	0.23	0.48	±	0.20	0.77	±	0.16	0.79	±	0.16	0.34
1,6-Dimethyl-1,5-cyclooctadiene	68	65	13.09	4.36	±	0.79	4.05	±	0.85	4.40	±	0.97	3.39	±	0.77	4.07	±	0.73	3.87	±	0.41	0.48
Methyl phenethyl sulfoxide	91	71	14.43	n.d.	±	-	n.d.	±	-	0.11	±	0.05	0.02	±	0.01	0.06	±	0.02	0.23	±	0.14	0.51
2-Neopentylacrolein	57	62	14.96	n.d.	±	-	n.d.	±	-	7.77	±	1.98	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.00
4-Ethylstyrene	117	69	15.00	n.d.	±	-	0.34	±	0.10	1.54	±	0.43	1.12	±	0.34	n.d.	±	-	n.d.	±	-	0.31
Undecane	57	87	15.45	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	2.09	±	0.45	0.01
3,3,6-Trimethyl-1,5-heptadien-4-one	83	82	15.64	0.05	±	0.02	n.d.	±	-	0.25	±	0.07	n.d.	±	-	0.28	±	0.09	n.d.	±	-	0.00
5-Butylnonane	71	63	17.29	0.30	±	0.11	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.48	±	0.17	n.d.	±	-	0.10
2,3-Dimethyloctane	57	64	17.29	3.28	±	1.98	0.26	±	0.10	n.d.	±	-	2.11	±	0.82	n.d.	±	-	n.d.	±	-	0.12
3,5,5-Trimethyl-1-hexene	57	64	17.51	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.31	±	0.09	0.04
3,8-Dimethyldecane	57	78	17.76	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.22	±	0.05	0.00
Dodecane	57	64	18.61	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	5.40	±	1.72	2.50	±	0.51	0.02
3,4,-Epoxy-2-hexanone	43	70	19.32	0.11	±	0.04	0.10	±	0.06	0.09	±	0.02	0.65	±	0.33	n.d.	±	-	0.05	±	0.02	0.19
1,1,3,5-tetramethylcyclohexane	69	74	21.38	1.43	±	0.49	0.19	±	0.05	0.35	±	0.10	0.11	±	0.04	0.36	±	0.13	0.46	±	0.14	0.19
2,4-Dimethyldecane	43	63	21.59	0.53	±	0.19	0.18	±	0.05	0.58	±	0.29	n.d.	±	-	3.91	±	1.56	0.72	±	0.24	0.19
Isoterpinolene	121	76	22.64	n.d.	±	-	0.24	±	0.07	2.56	±	0.91	2.14	±	0.62	0.46	±	0.16	1.32	±	0.43	0.17
2,4,4-Trimethyl-2-pentene	97	81	23.01	6.83	±	2.34	0.93	±	0.33	3.54	±	0.79	4.09	±	1.16	7.11	±	3.48	1.66	±	0.40	0.66
(E)-5-butoxy-2-pentene	57	73	24.73	0.44	±	0.15	0.07	±	0.03	0.15	±	0.04	0.39	±	0.14	0.52	±	0.30	0.12	±	0.03	0.56
Caryophyllene	93	85	25.05	3.96	±	0.87	1.80	±	0.71	5.10	±	1.20	11.28	±	2.44	2.08	±	0.58	5.63	±	3.13	0.47
2-Norpinene	93	68	25.99	n.d.	±	-	n.d.	±	-	n.d.	±	-	1.18	±	0.31	n.d.	±	-	1.23	±	0.48	0.17
(E,E)-2,4-Heptadien-6-ynal	105	64	26.08	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.07	±	0.03	0.06

Table 2.3. Tentatively identified VOCs emitted from flowers of *AtCYP82G1* and EV control plants. Average percentage peak area (Avg) along with standard error of the mean (SEM) are listed for each feature by retention time (RT). Each feature reported had a mass spectral match percentage (%M) of at least 60%. A minimum of six biological replicates were collected for each line. P-Val indicates p-value returned from a one-way ANOVA test conducted on each feature, with values less than .05 considered significant. Compounds that below detection limits in a given line are marked as n.d.

Table 2.3				**EV**			**1**			**5**			**4.2**			**4.1**			**3**			
Tentative Compound ID	**m/z**	**%M**	**RT**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**p-val**
2-Tert-butyl-3-methoxyirane	55	67	4.74	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	tr.	±	-	n.d.	±	-	0.01
3-Methyl-2-butenal	84	60	4.76	0.02	±	0.01	0.02	±	0.00	n.d.	±	-	0.02	±	0.00	0.02	±	0.00	0.02	±	0.01	0.04
Toluene	91	83	5.25	0.03	±	0.01	0.04	±	0.01	0.04	±	0.01	0.03	±	0.01	0.03	±	0.01	0.01	±	0.00	0.63
2,2,4-trimethylpentane	57	71	5.51	n.d.	±	-	0.10	±	0.04	0.08	±	0.02	0.02	±	0.01	0.05	±	0.02	0.04	±	0.01	0.17
Hexanal	44	87	6.20	1.66	±	0.15	1.32	±	0.22	2.09	±	0.26	0.85	±	0.11	2.20	±	0.56	1.38	±	0.43	0.05
(R)-(+)-3-Methylcyclopentanone	69	90	7.11	n.d.	±	-	0.09	±	0.02	0.19	±	0.04	n.d.	±	-	0.14	±	0.03	0.12	±	0.03	0.00
3,4-Dimethyl-2-hexanone	43	65	7.35	0.11	±	0.04	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.06	±	0.02	0.01
Propylcyclopropane	56	85	7.86	0.20	±	0.05	0.14	±	0.03	0.22	±	0.06	0.13	±	0.02	0.23	±	0.06	0.12	±	0.03	0.54
p-Xylene	91	64	7.95	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.01	±	0.00	n.d.	±	-	0.01
Styrene	104	80	8.58	0.19	±	0.03	0.18	±	0.04	0.18	±	0.02	0.14	±	0.04	0.12	±	0.02	0.12	±	0.02	0.81
α-Pinene	93	98	9.96	85.50	±	2.38	82.51	±	4.69	82.90	±	2.14	91.05	±	0.34	84.17	±	2.67	82.97	±	5.54	0.18
α-Thujene	93	71	10.22	0.46	±	0.16	7.06	±	4.84	1.85	±	1.40	0.45	±	0.27	3.18	±	1.91	5.98	±	4.96	0.54
Benzaldehyde	77	84	10.81	0.11	±	0.03	0.04	±	0.03	0.08	±	0.02	n.d.	±	-	0.03	±	0.01	n.d.	±	-	0.04
Bicyclo[3.2.0]hepta-2,6-diene	91	70	10.82	0.13	±	0.05	0.05	±	0.02	0.04	±	0.01	0.09	±	0.04	0.83	±	0.60	0.05	±	0.01	0.44
Cyclofenchene	93	61	11.06	3.02	±	1.91	0.66	±	0.10	3.69	±	1.62	0.62	±	0.27	3.46	±	1.91	2.70	±	1.18	0.63
2-Propenylidene-cyclobutene	91	63	11.11	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.15	±	0.06	0.34
6-Methyl-5-hepten-2-one	43	77	11.62	n.d.	±	-	n.d.	±	-	0.06	±	0.02	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.06
5-Methyl-1-phenyl-1-hexanone	105	72	11.84	n.d.	±	-	0.01	±	0.00	n.d.	±	-	0.01	±	0.00	n.d.	±	-	n.d.	±	-	0.17
β-Cymene	119	87	13.00	0.68	±	0.11	0.62	±	0.08	0.75	±	0.11	0.64	±	0.06	0.56	±	0.07	0.47	±	0.04	0.40
1,6-Dimethyl-1,5-cyclooctadiene	68	65	13.09	4.81	±	0.47	4.32	±	0.24	5.05	±	0.46	4.82	±	0.20	4.43	±	0.33	4.05	±	0.25	0.26
o-Propyltoluene	105	72	14.08	0.06	±	0.02	0.03	±	0.01	0.04	±	0.01	0.03	±	0.01	0.03	±	0.01	0.02	±	0.01	0.19
2,3,3-Trimethyloctane	43	66	14.22	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.13	±	0.03	0.01
Methyl phenethyl sulfoxide	91	71	14.43	n.d.	±	-	0.01	±	0.01	n.d.	±	-	0.22	±	0.10	0.36	±	0.16	0.11	±	0.07	0.55
Dihydromyrcenol	59	78	14.51	0.51	±	0.19	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.01
4-Ethylstyrene	117	69	15.00	0.05	±	0.01	n.d.	±	-	0.06	±	0.01	0.01	±	0.00	0.04	±	0.01	n.d.	±	-	0.01
5-Methyl-1-hexanol	43	62	15.02	n.d.	±	-	n.d.	±	-	0.35	±	0.11	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.05
Undecane	57	87	15.45	n.d.	±	-	0.26	±	0.10	0.21	±	0.04	0.04	±	0.01	0.17	±	0.10	0.17	±	0.07	0.13

Table 2.3 Cont.				EV			1			5			4.2			4.1			3			
Tentative Compound ID	**m/z**	**%M**	**RT**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**p-val**
Nonanal	57	66	15.55	0.27	±	0.09	0.19	±	0.05	0.20	±	0.04	0.07	±	0.02	0.19	±	0.05	0.10	±	0.03	0.93
3,3,6-Trimethyl-1,5-heptadien-4-one	83	82	15.64	0.08	±	0.02	n.d.	±	-	n.d.	±	-	0.02	±	0.01	n.d.	±	-	n.d.	±	-	0.03
5-Butylnonane	71	63	17.29	0.01	±	0.00	0.03	±	0.01	0.04	±	0.01	0.03	±	0.02	0.05	±	0.02	0.03	±	0.01	0.83
2,3-Dimethyloctane	57	64	17.29	n.d.	±	-	0.07	±	0.03	0.07	±	0.01	0.04	±	0.01	0.03	±	0.01	0.13	±	0.07	0.10
3,5,5-Trimethyl-1-hexene	57	64	17.51	n.d.	±	-	0.11	±	0.03	0.11	±	0.03	0.05	±	0.02	0.06	±	0.02	0.13	±	0.04	0.17
3,8-Dimethyldecane	57	78	17.76	0.18	±	0.08	0.12	±	0.03	0.62	±	0.18	0.08	±	0.02	0.06	±	0.02	n.d.	±	-	0.00
Methyl salicylate	120	84	18.31	0.04	±	0.01	0.04	±	0.01	0.14	±	0.03	0.05	±	0.02	0.19	±	0.06	0.03	±	0.01	0.10
Dodecane	57	64	18.61	n.d.	±	-	0.21	±	0.05	0.23	±	0.06	n.d.	±	-	0.21	±	0.09	0.16	±	0.06	0.22
3,4,-Epoxy-2-hexanone	43	70	19.32	0.06	±	0.03	0.01	±	0.01	0.07	±	0.03	tr.	±	-	tr.	±	-	0.01	±	0.01	0.11
2,2,3,3,4,4-Hexamethyltetrahydrofuran	83	60	20.74	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.08	±	0.02	0.11	±	0.03	0.04	±	0.01	0.00
1,1,3,5-Tetramethylcyclohexane	69	74	21.38	0.06	±	0.02	0.04	±	0.02	0.07	±	0.03	0.05	±	0.01	0.02	±	0.01	0.06	±	0.02	0.87
2,4-dimethyldecane	43	63	21.59	0.10	±	0.03	0.03	±	0.01	0.12	±	0.02	0.06	±	0.01	0.02	±	0.01	0.08	±	0.03	0.20
Acetophenone	121	76	22.63	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.02	±	0.01	n.d.	±	-	0.04
Isoterpinolene	121	76	22.64	n.d.	±	-	0.63	±	0.23	n.d.	±	-	0.10	±	0.03	0.16	±	0.06	0.32	±	0.11	0.12
2,4,4-Trimethyl-2-pentene	97	81	23.01	0.63	±	0.29	0.34	±	0.08	0.50	±	0.10	0.24	±	0.07	0.25	±	0.06	0.35	±	0.11	0.56
(E)-5-Butoxy-2-pentene	57	73	24.73	0.07	±	0.02	0.02	±	0.00	0.03	±	0.01	0.01	±	0.00	0.01	±	0.00	0.04	±	0.01	0.08
Caryophyllene	93	85	25.05	1.32	±	0.42	1.24	±	0.30	0.88	±	0.22	0.62	±	0.19	0.75	±	0.20	1.10	±	0.46	0.48
Cis-muurola-4(14),5-diene	161	62	25.42	0.12	±	0.05	n.d.	±	-	n.d.	±	-	0.01	±	0.00	n.d.	±	-	n.d.	±	-	0.35
2-Norpinene	93	68	25.99	0.11	±	0.03	0.21	±	0.06	0.09	±	0.02	0.07	±	0.02	0.10	±	0.03	0.19	±	0.08	0.25
(E,E)-2,4-Heptadien-6-ynal	105	64	26.08	0.01	±	0.00	n.d.	±	-	tr.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.18
Pentadecane	57	87	27.12	0.91	±	0.27	0.45	±	0.12	0.97	±	0.22	n.d.	±	-	0.24	±	0.07	0.52	±	0.20	0.11

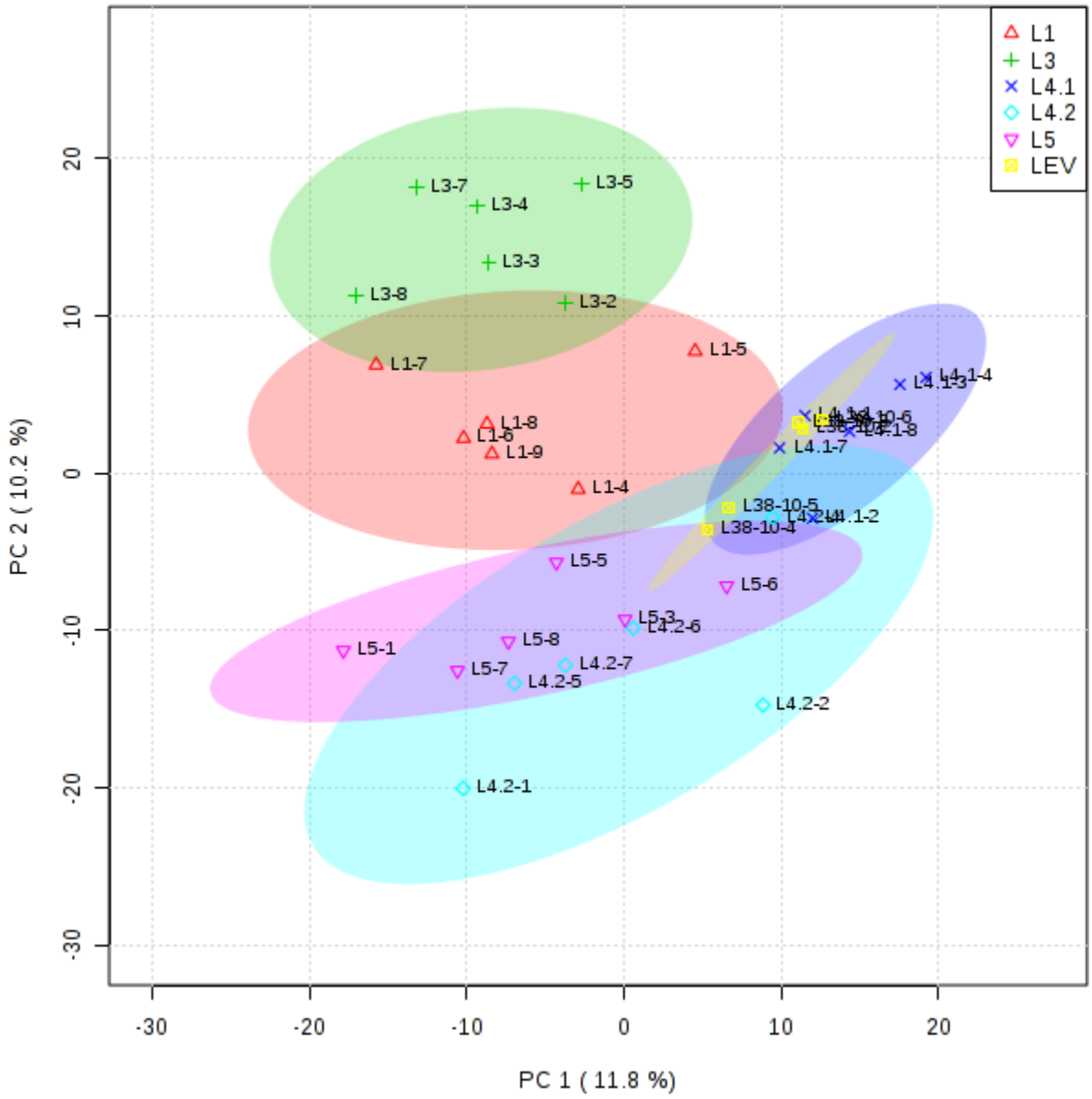

Figure 2.4. Principle component analysis of all detected chromatographic features from leaves of empty vector (EV) control and five *AtCYP82G1* lines. Each point within a plot is representative of a single plant for a total of six biological replicates per line. Differences can be seen between EV and all transgenic lines besides 4.1, as the EV group and 4.1 group overlap.

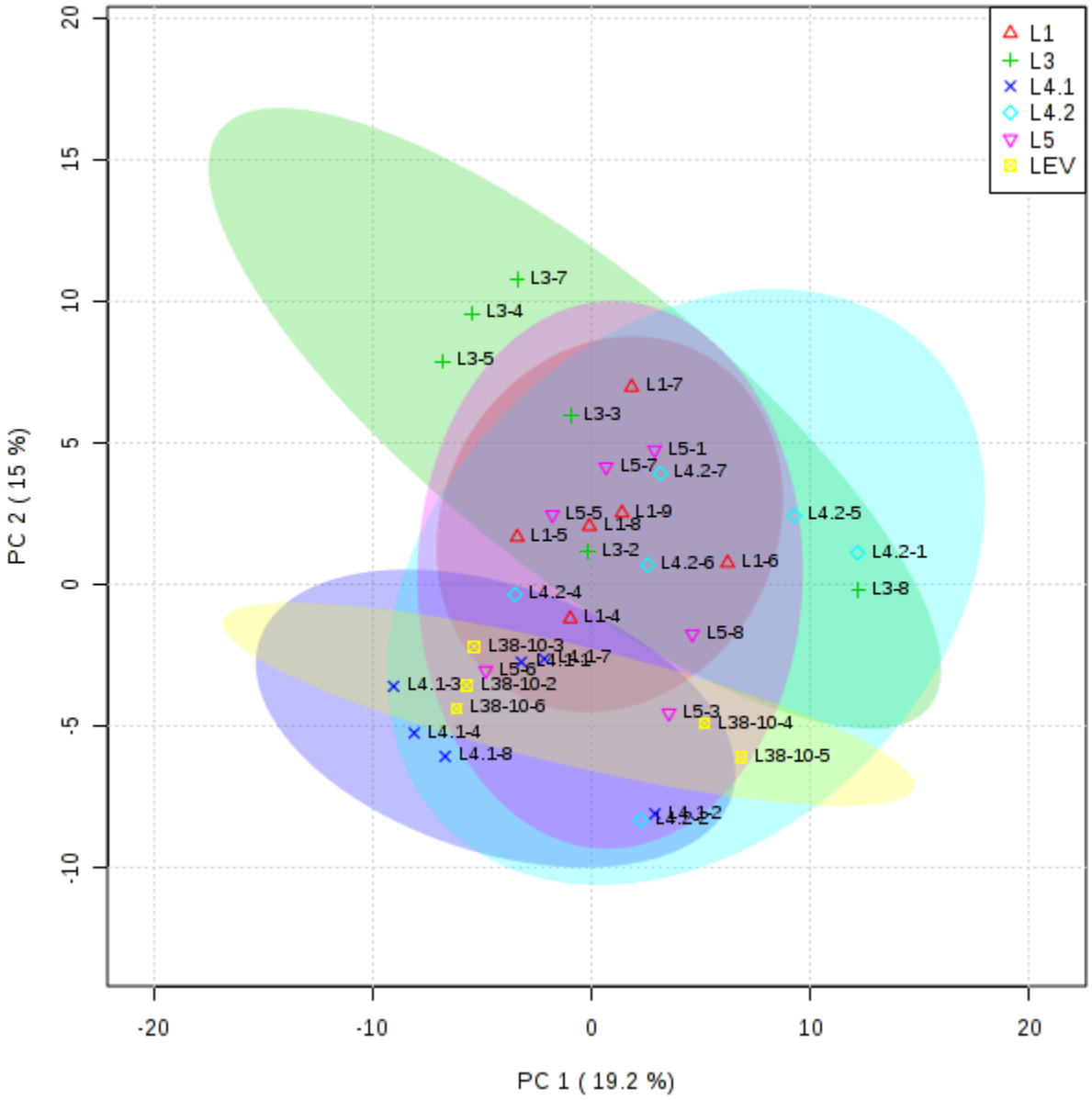

Figure 2.5. Principle component analysis of tentatively identified chromatographic features from leaves of empty vector (EV) control and five *AtCYP82G1* lines. Each point within a plot is representative of a single plant for a total of six biological replicates per line. When only the identified features are used for plot construction, the differences between lines are no longer as apparent as in figure 2.4.

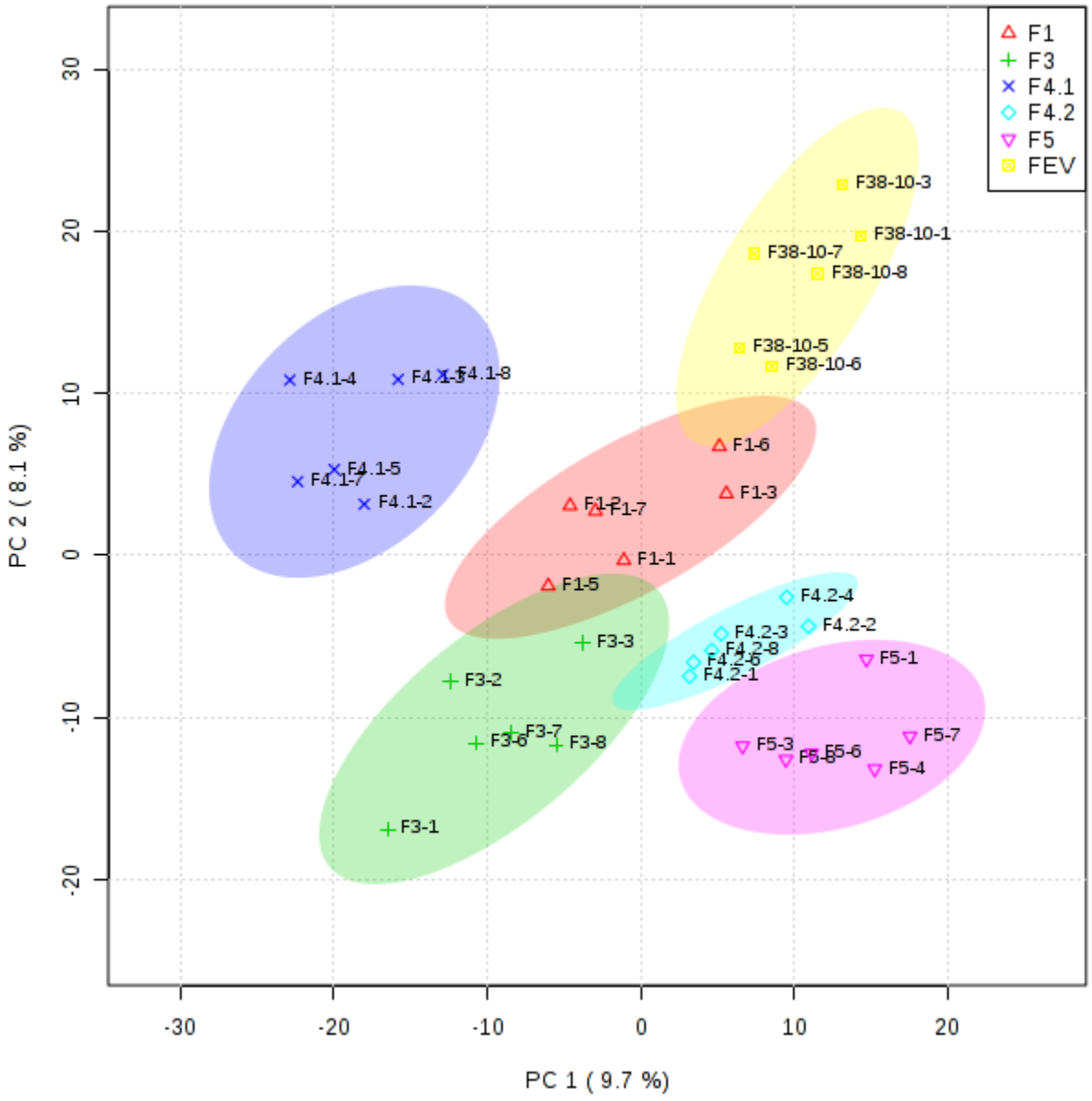

Figure 2.6. Principle component analysis plot of all detected flower chromatographic features from empty vector (EV) control and five *AtCYP82G1* lines. Each point within a plot is representative of a single plant for a total of six biological replicates per line. Differences are evident between all transgenic lines and EV.

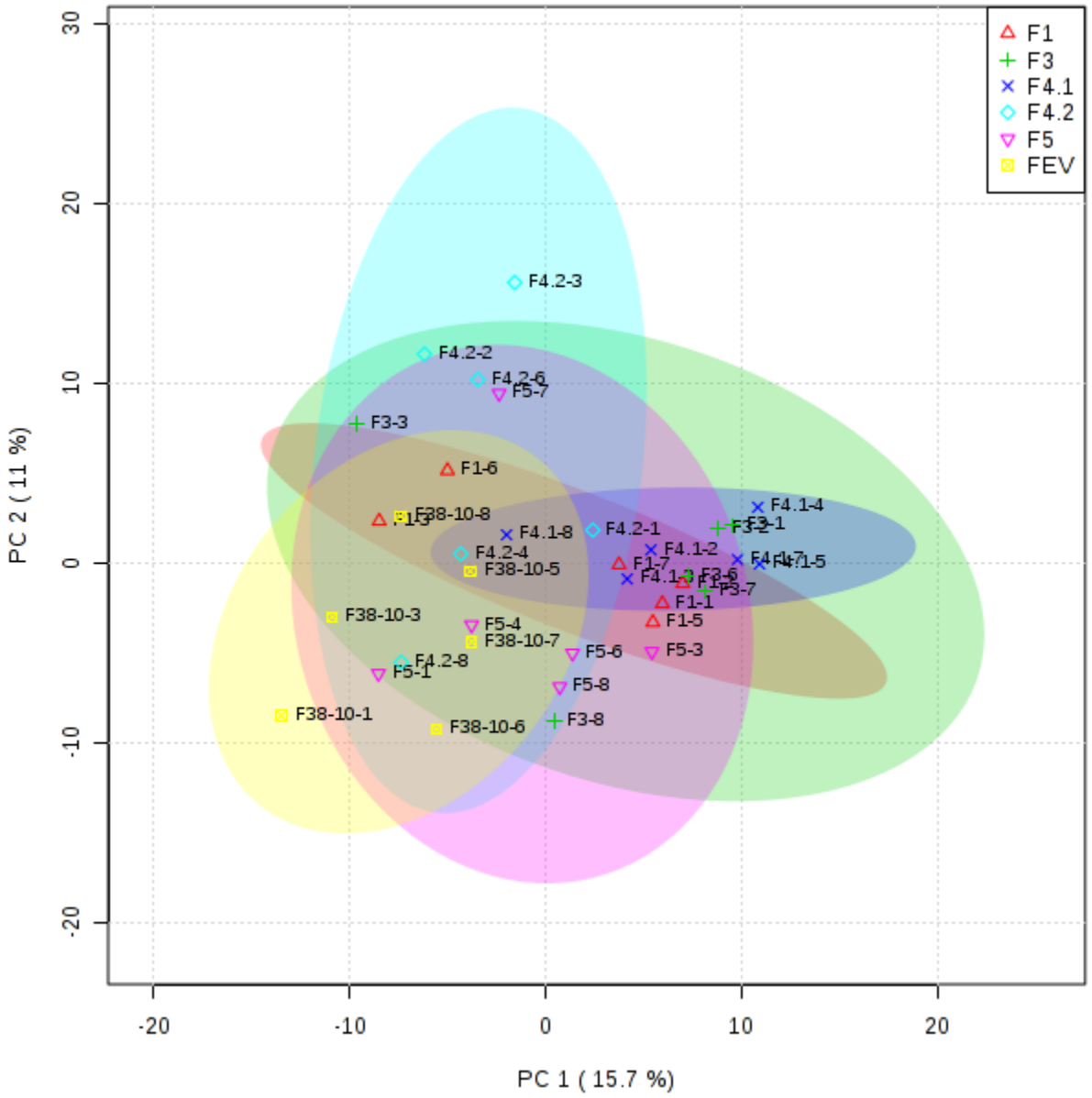

Figure 2.7. Principle component analysis plot of all tentatively identified flower chromatographic features from empty vector (EV) control and five *AtCYP82G1* lines. Each point within a plot is representative of a single plant for a total of six biological replicates per line. Differences are much less obvious when only identified compounds are used for plot construction, compared to figure 2.6 where all features are used.

2.4 Discussion and Conclusion

MicroTom tomato plants expressing *AtCYP82G1* were successfully generated by *Agrobacterium*-mediated transformation. In total 30 leaf and 48 flower VOCs were tentatively identified by mass spectra. Expression of *AtCYP82G1* was expected to induce emission of one or both of the homoterpenes DMNT and TMTT, but neither could be detected from the flowers or leaves of the transgenic plants. While it cannot be ruled out that the expression levels achieved are too low or that the CYP82G1 enzyme produced by the transgene is non-functional in our mutant lines, it is also possible that the lack of DMNT and TMTT detection is due to a number of other factors.

It has been reported previously that both TMTT and DMNT are emitted at low levels from healthy tomato leaves, albeit in the cultivar Moneymaker (Ament et al., 2006; Silva et al., 2017). In two studies which report the emissions of these compounds, volatiles were collected for extended periods of time in dynamic headspace collection systems (Ament et al., 2006; Silva et al., 2017). In the current study, a rapid static headspace collection system using SPME was employed. The differences in VOC collection methods are one potential reason for the differences between the results seen here and the previous studies. Because of the diverse biochemical makeup of VOCs emitted by plants, collecting volatiles with different methods can have a drastic impact on the final results. For static headspace extractions alone, different SPME fibre coatings have varied affinities for each class of molecules, resulting in differing extraction efficiencies (Caceres et al., 2015). While SPME-based static headspace sampling is much quicker and easier to conduct, dynamic headspace sampling using trapping mechanisms can be a more exhaustive approach, as extended collection times allow any VOCs that may be emitted in minute quantities to build up over time on the adsorbent material (Tholl et al., 2006). The static system employed in this work also only collected VOC emissions from single leaves and flowers, while the dynamic systems used in the previous studies collected VOC emissions from entire Moneymaker plants, which do not have the severe dwarf phenotype that is present in the MicroTom cultivar.

These factors, compounded by the fact that volatile emissions may differ naturally between cultivars, could all feasibly contribute in some way to the lack of DMNT and TMTT detection in

this study. It is also possible that feedback control or lack of accessible substrate could be responsible for the lack of TMTT or DMNT emissions, which has been suggested as a potential reason for the lack of increased TMTT emission by *Arabidopsis* plants overexpressing *AtCYP82G1* (Lee et al., 2010). It may be prudent in future studies to express *nerolidol synthase* (*NES*) and *geranyllinalool synthase* (*GES*) alongside *CYP82G1* in an attempt to increase the total flux through the synthesis pathway of these homoterpenes.

In the study describing the function of *AtCYP82G1*, Lee et al. (2010) utilized the fungal elicitor alamethicin which increased the emission of TMTT and its precursor geranyllinalool as well as the expression of *CYP82G1* and *GES*. When treated with alamethicin, the mutant lines overexpressing *AtCYP82G1* showed an increased emission of TMTT compared to treated WT controls (Lee et al., 2010). Although in that case the increase was statistically insignificant, it could be beneficial to treat the lines used in this study with alamethicin in order to determine whether or not MicroTom plants can produce either of the homoterpenes in addition to determining if the CYP82G1 enzyme produced by the transgene is functional (Lee et al., 2010). If an increase in the emission of TMTT by the mutant is observed with an elicitor, it is also possible that TMTT emissions would be heightened after herbivory, potentially improving the defense capabilities of the plant.

Although DMNT and TMTT were not detected, the remainder of the VOC emission profile was examined using PCA. PCA allowed for visualization of the differences between the transgenic and EV control plants. While the PCA plots that included all detected features showed distinct separation between transgenic and EV lines, these differences disappeared when only identified compounds were used for plot construction. This strongly suggests that the separation between the transgenic and non-transgenic plants is driven largely by unidentified features. This work did not attempt to determine the identity of any features which may be causing the separation between transgenic and non-transgenic groups, as the focus of the project was to produce plants that emitted DMNT and TMTT through expression of *AtCYP82G1*. It should also be noted however that the components calculated through PCA only account for at most 19.2% of the leaf volatile variation and 15.7% of the flower volatile variation. This suggests a sizeable amount of variability between members of each line as well as between sibling plants, which could be due to a variety of factors. This variability could be attributed to minor water or nutrient status

differences between plants, as they were manually watered and fertilized as opposed to computer supervised monitoring. Plants of the same line were kept in like groups in the growth chamber, so subtle differences in light availability or watering could contribute to the differences seen between members of each line. Finally, it is also likely that the lack of normalization contributed greatly to the variation seen. While sampling leaves and flowers of the same size and at the same stage was done, it is not realistically possible to ensure that each leaf or flower is exactly identical. This can lead to discrepancies in the amount of VOCs emitted in the given collection timeframe. In addition, the manual nature of the sampling technique used here is less than ideal for SPME, which benefits greatly from automation, normalization and calibration for quantitative analyses. In the future, the static headspace approach used here could be improved by normalization either by the inclusion of an internal standard into the sampled headspace or by collecting and weighing the leaf or flower of interest after VOC collection for normalization by weight.

In conclusion, tomato plants expressing *AtCYP82G1* were generated but DMNT and TMTT emissions were undetectable, suggesting that genetically engineering the biosynthesis of these two important homoterpenes requires more optimization. Currently, there is no direct evidence that the enzyme product is functioning properly, but the inability to detect the homoterpenes may also be due to other factors with the VOC collection system. Future experiments will be conducted in order to confirm the enzymes proper function as well as reduce the variability in the volatile collection system. In the longer term, expression of genes involved in the production of the homoterpene precursors geranyllinalool and nerolidol in the current mutant lines may increase their availability and result in the desired increased emissions of DMNT and TMTT.

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Chapter 3. Molecular, Chemical, and Entomological Analysis of the Effects of *AtCCD4* Expression in Tomato

3.1 Introduction

Carotenoids are a large subclass of pigments that fall within the terpenoid family of compounds (Stange, 2016). Plants and algae are the main sources of carotenoids, but they are also produced by various species of fungi and bacteria (Hannoufa and Hossain, 2012; Stange, 2016). In humans as well as other animals, dietary carotenoid compounds contribute to healthy eyesight as well as the prevention of some chronic diseases (Beatty et al., 2004; Collins, 1999; Hadley et al., 2002). In plants, these pigments are crucial for proper maintenance of photosynthetic machinery, acting as scavengers of reactive oxygen species (ROS), especially singlet oxygen (Niyogi, 1999). The conjugated double bond structure of carotenoids is a target for singlet oxygen, which reacts to form apocarotenoids via oxidative cleavage (Stange, 2016). The resulting apocarotenoids can act as signals to the cell of photooxidative stress, influencing expression of genes involved in photoprotection (Ramel et al., 2012). Many apocarotenoids also function as important signals in other parts of a plant's physiology. Two of these apocarotenoids, abscisic acid (ABA) and strigolactone (SL), are important phytohormones.

ABA is produced from the cleavage and modification of carotenoids by the nine-*cis*-epoxy carotenoid dioxygenases (NCED), a group of enzymes within the carotenoid cleavage dioxygenase (CCD) family (Tan et al., 2003). ABA contributes to proper plant development and is also an important regulator of plant response to abiotic stress (Dong et al., 2015; Tan et al., 2003). Strigolactone (SL) is also derived from carotenoid cleavage products, and is involved in regulating shoot branching (Alder et al., 2012; Hou et al., 2016; Shinohara et al., 2013). Two CCD enzymes, namely CCD7 and CCD8, are involved in SL synthesis (Alder et al., 2012). In addition to controlling shoot branching, SL is a major player in communication between roots and mycorrhizal fungi (Alder et al., 2012; Hou et al., 2016; Schwartz et al., 2004).

A number of other carotenoids and apocarotenoids also play important roles in the interactions between plants and their environment. This includes interactions with humans, who have over long periods of time selected for plants with interesting colourations and tastes that can be

directly attributed to carotenoid content (Brandi et al., 2011; Ohmiya et al., 2006; Simkin et al., 2004).

As an example, the deep red colour of a tomato is due to the high lycopene content of the fruit, while the flavour and aroma are heavily influenced by the presence or absence of the apocarotenoids β-cyclocitral, β-ionone, 6-methyl-5-hepten-2-one and geranylacetone (Fraser et al., 1994; Vogel et al., 2010). While obviously important cues for humans, the colours and smells provided by carotenoids and apocarotenoids are also sensed by insects in search of food and shelter. Volatile organic compound (VOC) emissions are vital to both beneficial and negative interactions between plants and insects. While Plant VOC profiles generally include some apocarotenoids, they also contain a number of different compounds of various chemistries (Dicke, 2016; Hou et al., 2016). A plant's VOC profile is quite plastic, as plants are able to rapidly change the ratios of the VOCs present as well as induce or attenuate the emission of specific compounds in response to a sudden change in the plant's surroundings (Dicke, 2016). This includes responses to pollination status as well as herbivory and oviposition (Grison-Pigé et al., 2002; Hegde et al., 2011; Hilker and Fatouros, 2015; Kolosova et al., 2001; Rodriguez-Saona et al., 2011).

Herbivore-induced plant volatiles (HIPVs) produced in response to attack by herbivorous insects can directly prevent further colonization of a plant by pests (Hegde et al., 2011). HIPVs can also act to indirectly protect plants by attracting herbivore enemies including predators and parasitoids of herbivores and their eggs (Dicke, 2016; Hilker and Fatouros, 2015). Often, these too contain volatile apocarotenoids, which previous studies have shown to be active in interactions between plants and their pests (Hou et al., 2016). Emission of the apocarotenoid β-cyclocitral from strawberries has been linked to the attraction of the fruit feeding spotted wing drosophila (*Drosophila suzukii* (Matsmura)) (Keesey et al., 2015). Conversely, the related apocarotenoid β-ionone has been implicated in the repellence of crucifer flea beetles (*Phyllotreta cruciferae* (Goeze)) from *Brassica napus* (L.) leaves (Gruber et al., 2009). While these two volatile apocarotenoids are products of uncontrolled photooxidation of carotenoids, their synthesis is also catalyzed by CCD1 and CCD4 (Ramel et al., 2012; Simkin et al., 2004; Song et al., 2016; Wei et al., 2011). These two enzymes have a wide substrate specificity compared to other CCD family members, as they are mainly involved in carotenoid catabolism, which may

influence the plant's fragrance and pigmentation (Bruno et al., 2016; Gonzalez-Jorge et al., 2013; Stange, 2016; Vogel et al., 2010). In *Arabidopsis thaliana*, CCD4 is also heavily involved in carotenoid turnover, maintaining carotenoids at a steady-state level by producing a variety of apocarotenoids that are stored as glycosides (Lätari et al., 2015).

Due to their role in influencing agriculturally important pests, VOCs and HIPVs in particular are increasingly important for researchers seeking to improve crop protection. One method employing VOCs from different plants in order to reduce pest insect damage is the "Push-Pull" strategy. Developed for small African farms struggling with lepidopteran pests, this system uses a combination of border crops with attractive VOC emissions and intercrops with repellent VOC emissions to reduce pest damage and improve the yield of the main crop (Khan et al., 2016; Khan et al., 2008). With improvements in molecular breeding and biotechnology, production of transgenic crops with the goal of improving pest control through volatile emissions has been attempted in a lab setting with some success. Transgenic *A. thaliana* plants with enhanced emission of linalool and nerolidol repelled green peach aphids (*Myzus persicae* (Sulzer)) (Aharoni et al., 2003). Also in *A. thaliana*, overexpression of *AtCCD1* resulted in enhanced β-ionone emission and reduced feeding damage by crucifer flea beetle herbivory (Wei et al., 2011). In addition, β-ionone has been shown to function as a repellent in isolation, repelling two-spotted spider mites (*Tetranychus urticae* (Koch)) and silverleaf whitefly (*Bemisia tabaci* (Gennadius)) oviposition (Caceres et al., 2016). While useful for demonstrating feasibility of concepts, *in vitro* assays and *A. thaliana*-based studies of VOCs for pest management must be repeated in crop plants in order to confirm their potential for use as tools in integrated pest management (IPM) programs. This chapter explores the possibility of altering the volatile emission of tomatoes *Solanum lycopersicum* (L.) cv. MicroTom by transformation with *AtCCD4*, which has been shown to produce apocarotenoid volatiles including β-ionone both *in vitro* and *in planta* (Bruno et al., 2016; Song et al., 2016). The effects of *AtCCD4* expression on leaf carotenoid content was examined, and the VOC blends of transgenic and non-transgenic plants were compared using gas chromatography-mass spectrometry (GC-MS). Additionally, oviposition preference bioassays were conducted to examine if control or mutant VOC blends were preferable to greenhouse whiteflies (*Trialeurodes vaporariorum* (Westwood)).

3.2 Materials and Methods

3.2.1 Plant Material and Generation of Tomato Lines Expressing *AtCCD4*

Seeds of *Solanum lycopersicum* (tomato) cv. MicroTom were sterilized, germinated and grown as detailed in section 2.2.1 Cloning of *CCD4* (AT4G19170) from *A. thaliana* was conducted by previous members of the Hannoufa lab at Agriculture and Agri-Food Canada's London Research and Development Centre. RNA was extracted from *A. thaliana* leaves with a PowerPlant RNA Isolation Kit (MoBio Laboratories, Carlsbad, CA) and reverse transcribed using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Mississauga, ON). PCR amplification of *AtCCD4* from the resulting cDNA was conducted using gene-specific primers designed for gateway cloning (Table 3.1). The amplified fragment was then cloned into the entry vector pENTR™/D-TOPO® (ThermoFisher, Mississauga, ON) and subsequently subcloned into the pMDC-32 expression vector and transferred into *Agrobacterium tumefaciens* strain EHA105 (Curtis and Grossniklaus, 2003). Tomato transformation was carried out according to the tomato transformation protocols provided by Cruz-Mendivil et al. (2011) and Sun et al. (2006). A schematic of the transgene insert is depicted in Figure 3.1. Tissue culture media recipes are detailed in appendix A. Hygromycin was used as an initial screen for positive transformants. These putatively transgenic plants were grown to maturity for seed collection. The seeds were then sterilized and sown on germination media supplemented with 40 mg/L hygromycin. The resulting T1 plants were then transplanted to soil and DNA was extracted using the DNasy Plant Mini Kit (QIAGEN) in order to confirm the presence of the transgene via PCR. Genotyping PCRs were carried out using the 35S-F3 and CCD4-Int-Rev primers (Table 3.1). This process was repeated and the resulting T2 plants were used for subsequent analysis. Plants successfully recovered from tissue culture were grown to maturity for seed collecation. The seeds were sterilized as detailed previously and sown on germination media supplemented with 40 mg/L hygromycin to select for transgenic plants followed by transplantation to soil for use in subsequent analyses.

Primer Name	Primer Sequence	Purpose	Amplicon	GenBank Accession
35S-F3 (pMDC) NOS-R2 (pMDC)	CAATCCCACTATCCTTCGCAAGACCC ATAATCATCGCAAGACCGGCAAC	PCR confirmations, sequencing	Variable	-
M13 F (pentr) M13 R (pentr)	GTAAAACGACGGCCAG CAGGAAACAGCTATGAC	PCR confirmations, sequencing	Variable	-
CCD4-Int-For CCD4-Int-Rev	TCACGCCATAAAAATCCACAACG CGTGAATGATATTGAATCCAGGAACTTC	PCR confirmations, sequencing	716	NM_118036
35S-F3 (pMDC) CCD4-Int-Rev	CAATCCCACTATCCTTCGCAAGACCC CGTGAATGATATTGAATCCAGGAACTTC	PCR confirmations, sequencing	355	NM_118036
Ath-CCD4-Fq Ath-CCD4-Rq	AAGATCTCCGGTGTGGTGAAGC CCGGATTACCAGGATCCCTAGC	RT-qPCR	133	NM_118036
Sl-ExpSq-Fq Sl-ExpSq-Rq	GCTAAGAACGCTGGACCTAATG TGGGTGTGCCTTTCTGAATG	RT-qPCR Reference Gene	183	XM_004242916
Sl-CAC-Fq Sl-CAC-Rq	CCTCCGTTGTGATGTAACTGG ATTGGTGGAAAGTAACATCATCG	RT-qPCR Reference Gene	173	NM_001324017
SlACTINF163 SlACTINR347	CATGCCATTCTTCGTTTGGA GAGCTGCTCCTGGCAGTTTC	RT-qPCR Reference Gene	184	NM_001321306

Table 3.1. Primers used for genotyping, sequencing and expression analysis of plants expressing *AtCCD4*. Primers with variable amplicons are primers designed for the plasmid backbone listed in brackets that would amplify any insert.

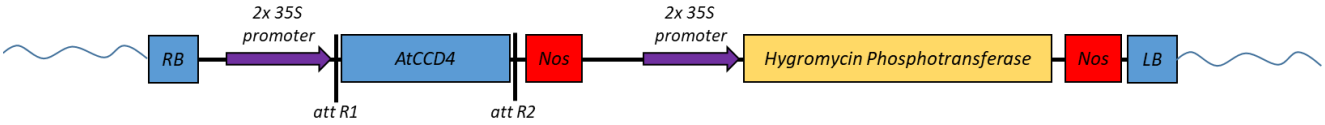


Figure 3.1. Schematic of transgene insertion cassette for *AtCCD4* expression in tomato. The *attR1* and *attR2* sites within the pMDC-32 expression vector allow for gateway cloning of the gene of interest between a 2x35S promoter and *Nos* terminator (Curtis and Grossniklaus, 2003). *Hygromycin phosphotransferase* is also expressed similarly for selection of transformed plants. The flanking border sequences (*RB, LB*) are cut from the expression vector and randomly inserted into the plant genome following infection with *Agrobacterium tumefaciens.*

3.2.2 *AtCCD4* Expression Analysis

To determine the level of transgene expression in T2 tomato lines expressing *AtCCD4*, reverse transcription quantitative PCR (RT-qPCR) experiments were conducted. Expression analysis was carried out in accordance with the methods described in section 2.2.3.

3.2.3 Collection and Analysis of *In vivo* and Ground Tissue VOCs

VOCs were collected from EV control and transgenic *AtCCD4* leaves and flowers *in vivo* to determine the effect of *AtCCD4* expression on undamaged plant VOC profiles. The static headspace collection method used was modified from Caceres et al. (2015) as detailed in section 2.2.3. The first expanded leaf of 4-week-old plants was carefully placed within a 27 mL glass adaptor open at both ends and sealed using parafilm to generate a static headspace (Figure 3.2). Before sealing both sides, 1 µL of 100 ppm 2-octanone was added to the headspace in order to aid in normalization between samples. The same procedure was conducted on the same plants at approximately six weeks for flower VOC collection.

A similar approach was undertaken for ground leaf tissue. An expanding leaf from a four-week-old tomato plant was weighed and frozen in liquid nitrogen and stored at -80°C until time of analysis. Leaves were placed in 2 mL screw cap tubes (ThermoFisher) containing 8-12 2.3 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK). At time of analysis, tubes were taken out of the freezer and subsequently homogenized for 2 min at 1500 rpm using a bead homogenizer (MoBio Laboratories). The tubes containing the homogenized tissue were briefly pulsed in a centrifuge to loosely pellet the tissue at the bottom of the tube, at which point the caps were removed and replaced with parafilm. VOCs were then accumulated in the re-sealed tubes for 40 min, followed by insertion and exposure of a 65 µm PDMS/DVB SPME fibre into the headspace for a 20 min collection period. After 20 min, the SPME fibres were sequentially run on the GC-MS using the same settings as those detailed for the *in vivo* analysis. For both *in vivo* and ground tissue VOCs, analysis was carried out using AMDIS, PARADISe and SpectConnect as detailed in section 2.2.3.

Figure 3.2. *In vivo* collection system for MicroTom VOC emissions. Each plant was carefully enclosed within the 27 mL glass adapter for 40 min, at which point an SPME fibre was introduced through the top layer of parafilm and exposed to the headspace for 20 min. After 20 min of collection, the fibre was withdrawn and inserted into the GC-MS for analysis.

3.2.5 Leaf Carotenoid Analysis

In addition to assessing the VOC profiles of tomatoes expressing *AtCCD4*, the two main carotenoid compounds present in tomato leaves were extracted and measured using a rapid high performance liquid chromatography (HPLC) protocol (Kormendi et al., 2016). After *in vivo* VOC collection, the same leaves were excised from the plant, weighed, placed in 2 mL screw cap tubes (ThermoFisher) containing 8-12 2.3 mm zirconia/silica beads (BioSpec Products, Bartlesville, OK) and snap frozen in liquid nitrogen. 500 µL of a mixture of HPLC grade extraction solvents (2:1:1 hexane:acetone:ethanol, ThermoFisher) was added to the frozen tissue and subsequently homogenized for 2 min at 2500g using a bead homogenizer (MoBio Laboratories). The tubes were transferred to an Eppendorf™ thermomixer (Eppendorf Canada, Mississauga, ON) and mixed for 2 min at 1000 rpm followed by centrifugation at 2655g for 2 min at 4°C, at which point the supernatant was transferred to a second tube. The extraction was then repeated on the pellet two additional times, pooling the supernatants after each repetition. After pooling, the extracts were dried under a stream of nitrogen, reconstituted in 1 mL of 5:4:1 acetonitrile:dichloromethane/methanol (ACN/DCM/MeOH, ThermoFisher) containing 0.5% (w/v) 2,6-di-*tert*-butyl-4-methylphenol (BHT, SigmaAldrich), and passed through 0.2 µm nylon syringe driven filter units.

An Agilent 1200 Series HPLC coupled with a G1315D diode array detector (DAD) was used for all analyses (Agilent Technologies, Mississauga, ON). Carotenoids were separated on a Poroshell 120 EC-C18, 4.6 x 75 mm, 2.7 µm particle size column behind a poroshell 120 Fast Guard EC-C18 guard column (Agilent Technologies). A mix of MeOH, methyl tert-butyl ether (MTBE), and water were used for carotenoid elution. The gradient parameters are noted in Table 3.2. The identities of the lutein and β-carotene peaks detected were validated by comparison to the retention times and absorption spectra of the respective commercial standards (CaroteNature, Switzerland).

Table 3.2. Solvent gradient parameters for HPLC analysis of lutein and β-carotene.

Time (Minutes)	MeOH%	MTBE%	Water%
0	80	0	20
7	100	0	0
7.5	100	0	0
8.5	5	95	0
10.5	5	95	0
11	100	0	0
11.5	100	0	0
12	80	0	20
14	80	0	20

3.2.6 *Trialeurodes vaporariorum* Oviposition Preference Bioassay

To determine if the VOC emissions from the tomato lines expressing *AtCCD4* had an effect on greenhouse whiteflies, choice oviposition preference bioassays were conducted. Greenhouse whiteflies *T. vaporariorum* were reared on tomato cv. Foronti in a greenhouse at the Harrow Research and Development Centre, Harrow, ON. The greenhouse was maintained at 20-26°C with a relative humidity of 55-70%. The greenhouse was kept on a 16:8 hr light:dark photoperiod with supplemental lighting provided when levels fell below 200-300 w/m^2. Whiteflies were collected by gently shaking them off of tomato leaves near the shoot apex and into perforated plastic bags. Bags containing whiteflies were then placed into insulated Styrofoam containers for transport to the London Research and Development Centre, where they were released in a 60 cm x 60 cm x 60 cm mesh insect rearing cage (BugDorm, MegaView Science Co., Taiwan) containing two 3-week-old Foronti plants. Whiteflies were held in the rearing cage for no longer than seven days before being used in an assay. The growth chamber used for holding whiteflies and oviposition bioassays was held at 23°C and 70% relative humidity, with a 16:8 hr light:dark cycle.

Each assay was set up in a 30 cm x 30 cm x 30 cm insect rearing cage (BugDorm). One mutant and one EV control plant were placed in opposite corners of each cage (Figure 3.3). Both plants were approximately six weeks old and were matched visually in an attempt to ensure plant size, flower numbers, leaf numbers and leaf area were as similar as possible for each assay. Aluminum foil was placed over the soil of each pot to reduce soil water loss over the duration of the assay as well as aid in the recovery of whiteflies after assay completion. Forty mixed age, mixed sex whiteflies were added to each cage by gently tapping them off of the tomato leaves and into a 250 mL Erlenmeyer flask, which was then placed in the middle of the assay cage between the two choice plants. The assay cages were sealed and the whiteflies allowed to oviposit for a total of 72 hr, at which point the assay cages were transferred to 4°C to arrest oviposition. Leaves and flowers were removed from each plant and examined under a dissecting microscope in order to count whitefly eggs. A minimum of four choice assays were conducted for each line.

Figure 3.3. Cage trial setup for *T. vaporariorum* oviposition preference bioassay. One EV control and one mutant plant expressing *AtCCD4* were placed in a cage for three days with 40 mixed age, mixed sex *T. vaporariorum* adults. After three days, the eggs on the leaves and flowers were counted and trial totals were averaged for each line.

3.3 Results

3.3.1 Analysis of *AtCCD4* Expression Levels in Transgenic Tomato

Initially, six T2 lines expressing *AtCCD4* (24, 27, 28, 29, 41, 44) were chosen for expression level analysis alongside EV plants. The T2 plants selected for analysis were confirmed to be transgenic through PCR and were visually indistinguishable from EV plants (Figure 3.4A). After the plants were confirmed transgenic, the expression level of the transgene was assessed using RT-qPCR. Of the reference genes tested, *SlActin* (CV=0.2329, M-value=0.5125) and *SlCAC* (CV=0.1527, M-value=0.4490) were the most stable and were used for expression analysis while *SlEXP* (CV=0.3025, M-value=0.7277) was disregarded. Each primer used was tested for non-specific binding potential using NCBI Primer-BLAST, which suggested that only one target would be amplified for each primer pair. This was confirmed by post RT-qPCR melt curve analysis, where a single melt curve was observed for each target gene. The *AtCCD4* transcript was detected in all six lines tested, but not in any EV or no template controls (Figure 3.4B). Of the lines tested, lines 24 and 29 had the lowest levels of expression while lines 27 and 41 had the highest levels of expression, albeit with more variation. From this analysis, lines 24, 28, 41, and 44 were chosen for follow-up expression analysis, where the entire process was repeated to confirm the expression patterns of the four lines. Due to a lack of seeds for lines 24-15 and 41-13, the related lines 24-5 and 41-5 were used as replacements for the second round of expression analysis. This repetition confirmed the expression patterns seen in the previous analysis (Figure 3.4C), with the replacement lines 24-5 and 41-5 showing similar expression to 24-15 and 41-13 respectively. As such, lines 24-5, 28-13, 44-12, and 41-5 were selected for further analysis through VOC and oviposition trials.

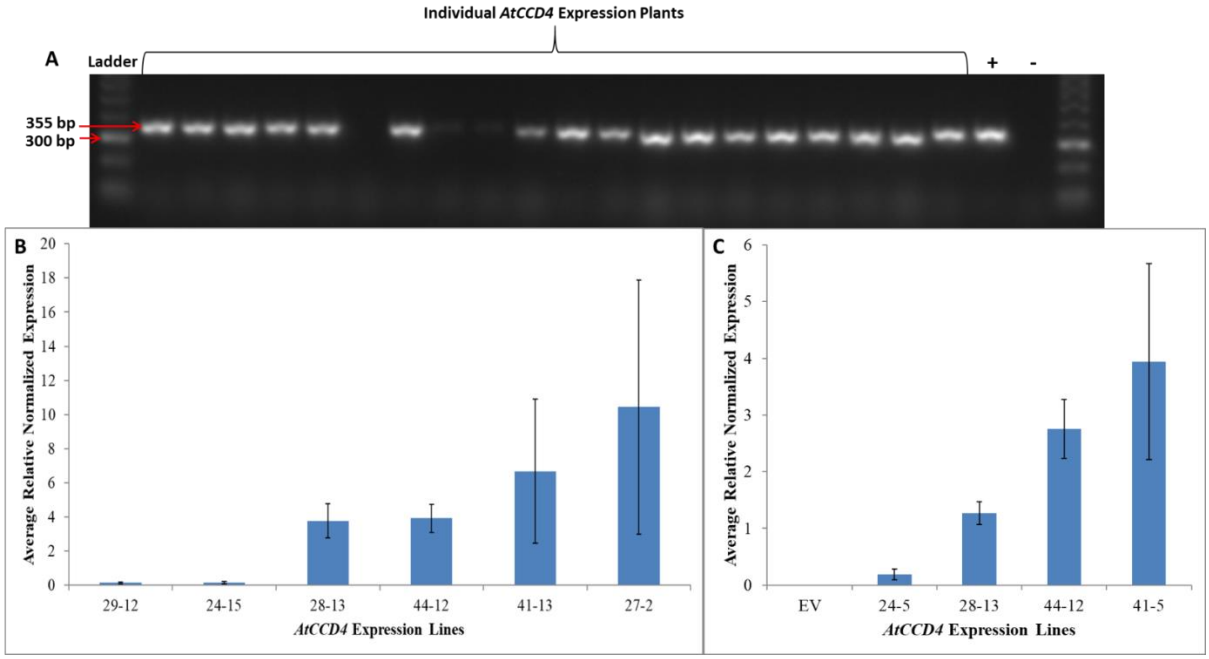


Figure 3.4. Genotyping and expression analysis in leaves of transgenic tomatoes expressing *AtCCD4*. Individuals were genotyped to confirm the presence of the transgene (A). An EV plant was used as a negative control (-) while the pMDC-32 expression vector containing the *AtCCD4* insert was used as a positive control (+). Six lines (24, 27, 28, 29, 41, 44) were initially screened for levels of expression (B). Four lines (24, 28, 41, 44) were selected for repeated analysis to confirm the expression pattern (C). A minimum of two (B) or four (C) biological replicates were averaged, with the error bars representing the standard error of the mean. Lines 24-5, 28-13, 41-5, and 44-12 were advanced for VOC analysis and oviposition preference assays.

3.3.2 Analysis of Tomato VOCs *in vivo*

Leaf and flower VOC emissions were collected *in vivo* to determine if *AtCCD4* expression had an effect on the non-damaged VOC profile of MicroTom plants. While changes to the apocarotenoid volatiles were expected, the untargeted approach to VOC collection and analysis allowed for the observation of all classes of VOCs. In both leaf and flower collections, 1 μL of 2-octanone was added as an internal standard. The resulting 2-octanone peak area was recorded from each sample along with which individual SPME fibre was used for the collection. This data is depicted in Figure 3.5, which illustrates the inherent variability that accompanies the sampling system used. In addition, comparison of 2-octanone peaks using ANOVA suggests a significant statistical difference exists in the raw peak areas of the lines tested (Table 3.3). Due to this inconsistency, 2-octanone was not used for normalization and the raw percentage peak areas of the tentatively identified features are reported below.

From leaves, the major compound that was consistently detected at high levels was the monoterpene α-pinene, which made up between 34.39% (41-13) and 59.60% (line 24-5) of the entire emission profile (Table 3.3). The second most common compound was the green leaf volatile (GLV) 3-hexanol, which comprised between 9.11% (24-5) and 15.75% (28-13) of emissions. Other minor VOCs were D-limonene, o-cymene, isocaryophyllene, and α-thujene. In total, 52 compounds were tentatively identified from leaf VOC emissions. The only apocarotenoid compound detected from *in vivo* leaf collections was 6-methyl-5-hepten-2-one, which was only found in minute amounts in the VOCs from lines 24-5 (0.11%) and 41-5 (0.13%).

Principle component analysis (PCA) of the *in vivo* leaf volatile profiles, which consisted of 356 features, revealed differences between EV and *AtCCD4* expressing plants along principle component (PC) 1 (12.5%) (Figure 3.6). Line 41-5, which expressed *AtCCD4* at the highest level relative to the other lines, was the most different from the EV control plants. Transgenic lines 24-5 and 44-12 were also separated from the EV line along principle component 2 (11.2%). Similar analysis was done on only the 52 identified compounds revealed separation between line 41-5 and the EV controls, as well as some separation between line 24-5 (Figure 3.7). Both lines were slightly separated from EV controls along PC1 (18.7%), as well as in different directions along

PC2 (15.0%). However, the differences between lines were not as pronounced when only tentatively identified compounds were used for PCA construction.

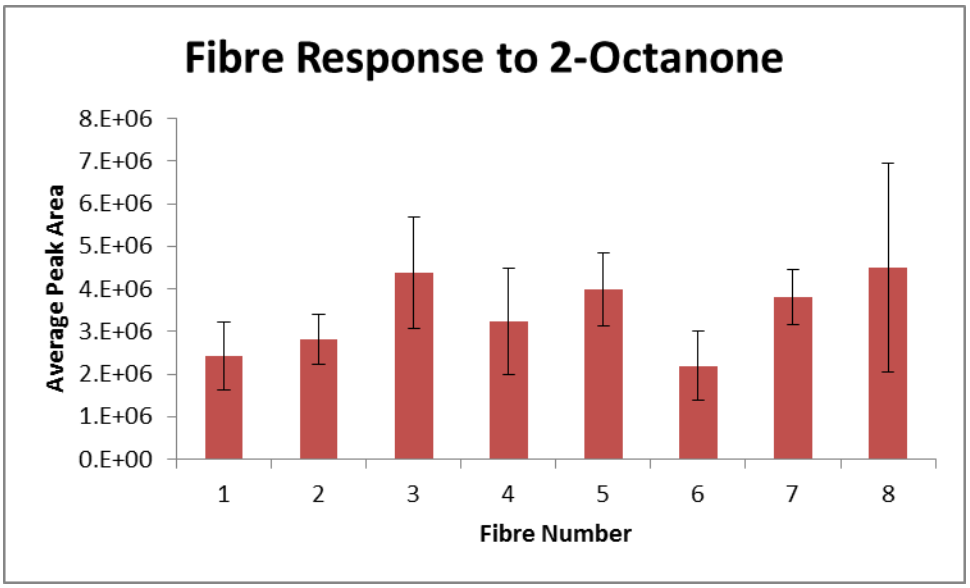


Figure 3.5. Response of the SPME fibres used for *in vivo* VOC collection to the internal standard 2-octanone. Average peak area (± Standard Error of the Mean (SEM)) of 2-octanone from each leaf and flower collection were compiled according to which fibre was used for collection. Depending on the fibre the peak area varied greatly, giving an indication of the variability within the manual SPME collection system used for subsequent analysis.

Table 3.3. Tentatively identified VOCs emitted from tomato lines expressing *AtCCD4* and EV control leaves. Features are listed by retention time (RT) and average (Avg) of the peak areas along with standard error of the mean (SEM) are shown, with each calculated from a minimum of five biological replicates. P-Val is indicative of the p-value returned from a one-way ANOVA test conducted on each feature, with values less than .05 considered significant. A minimum of 60% mass spectral match (%M) was set for tentative identification of the compounds listed. Compounds that were not detected in the profiles of a specific line are marked as n.d.

Table 3.3				**EV**		**24-5**		**28-13**		**44-12**		**41-5**		
Tentative Compound ID	**m/z**	**%M**	**RT**	**Avg**	**± SEM**	**Avg**	**± SEM**	**Avg**	**± SEM**	**Avg**	**± SEM**	**Avg**	**± SEM**	**p-val**
2-Methyl-2-pentanol	59	97	4.56	0.81	± 0.23	0.62	± 0.17	1.18	± 0.48	0.73	± 0.09	0.54	± 0.06	0.81
3-Methyl-3-pentanol	73	97	5.03	1.22	± 0.36	0.84	± 0.28	1.74	± 0.59	1.56	± 0.61	0.71	± 0.12	0.74
Bicyclo[3.2.0]hepta-2,6-diene	91	89	5.22	0.09	± 0.04	0.11	± 0.04	0.24	± 0.11	0.23	± 0.07	0.06	± 0.02	0.49
5-Hexen-2-one	43	66	5.45	n.d.	± -	n.d.	± -	0.38	± 0.12	0.36	± 0.16	0.58	± 0.21	0.01
1,2-Dimethoxypropane	59	63	5.51	0.12	± 0.04	n.d.	± -	0.40	± 0.19	1.76	± 1.25	1.07	± 0.73	0.64
3-Methoxy-2-butanol	59	73	5.56	0.06	± 0.02	0.05	± 0.01	0.13	± 0.03	1.81	± 0.95	3.27	± 1.72	0.55
2-Methyl-3-pentanol	59	73	5.62	0.15	± 0.04	4.85	± 1.61	2.76	± 2.32	0.16	± 0.04	2.16	± 1.47	0.45
3-Hexanol	59	97	5.96	12.98	± 1.97	9.11	± 2.20	15.75	± 3.68	13.28	± 1.66	15.43	± 2.57	0.49
2-Hexyl hydroperoxide	43	65	6.06	3.59	± 0.72	1.75	± 0.42	3.78	± 0.82	2.68	± 0.37	3.28	± 0.57	0.48
Hexanal	44	90	6.71	n.d.	± -	0.18	± 0.05	0.51	± 0.23	n.d.	± -	0.19	± 0.06	0.12
4-Hydroxy-4-methyl-2-pentanone	43	70	7.07	3.99	± 2.00	0.05	± 0.01	0.14	± 0.04	0.11	± 0.03	0.08	± 0.02	0.13
3,4-Dimethyl-2-hexanone	43	66	7.47	0.34	± 0.22	n.d.	± -	n.d.	± -	n.d.	± -	0.06	± 0.02	0.09
p-Xylene	91	83	7.95	0.08	± 0.02	n.d.	± -	0.06	± 0.03	0.13	± 0.03	n.d.	± -	0.01
α-Pinene	93	98	9.96	42.00	± 6.79	59.60	± 5.59	40.06	± 11.03	42.31	± 3.65	34.39	± 4.23	0.11
6-Ethyl-o-cresol	121	63	10.14	n.d.	± -	7.68	± 3.96	n.d.	± -	n.d.	± -	n.d.	± -	0.40
α-Thujene	93	63	10.23	4.99	± 3.82	1.87	± 1.24	0.29	± 0.17	4.08	± 3.31	4.90	± 3.52	0.77
(E,E)-2,4-Heptadien-6-ynal	105	67	11.06	0.13	± 0.05	0.04	± 0.01	0.03	± 0.01	0.02	± 0.01	0.04	± 0.01	0.31
2-Pentanone	43	90	11.09	n.d.	± -	n.d.	± -	n.d.	± -	n.d.	± -	0.20	± 0.05	0.01
Valeraldehyde	43	64	11.43	n.d.	± -	n.d.	± -	n.d.	± -	n.d.	± -	16.03	± 6.06	0.08
6-Methyl-5-hepten-2-one	43	77	11.61	n.d.	± -	0.11	± 0.03	n.d.	± -	n.d.	± -	0.13	± 0.04	0.06
5-Methyl-1-phenyl- 1-hexanone	105	73	11.74	0.51	± 0.27	0.11	± 0.04	0.15	± 0.05	n.d.	± -	n.d.	± -	0.01

Table 3.3 Cont.				EV		24-5		28-13		44-12		41-5		
Tentative Compound ID	**m/z**	**%M**	**RT**	**Avg**	**± SEM**	**Avg**	**± SEM**	**Avg**	**± SEM**	**Avg**	**± SEM**	**Avg**	**± SEM**	**p-val**
2-Octanone	45	94	12.19	5.48	± 1.75	1.16	± 0.40	4.55	± 1.14	2.27	± 0.46	1.77	± 0.46	0.01
2,2,4,6,6-Pentamethyl-3-heptene	57	96	12.39	2.73	± 1.02	1.71	± 0.47	4.80	± 1.05	4.26	± 0.92	4.15	± 1.08	0.30
2,2,4-Trimethylpentane	57	63	12.59	0.12	± 0.03	0.06	± 0.02	0.13	± 0.03	0.24	± 0.07	0.19	± 0.05	0.85
Mesitylene	105	63	12.75	0.16	± 0.06	0.08	± 0.02	0.14	± 0.04	0.11	± 0.04	0.11	± 0.01	0.85
Artemisia ketone	83	72	12.81	n.d.	± -	n.d.	± -	0.16	± 0.06	n.d.	± -	n.d.	± -	0.01
1-Methyl-3-propyl-cyclooctane	69	60	12.83	0.90	± 0.33	0.29	± 0.08	0.74	± 0.19	0.72	± 0.11	0.73	± 0.14	0.24
o-Cymene	119	96	12.96	0.88	± 0.13	1.16	± 0.24	1.06	± 0.24	1.02	± 0.30	0.87	± 0.06	0.10
D-Limonene	68	71	13.08	3.41	± 0.75	4.18	± 0.68	2.99	± 0.87	2.89	± 0.51	3.18	± 0.19	0.25
Trans-1-ethyl-1,3-dimethyl-cyclohexane	69	67	13.13	0.41	± 0.07	0.23	± 0.06	0.44	± 0.05	0.37	± 0.07	0.42	± 0.07	0.26
2-Ethyl-1-hexanol	57	61	13.31	n.d.	± -	n.d.	± -	1.01	± 0.39	n.d.	± -	0.45	± 0.11	0.13
2,6-Dimethyloctane	57	60	13.61	0.27	± 0.07	n.d.	± -	0.27	± 0.07	0.21	± 0.06	0.35	± 0.09	0.18
2,4,4-Trimethyl- 1-pentene	57	77	13.95	2.54	± 1.02	1.34	± 0.38	3.76	± 0.87	3.98	± 0.81	3.40	± 0.92	0.29
1-Methyl-2-propyl-benzene	105	71	14.08	0.17	± 0.04	0.07	± 0.02	0.14	± 0.03	0.18	± 0.05	0.16	± 0.06	0.77
2,3-Dimethyldecane	43	76	14.49	0.60	± 0.24	0.21	± 0.05	0.64	± 0.13	0.51	± 0.14	0.47	± 0.09	0.39
2,4,4-Triethyl- 1-hexene	57	78	14.97	1.03	± 0.49	0.70	± 0.26	1.71	± 0.41	1.86	± 0.34	1.66	± 0.50	0.38
2,2,4-trimethyl-1-pentanol	57	60	15.10	n.d.	± -	n.d.	± -	n.d.	± -	n.d.	± -	1.68	± 0.60	0.09
1-Methylindan	117	61	15.31	n.d.	± -	0.09	± 0.03	n.d.	± -	n.d.	± -	n.d.	± -	0.01
Undecane	57	88	15.46	6.41	± 3.26	1.91	± 0.40	4.17	± 0.93	3.93	± 0.69	3.19	± 0.52	0.81
β-Cymene	119	66	16.68	0.22	± 0.12	0.07	± 0.02	0.19	± 0.05	0.13	± 0.03	0.14	± 0.04	0.41
1,3-Diethyl-5-methyl-benzene	119	68	16.86	n.d.	± -	n.d.	± -	0.05	± 0.01	n.d.	± -	0.07	± 0.02	0.00
3,5,5-Trimethyl-1-hexene	57	61	17.59	1.14	± 0.49	0.46	± 0.14	0.98	± 0.20	0.97	± 0.23	0.69	± 0.17	0.55
2,3-Dimethyloctane	57	60	17.79	n.d.	± -	0.11	± 0.05	0.34	± 0.10	n.d.	± -	0.09	± 0.03	0.00
Methyl salicylate	120	92	18.31	n.d.	± -	0.15	± 0.05	n.d.	± -	0.34	± 0.08	0.19	± 0.07	0.15
2,4-Dimethyldecane	43	77	18.61	3.12	± 1.22	1.41	± 0.33	2.53	± 0.63	2.14	± 0.36	1.73	± 0.37	0.91
1-(3-Ethyloxiranyl)-ethanone	43	68	20.27	n.d.	± -	0.01	± 0.00	n.d.	± -	n.d.	± -	0.02	± 0.01	0.02
Diisobutylene	97	64	20.70	0.29	± 0.10	0.23	± 0.07	0.22	± 0.07	0.21	± 0.04	0.18	± 0.06	0.36
cis-1,1,3,5-Tetramethylcyclohexane	69	73	21.40	0.14	± 0.03	0.13	± 0.04	0.14	± 0.05	0.18	± 0.07	n.d.	± -	0.24
δ-EIemene	121	81	22.66	n.d.	± -	0.86	± 0.43	n.d.	± -	n.d.	± -	n.d.	± -	0.25
(E)-5-Butoxy-2-pentene	57	75	24.75	0.14	± 0.03	0.08	± 0.03	0.11	± 0.03	0.11	± 0.04	0.06	± 0.02	0.64
Isocaryophyllene	41	86	25.06	1.93	± 0.49	2.25	± 1.00	4.68	± 1.61	10.36	± 4.14	1.04	± 0.28	0.68
Santolina triene	93	73	26.03	0.28	± 0.09	0.40	± 0.18	n.d.	± -	n.d.	± -	n.d.	± -	0.35

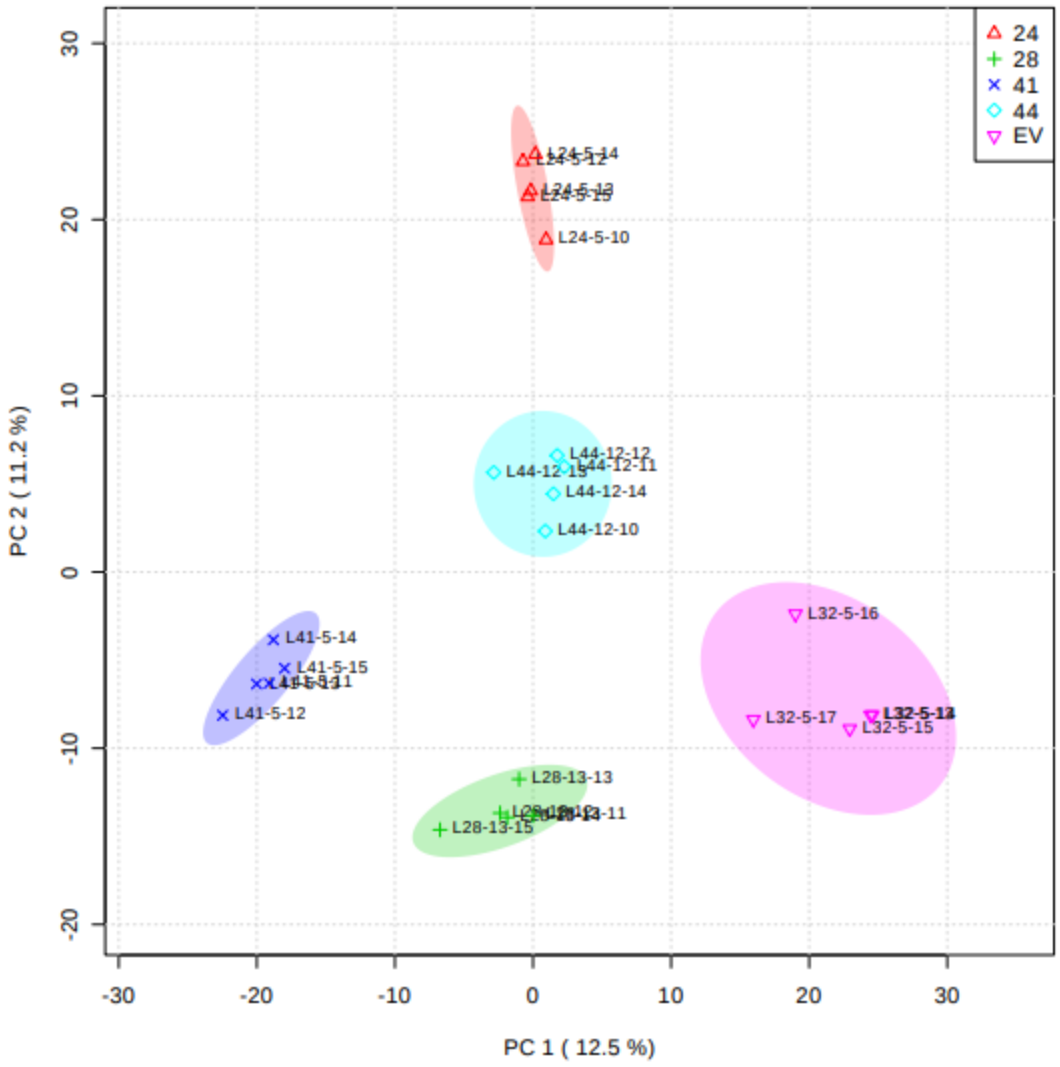


Figure 3.6. Principle component analysis of all detected leaf VOC features from EV and *AtCCD4* expression lines. Each point is one biological replicate consisting of the first expanded leaf of a single 4-week-old tomato plant. Line 41-5 was the most different than EV along PC1, while line 24-5 was the most different relative to EV along PC2.

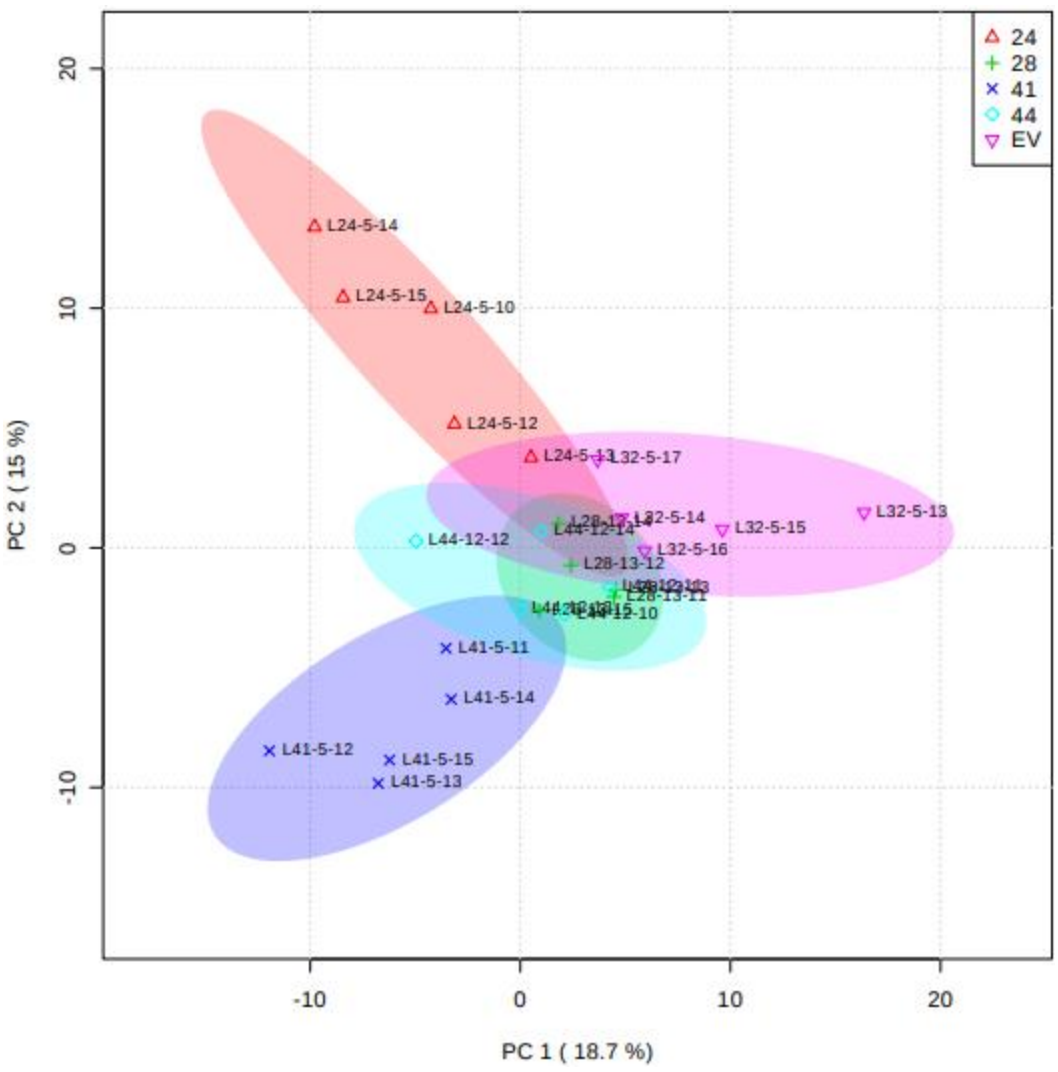


Figure 3.7. Principle component analysis of tentatively identified leaf VOC features from plants expressing *AtCCD4* and EV controls. Each point is one biological replicate consisting of the first expanded leaf of a single 4-week-old tomato plant. Line 41-5 differed the most from EV, followed by 24-5. In general, the remaining lines overlapped with the EV controls. The differences are not as pronounced as those present when all features are taken into consideration for PCA construction.

From the flower VOC profiles, 43 compounds were tentatively identified, with α-pinene again making up the bulk of the VOC profile. α-pinene accounted for between 73.16% (line 44-12) and 83.00% (line 24-5) of VOC emissions (Table 3.4). The monoterpenes D-limonene and α-thujene along with the sesquiterpene isocaryophyllene were also present in relatively high amounts compared to compounds other than α-pinene. As was the case with the leaf VOC profiles the only apocarotenoid detected was 6-methyl-5-hepten-2-one. In contrast to the leaves however, 6-methyl-5-hepten-2-one was detected in all lines, comprising between 0.18% (44-12) and 0.61% (24-5) of emissions. Principle component analysis of 255 flower features revealed lines 41-5 and 44-12 to be the most different relative to EV control plants (Figure 3.8). These two lines separate from EV controls along principle component 1 (10.2%) and separate from each other along principle component 2 (9.8%). Flower VOCs from lines 24-5 and 28-13 appear to have some overlap with EV control profiles, as the separation is not as prominent as for lines 41-5 and 44-12. When only tentatively identified features were used for PCA plot construction, the separation between the transgenic and non-transgenic lines was reduced substantially (Figure 3.9). Only a few individuals from lines 44-12 and 41-5 showed any separation from EV along PC1 (14.7%). All lines had individuals scattered along PC2 (10.4%), and no distinct pattern could be discerned.

Table 3.4. Tentatively identified VOCs emitted from plants expressing *AtCCD4* and EV control flowers. All identified features are listed by retention time (RT). Averages (Avg) of the peak areas along with standard error of the mean (SEM) are shown, with each calculated from a minimum of eight biological replicates. P-Val is indicative of the p-value returned from a one-way ANOVA test conducted on each feature, with values less than .05 considered significant. A minimum of 60% mass spectral match (%M) was set for tentative identification of the compounds listed. Compounds that were not detected in the profiles of a specific line are marked as n.d.

Table 3.4				**EV**			**24-5**			**28-13**			**44-12**			**41-5**			
Tentative Compound ID	**m/z**	**%M**	**RT**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**p-val**
Bicyclo[3.2.0]hepta-2,6-diene	91	89	5.22	0.15	±	0.07	0.21	±	0.11	0.09	±	0.02	0.08	±	0.02	0.05	±	0.01	0.44
5-Hexen-2-one	43	66	5.45	n.d.	±	-	n.d.	±	-	0.30	±	0.07	0.12	±	0.03	0.39	±	0.11	0.13
1,2-Dimethoxypropane	59	63	5.51	n.d.	±	-	0.70	±	0.34	0.26	±	0.08	1.24	±	0.65	1.63	±	0.42	0.55
3-Methoxy-2-butanol	59	73	5.56	0.22	±	0.07	0.06	±	0.02	n.d.	±	-	n.d.	±	-	2.40	±	0.69	0.00
2-Methyl-3-pentanol	59	73	5.62	1.10	±	0.77	0.26	±	0.08	0.88	±	0.28	1.47	±	0.40	0.28	±	0.09	0.13
3-Hexanol	59	97	5.96	1.41	±	0.36	2.71	±	0.56	1.91	±	0.33	3.21	±	1.14	4.36	±	1.27	0.98
Hexanal	44	90	6.71	0.49	±	0.10	n.d.	±	-	1.02	±	0.20	n.d.	±	-	n.d.	±	-	0.00
4-Hydroxy-4-methyl-2-pentanone	43	70	7.07	n.d.	±	-	n.d.	±	-	0.05	±	0.01	n.d.	±	-	0.10	±	0.02	0.00
2-Hexenal	41	75	7.41	0.12	±	0.02	0.08	±	0.02	0.13	±	0.03	0.07	±	0.02	0.14	±	0.03	0.20
(E)-3-Hexen-1-ol	41	82	7.54	0.07	±	0.01	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.00
Propylcyclopropane	56	86	7.87	0.19	±	0.04	0.49	±	0.16	0.19	±	0.03	0.17	±	0.03	n.d.	±	-	0.13
p-Xylene	91	83	7.95	0.11	±	0.03	n.d.	±	-	n.d.	±	-	0.03	±	0.01	0.05	±	0.01	0.00
α-Pinene	93	98	9.96	79.24	±	2.31	83.00	±	1.25	78.04	±	3.82	73.16	±	4.34	73.54	±	3.06	0.31
α-Thujene	93	63	10.23	2.29	±	1.56	0.52	±	0.16	6.85	±	3.71	6.92	±	4.25	1.49	±	0.90	0.53
(E,E)-2,4-Heptadien-6-ynal	105	67	11.06	0.10	±	0.06	0.06	±	0.02	0.06	±	0.02	0.07	±	0.03	0.45	±	0.36	0.55
6-Methyl-5-hepten-2-one	43	77	11.61	0.53	±	0.13	0.61	±	0.29	0.22	±	0.05	0.18	±	0.03	0.35	±	0.08	0.51
5-Methyl-1-phenyl-1-hexanone	105	73	11.74	n.d.	±	-	0.04	±	0.01	0.07	±	0.02	0.06	±	0.02	0.08	±	0.03	0.17
2-Octanone	45	94	12.19	1.31	±	0.21	2.37	±	0.69	1.33	±	0.10	1.75	±	0.38	2.09	±	0.44	0.64
2,2,4,6,6-Pentamethyl-3-heptene	57	96	12.39	n.d.	±	-	n.d.	±	-	0.58	±	0.18	0.94	±	0.24	1.55	±	0.69	0.34
2,2,4-Trimethylpentane	57	63	12.59	n.d.	±	-	0.03	±	0.01	0.04	±	0.01	0.30	±	0.10	1.23	±	0.73	0.18
Mesitylene	105	63	12.75	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.08	±	0.01	n.d.	±	-	0.00

Table 3.4 Cont.				EV			24-5			28-13			44-12			41-5			
Tentative Compound ID	**m/z**	**%M**	**RT**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**p-val**
1-Methyl-3-propyl-cyclooctane	69	60	12.83	0.10	±	0.03	0.14	±	0.03	0.21	±	0.04	0.23	±	0.04	0.31	±	0.09	0.05
o-Cymene	119	96	12.96	1.49	±	0.15	1.55	±	0.10	1.20	±	0.08	1.28	±	0.08	1.51	±	0.16	0.32
D-Limonene	68	71	13.08	7.46	±	1.02	6.49	±	0.70	4.97	±	0.40	5.22	±	0.41	5.56	±	0.54	0.14
Trans-1-ethyl-1,3-dimethyl-cyclohexane	69	67	13.13	0.07	±	0.01	0.08	±	0.01	0.13	±	0.03	0.22	±	0.04	0.23	±	0.08	0.60
2,4,4-Trimethyl- 1-pentene	57	77	13.95	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.56	±	0.14	n.d.	±	-	0.03
1-Methyl-2-propyl-benzene	105	71	14.08	0.13	±	0.04	0.05	±	0.01	0.04	±	0.01	0.06	±	0.02	0.10	±	0.03	0.75
2,3-Dimethyldecane	43	76	14.49	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.23	±	0.06	n.d.	±	-	0.00
Undecane	57	88	15.46	1.02	±	0.36	n.d.	±	-	n.d.	±	-	1.46	±	0.31	2.45	±	0.60	0.00
Nonanal	57	80	15.56	0.40	±	0.20	0.33	±	0.06	0.42	±	0.13	0.21	±	0.04	0.16	±	0.04	0.32
β-Cymene	119	66	16.68	0.04	±	0.01	n.d.	±	-	0.04	±	0.01	0.03	±	0.01	0.06	±	0.02	0.13
3,5,5-Trimethyl-1-hexene	57	61	17.59	0.27	±	0.07	0.47	±	0.14	0.32	±	0.08	0.29	±	0.05	0.48	±	0.10	0.16
Methyl salicylate	120	92	18.31	0.14	±	0.03	0.11	±	0.02	0.23	±	0.06	0.22	±	0.09	0.74	±	0.29	0.41
2,4-Dimethyldecane	43	77	18.61	0.29	±	0.06	n.d.	±	-	0.33	±	0.08	0.43	±	0.13	n.d.	±	-	0.27
Decanal	43	80	18.77	0.29	±	0.08	0.58	±	0.15	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.00
1-(3-Ethyloxiranyl)-ethanone	43	68	20.27	0.01	±	0.00	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.15	±	0.06	0.13
Diisobutylene	97	64	20.70	0.09	±	0.02	0.05	±	0.01	0.07	±	0.01	0.08	±	0.02	0.16	±	0.04	0.78
cis-1,1,3,5-Tetramethylcyclohexane	69	73	21.40	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.03	±	0.01	0.03	±	0.01	0.01
δ-EIemene	121	81	22.66	0.38	±	0.09	0.31	±	0.07	0.44	±	0.11	0.56	±	0.13	0.70	±	0.18	0.79
(E)-5-Butoxy-2-pentene	57	75	24.75	0.05	±	0.01	n.d.	±	-	0.03	±	0.01	0.02	±	0.01	0.03	±	0.01	0.08
Isocaryophyllene	41	86	25.06	1.99	±	0.48	0.92	±	0.20	2.76	±	0.57	1.95	±	0.40	1.64	±	0.55	0.05
Santolina triene	93	73	26.03	0.26	±	0.08	n.d.	±	-	0.45	±	0.10	0.28	±	0.05	0.27	±	0.09	0.00
2,5,9-Trimethyldecane	57	83	27.13	0.68	±	0.34	0.71	±	0.21	0.27	±	0.08	0.88	±	0.24	1.65	±	0.45	0.41

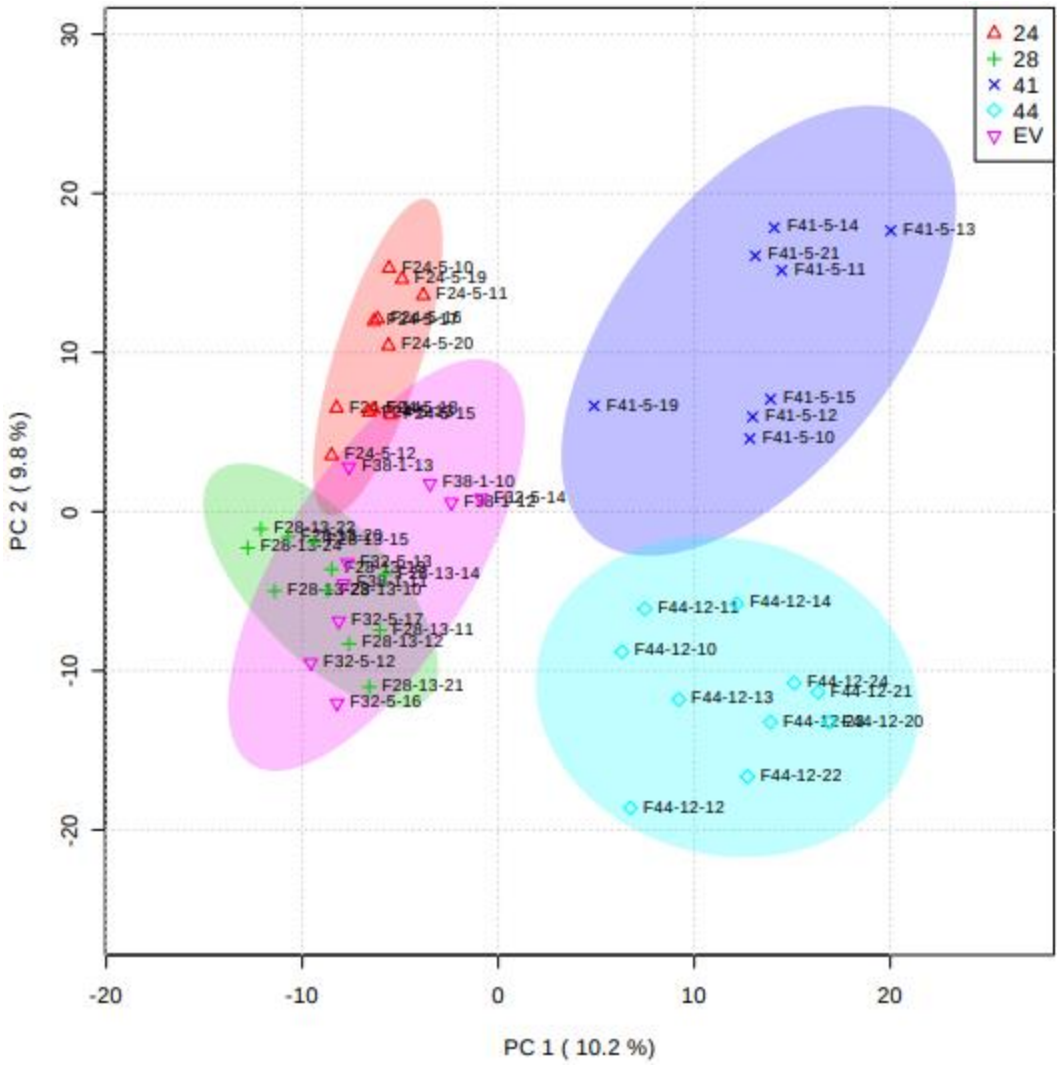


Figure 3.8. Principle component analysis of all detected flower VOC features from lines expressing *AtCCD4* and EV controls. Each point is one biological replicate consisting of a single open flower from a 6-week-old tomato plant. Lines 44-12 and 41-5 had the greatest differences relative to the other transgenic lines as well as EV controls. Overlap between EV, 24-5 and 28-13 suggest similarities in their VOC emission profiles.

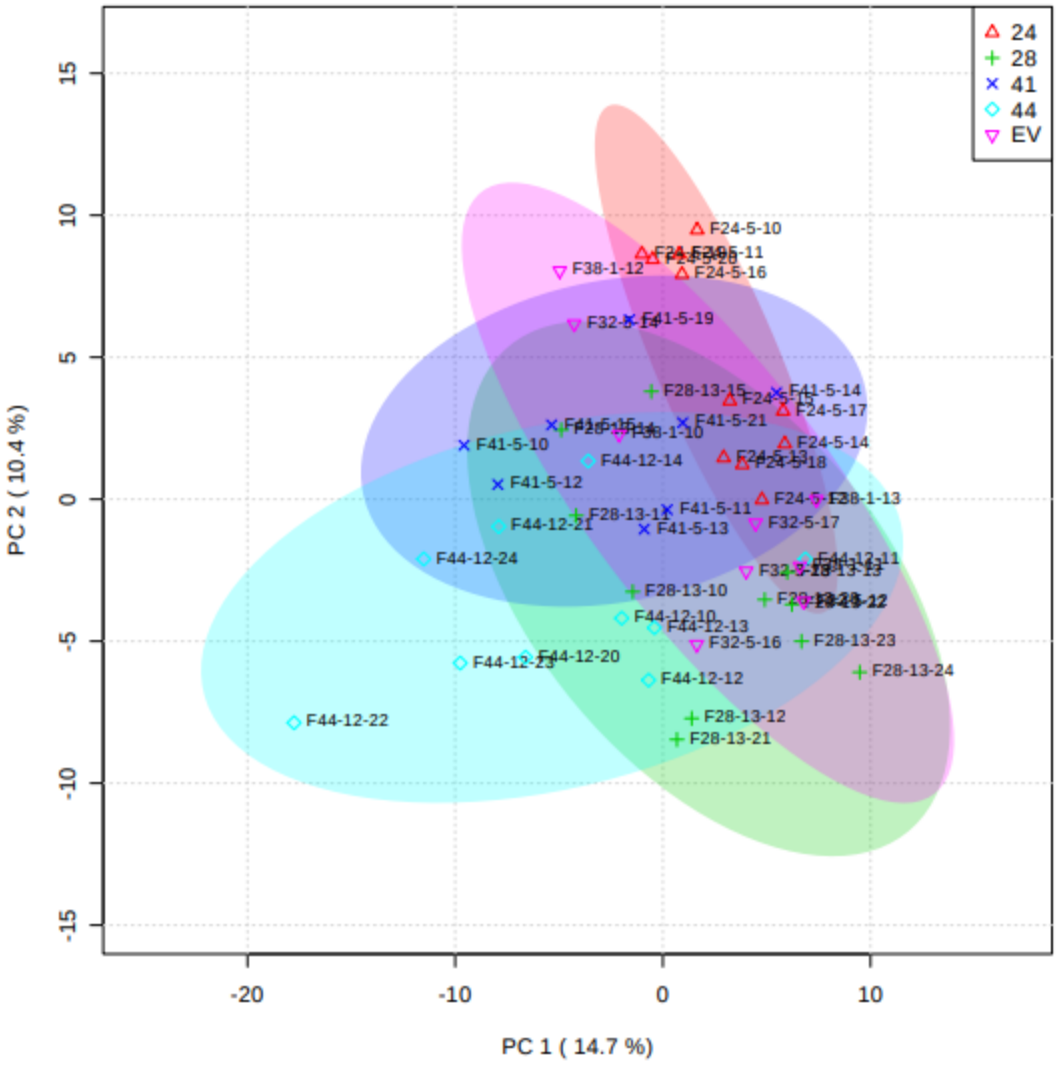


Figure 3.9. Principle component analysis of tentatively identified flower VOC features from lines expressing *AtCCD4* and EV controls. Each point is one biological replicate consisting of a single open flower from a 6-week-old tomato plant. Line 44-12 was the most different from the other transgenic lines as well as from EV controls, but most individuals within line 44-12 still overlapped with EV controls along with all the other transgenic lines.

3.3.3 Analysis of ground tomato leaf VOCs

Due to the lack of detection of any major volatile apocarotenoids besides 6-methyl-5-hepten-2-one from *in vivo* leaf and flower VOC collections, another experiment was required to determine if the expression of *AtCCD4* was having any direct effect on volatile apocarotanoid production. Since some volatile apocarotenoids are known to be sequestered as glycoside conjugates, leaf tissue was ground to promote cleavage of bound VOCs by native glycosidases (Lätari et al., 2015). In addition to liberating additional VOCs, the experiment was conducted in a 2 mL headspace instead of a 27 mL headspace in order to improve the chances of detection of any low concentration VOCs. Data were normalized to leaf weight prior to analysis.

After grinding the leaf tissue, headspace VOC analysis revealed that the most abundant components were the green leaf volatiles (GLVs) hexanal and 2-hexanal (Table 3.5). These two were present in nearly equal proportions, as hexanal comprised between 19.36% (line 24-5) and 32.22% (EV), while 2-hexanal accounted for 16.69% (line 24-5) and 36.54% (44-12). Another major component was α-pinene, although at lower proportions than *in vivo* collections, from 9.17% (line 44-12) to 30.82% (line 24-5). Other minor constituents were caryophyllene, β-thujene, σ-elemene, (E)-4-oxohex-2-enal, (E,E)-2,4-hexadienal, and methyl salicylate (MeSA). 86 VOCs were tentatively identified in total, vastly outnumbering those detected *in vivo*. This total also included two additional apocarotenoids, β-ionone and β-cyclocitral. Both were found in low amounts in every sample, yet no statistically significant differences in peak areas were found between the EV and transgenic lines (Figure 3.10).

Principle component analysis of 329 features from ground leaf VOC emission profiles illustrated differences between all four transgenic lines and the EV controls (Figure 3.11). Line 41-5 and 44-12 were the most similar to each other as well as to the EV controls, while lines 24-5 and 28-13 were the most different. In addition, these lines were different from the EV controls in different ways, as line 24-5 is separated along principle component 1 (20.6%), while line 28-13 is separated along principle component 2 (12.4%). When the same analysis was done using only the 86 features that had been tentatively identified, the differences between transgenic and non-transgenic lines was still evident (figure 3.12). A similar pattern could be seen with lines 24-5 and 28-13 separated from the EV control group by PC1 (19.3%) and PC2 (14.8%), respectively.

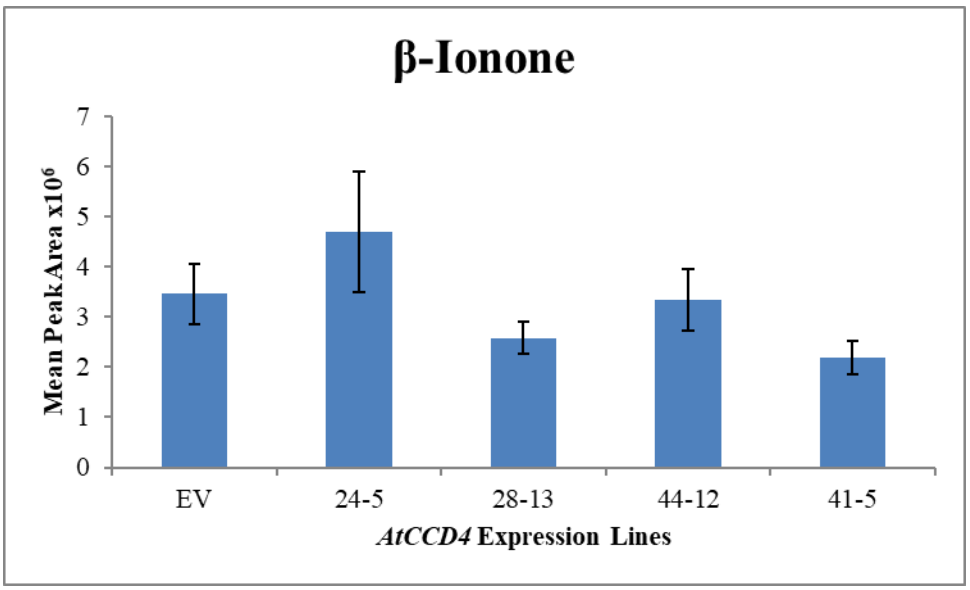
Also, lines 41-5 and 44-12 overlapped with the EV control group but trended away along PC2. Taken together, these results indicate that the differences that separate lines 24-5 and 28-13 are within the list of identified features. Looking at the ANOVA p-values, there are a large number of compounds that could be involved, including major VOCs such as α-pinene, β-thujene, D-limonene, as all these represent a larger percentage of the VOC profile in lines 24-5 and 28-13. There are also many other minor compounds which when combined together account for the separation seen in the PCAs.

Table 3.5. Tentatively identified VOCs collected from the headspace of ground leaves of plants expressing *AtCCD4*. Values reported are average percentage peak areas (±SEM) for a minimum of five biological replicates. P-vals were calculated using one-way ANOVA. VOCs are sorted by retention time (RT) and are listed with the mass-to-charge ratios (m/z) associated with their tentative identifications. A minimum of 60% mass spectral match (%M) was set for tentative identification of the compounds listed. Compounds that were not detected in the profiles of a specific line are marked as n.d., and any compounds that made up less than 0.01% of the peak area are marked as trace (t.r.)

Table 3.5				**EV**			**24-5**			**28-13**			**44-12**			**41-5**			
Tentative Compound ID	**m/z**	**%M**	**RT**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**p-val**
3,4-dihydro-2H-pyran	55	70	4.89	n.d.	±	-	0.05	±	0.01	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.06
(E)-2-Pentenal	55	95	4.98	0.22	±	0.03	0.07	±	0.02	0.14	±	0.02	0.33	±	0.12	0.17	±	0.02	0.26
3-Methyl-1-butanol	55	88	5.05	0.25	±	0.04	0.14	±	0.05	0.19	±	0.02	0.15	±	0.01	0.16	±	0.01	0.25
(Z)- 2-Penten-1-ol	57	94	5.34	0.44	±	0.09	0.08	±	0.02	0.20	±	0.05	0.49	±	0.08	0.23	±	0.04	0.36
Dimethylacetylacetone	43	65	5.45	0.05	±	0.01	0.02	±	0.01	0.04	±	0.01	0.04	±	0.01	0.02	±	0.00	0.31
3-Methyl-2-butenal	84	74	5.52	0.01	±	0.00	n.d.	±	-	t.r.	±	-	0.07	±	0.03	n.d.	±	-	0.11
Hexanal	44	97	6.11	32.22	±	2.13	19.36	±	2.71	27.79	±	2.65	29.59	±	1.46	30.99	±	2.85	0.80
1,3-Diazine	80	70	6.33	n.d.	±	-	n.d.	±	-	0.05	±	0.02	n.d.	±	-	n.d.	±	-	0.16
3-Hexenal	41	65	6.60	n.d.	±	-	n.d.	±	-	1.29	±	0.32	n.d.	±	-	n.d.	±	-	0.04
4-Methyl-1-pentanol	56	68	7.16	0.02	±	0.00	0.02	±	0.01	0.03	±	0.01	0.04	±	0.02	n.d.	±	-	0.83
2-Hexenal	41	97	7.57	26.26	±	2.84	16.69	±	1.79	24.17	±	3.31	36.54	±	1.75	31.58	±	3.27	0.17
Propylcyclopropane	56	65	7.63	n.d.	±	-	n.d.	±	-	n.d.	±	-	8.41	±	3.64	n.d.	±	-	0.05
1-Hexanol	56	92	7.87	0.22	±	0.08	0.10	±	0.01	0.25	±	0.12	0.12	±	0.02	0.09	±	0.01	0.56
2-Heptanone	43	72	8.56	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00	0.46
Styrene	104	98	8.58	0.04	±	0.01	0.05	±	0.01	0.04	±	0.01	0.03	±	0.01	0.01	±	0.00	0.01
(E)-2-Hepten-1-ol	57	72	8.94	0.04	±	0.01	0.04	±	0.00	0.02	±	0.00	n.d.	±	-	0.03	±	0.01	0.05
tert-Butylcarbinol	57	61	9.00	0.12	±	0.02	0.05	±	0.01	0.15	±	0.04	0.15	±	0.03	0.20	±	0.04	0.31
(E,E)-2,4-hexadienal	81	97	9.19	1.37	±	0.14	0.96	±	0.17	1.16	±	0.17	1.45	±	0.08	1.28	±	0.13	0.75
2-Ethyl-furan	81	70	9.28	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.41	±	0.12	n.d.	±	-	0.00
α-Pinene	93	96	9.97	19.14	±	3.00	30.82	±	0.97	24.07	±	3.18	9.17	±	1.03	19.93	±	3.86	0.04
Allo-Ocimene	121	64	10.10	n.d.	±	-	n.d.	±	-	1.77	±	0.65	n.d.	±	-	n.d.	±	-	0.20

Table 3.5 Cont.				EV			24-5			28-13			44-12			41-5			
Tentative Compound ID	**m/z**	**%M**	**RT**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**p-val**
Trimethyl(2-methyl-1-propenylidene)-cyclopropane	121	77	10.58	n.d.	±	-	0.34	±	0.08	1.39	±	0.46	n.d.	±	-	n.d.	±	-	0.01
7-exo-ethenyl-bicyclo[4.2.0]oct-1-ene	91	81	10.60	n.d.	±	-	0.04	±	0.01	0.03	±	0.01	n.d.	±	-	0.02	±	0.01	0.01
β-Thujene	93	69	10.64	3.87	±	1.62	7.50	±	2.73	0.72	±	0.26	0.96	±	0.35	1.61	±	0.39	0.36
(Z)-2-Heptenal	41	68	10.69	0.06	±	0.01	0.04	±	0.01	0.05	±	0.01	0.08	±	0.01	0.07	±	0.01	0.23
Benzaldehyde	77	96	10.81	0.13	±	0.01	0.10	±	0.02	0.11	±	0.01	0.18	±	0.03	0.14	±	0.02	0.67
(E)-4-Oxohex-2-enal	83	94	10.82	2.15	±	0.35	2.34	±	0.45	2.56	±	0.26	3.21	±	0.41	2.53	±	0.54	0.87
3,6,6-Trimethyl-2-norpinene	93	64	11.01	1.93	±	1.34	0.56	±	0.16	5.15	±	2.19	n.d.	±	-	n.d.	±	-	0.52
β-Sabinene	93	61	11.24	n.d.	±	-	0.20	±	0.04	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.04
L-β-Pinene	93	61	11.79	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.08	±	0.01	0.10
(E,E)-2,4-Heptadienal	81	94	12.04	0.25	±	0.04	0.09	±	0.02	0.20	±	0.03	0.34	±	0.03	0.36	±	0.04	0.02
β-Terpinene	93	64	12.34	n.d.	±	-	0.31	±	0.16	0.44	±	0.11	n.d.	±	-	n.d.	±	-	0.01
α-Phellandrene	93	62	12.68	0.55	±	0.21	0.49	±	0.14	n.d.	±	-	0.15	±	0.04	0.21	±	0.05	0.86
3,7,7-Trimethyl-1,3,5-cycloheptatriene	119	74	12.89	n.d.	±	-	n.d.	±	-	0.25	±	0.07	n.d.	±	-	n.d.	±	-	0.14
β-Cymene	119	98	12.92	0.24	±	0.05	0.62	±	0.09	0.38	±	0.07	0.14	±	0.04	0.33	±	0.10	0.06
D-Limonene	68	77	13.09	0.98	±	0.13	2.15	±	0.27	1.49	±	0.22	0.60	±	0.12	1.39	±	0.29	0.04
3,4-dimethyl-1,5-cyclooctadiene	68	64	13.09	n.d.	±	-	1.69	±	0.25	n.d.	±	-	n.d.	±	-	0.91	±	0.25	0.11
α-Ocimene	93	69	13.10	0.60	±	0.11	1.24	±	0.32	1.01	±	0.24	0.67	±	0.16	1.06	±	0.34	0.26
2,2,3-Trimethylhexane	57	64	13.11	n.d.	±	-	n.d.	±	-	0.20	±	0.08	n.d.	±	-	0.11	±	0.02	0.28
γ-Terpinene	93	65	13.48	0.46	±	0.13	1.45	±	0.34	0.66	±	0.20	0.15	±	0.04	0.74	±	0.36	0.12
α-Tolualdehyde	91	88	13.51	0.02	±	0.00	0.02	±	0.00	0.02	±	0.00	0.02	±	0.00	0.01	±	0.00	0.09
2,2-Dimethyl-1-pentanol	85	70	14.10	0.89	±	0.16	0.70	±	0.11	0.98	±	0.17	1.39	±	0.22	1.11	±	0.33	0.87
5-Methyl-1-phenyl-1-hexanone	105	82	14.22	0.01	±	0.00	0.01	±	0.00	0.01	±	0.00	0.02	±	0.00	0.01	±	0.00	0.28
(E,E)-2,4-Heptadien-6-ynal	105	64	14.63	n.d.	±	-	0.01	±	0.00	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.01
o-Guaiacol	109	93	14.82	n.d.	±	-	0.11	±	0.03	0.09	±	0.03	0.04	±	0.01	0.05	±	0.01	0.02
o-Cresol	121	73	14.82	0.12	±	0.02	0.27	±	0.05	0.13	±	0.03	0.05	±	0.01	0.19	±	0.07	0.19
1-Methylindan	117	72	14.99	0.03	±	0.01	0.03	±	0.01	n.d.	±	-	0.04	±	0.02	n.d.	±	-	0.78
4-Ethylstyrene	117	88	15.05	0.06	±	0.01	0.06	±	0.01	0.04	±	0.01	0.09	±	0.01	0.08	±	0.02	0.49
1-Dodecyne	81	61	15.17	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.21	±	0.05	0.06	±	0.02	0.00
α-Pinene oxide	67	90	15.38	n.d.	±	-	0.06	±	0.01	0.10	±	0.02	n.d.	±	-	0.03	±	0.01	0.01
β-Linalool	71	83	15.41	n.d.	±	-	0.07	±	0.01	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.01
Nonanal	57	89	15.55	0.06	±	0.01	0.19	±	0.03	0.06	±	0.01	0.04	±	0.01	0.04	±	0.00	0.00
3,4-Dimethylcyclohexanol	71	60	15.69	0.02	±	0.00	0.02	±	0.00	0.02	±	0.00	0.03	±	0.00	0.03	±	0.00	0.41
Benzylhydrazine	91	68	15.72	n.d.	±	-	0.03	±	0.00	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.00
3,3,6-Trimethyl-1,5-heptadien-4-one	83	74	15.88	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.49	±	0.27	0.02	±	0.01	0.04
6-Camphenol	93	78	16.26	n.d.	±	-	0.12	±	0.01	0.11	±	0.02	0.05	±	0.01	0.08	±	0.01	0.01
cis-Limonene oxide	43	84	16.39	n.d.	±	-	0.03	±	0.01	0.03	±	0.01	n.d.	±	-	n.d.	±	-	0.00
3,4-Dimethyl-2-hexanone	43	66	16.71	0.01	±	0.00	0.02	±	0.00	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.02

Table 3.5 Cont.				EV			24-5			28-13			44-12			41-5			
Tentative Compound ID	**m/z**	**%M**	**RT**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**Avg**	**±**	**SEM**	**p-val**
p-Xylene	119	61	17.22	n.d.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	t.r.	±	-	0.11
o-Hydroxyacetophenone	121	98	17.26	0.18	±	0.01	0.29	±	0.07	0.19	±	0.02	0.21	±	0.02	0.20	±	0.03	0.06
3-Ethyl-benzaldehyde	134	92	17.55	n.d.	±	-	0.01	±	0.00	0.03	±	0.01	0.03	±	0.01	0.02	±	0.00	0.58
Methyl salicylate	120	99	18.32	1.67	±	0.16	1.79	±	0.15	2.11	±	0.24	1.59	±	0.27	1.14	±	0.22	0.03
(S)-Verbenone	107	64	18.81	n.d.	±	-	0.01	±	0.00	0.02	±	0.00	n.d.	±	-	t.r.	±	-	0.00
β-Cyclocitral	41	86	19.15	0.03	±	0.00	0.03	±	0.01	0.05	±	0.00	0.05	±	0.01	0.06	±	0.01	0.55
cis-Geraniol	69	71	19.32	n.d.	±	-	n.d.	±	-	0.12	±	0.02	n.d.	±	-	n.d.	±	-	0.08
2-Isopropenyl-5-methylhex-4-enal	41	61	19.72	n.d.	±	-	0.01	±	0.00	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.01
3-Isopropylbenzaldehyde	133	68	20.41	n.d.	±	-	n.d.	±	-	t.r.	±	-	n.d.	±	-	n.d.	±	-	0.06
Decane, 2,4-dimethyl-	43	84	21.81	n.d.	±	-	0.02	±	0.00	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.01
2,4,6-Trimethyldecane	43	65	22.23	n.d.	±	-	0.02	±	0.00	0.01	±	0.00	0.02	±	0.00	n.d.	±	-	0.45
σ-Elemene	121	94	22.64	0.86	±	0.14	1.13	±	0.20	0.27	±	0.03	0.50	±	0.07	0.33	±	0.06	0.09
Diisobutylene	57	63	22.68	n.d.	±	-	0.02	±	0.01	0.01	±	0.00	0.02	±	0.00	0.02	±	0.01	0.19
α-2,5-Trimethyl-benzeneacetaldehyde	133	78	22.79	0.05	±	0.01	0.05	±	0.01	0.03	±	0.01	0.05	±	0.01	0.04	±	0.01	0.45
Disulfide, bis(1,1,3,3-tetramethylbutyl)	57	73	24.01	0.05	±	0.01	0.04	±	0.01	0.02	±	0.01	0.05	±	0.01	0.04	±	0.01	0.70
Caryophyllene	93	100	25.05	5.43	±	0.52	9.68	±	1.16	3.45	±	0.73	3.96	±	0.58	2.79	±	0.61	0.03
epi-10- γ-Eudesmol	189	66	25.34	n.d.	±	-	t.r.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.01
Isoledene	161	80	25.59	0.22	±	0.02	0.44	±	0.04	0.12	±	0.02	0.16	±	0.02	0.19	±	0.06	0.04
β-Ionone	177	92	26.55	0.07	±	0.01	0.12	±	0.02	0.07	±	0.01	0.09	±	0.01	0.08	±	0.01	0.18
D-Germacrene	161	84	26.63	0.06	±	0.01	0.11	±	0.01	0.03	±	0.01	0.03	±	0.01	0.02	±	0.00	0.01
δ-Selinene	161	68	26.81	0.02	±	0.00	0.02	±	0.00	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.09
Cis-muurola-4(14),5-diene	161	71	26.94	t.r.	±	-	t.r.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.16
4,11-Selinadiene	189	76	27.09	n.d.	±	-	0.03	±	0.00	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.00
3,8-Dimethylundecane	57	86	27.39	0.05	±	0.01	0.07	±	0.02	0.05	±	0.01	0.08	±	0.01	0.05	±	0.01	0.86
2-Ethyl-1-decanol	57	65	27.63	n.d.	±	-	0.02	±	0.00	0.01	±	0.00	0.02	±	0.00	n.d.	±	-	0.39
Caryophyllene oxide	43	91	29.30	0.23	±	0.04	0.37	±	0.06	0.21	±	0.05	0.21	±	0.04	0.14	±	0.04	0.07
Santolina triene	93	62	29.71	0.02	±	0.00	0.03	±	0.01	0.02	±	0.00	0.01	±	0.00	0.01	±	0.00	0.02
(E)-5-Butoxy-2-pentene	57	68	30.34	n.d.	±	-	t.r.	±	-	n.d.	±	-	n.d.	±	-	n.d.	±	-	0.04



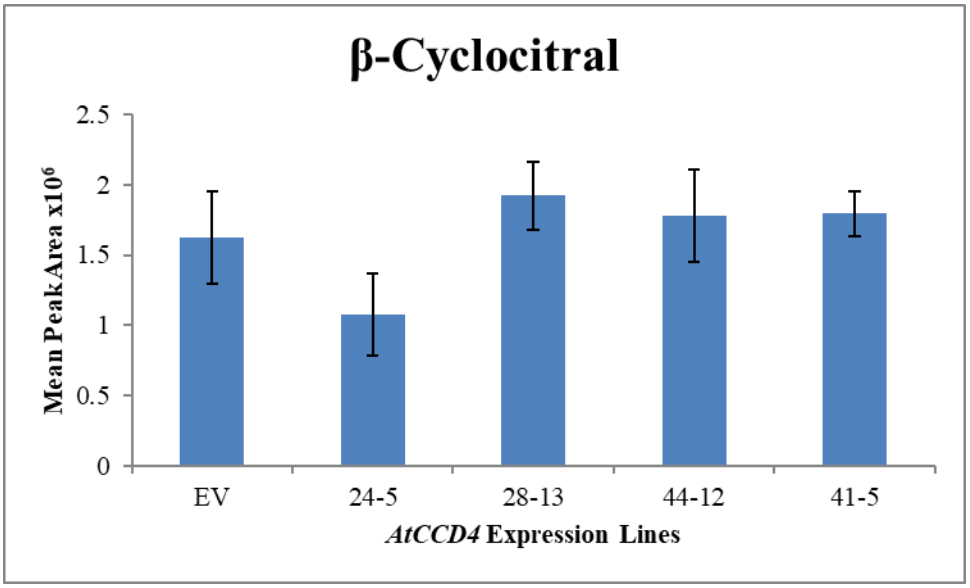


Figure 3.10. Average peak area of β-ionone and β-cyclocitral detected from ground leaf tissue VOC collections. Leaves were flash frozen in liquid nitrogen and ground in 2 mL bead tubes, after which VOCs were accumulated for 40 min and collected via SPME for 20 min. Each bar is an average of a minimum of five biological replicates (±SEM).

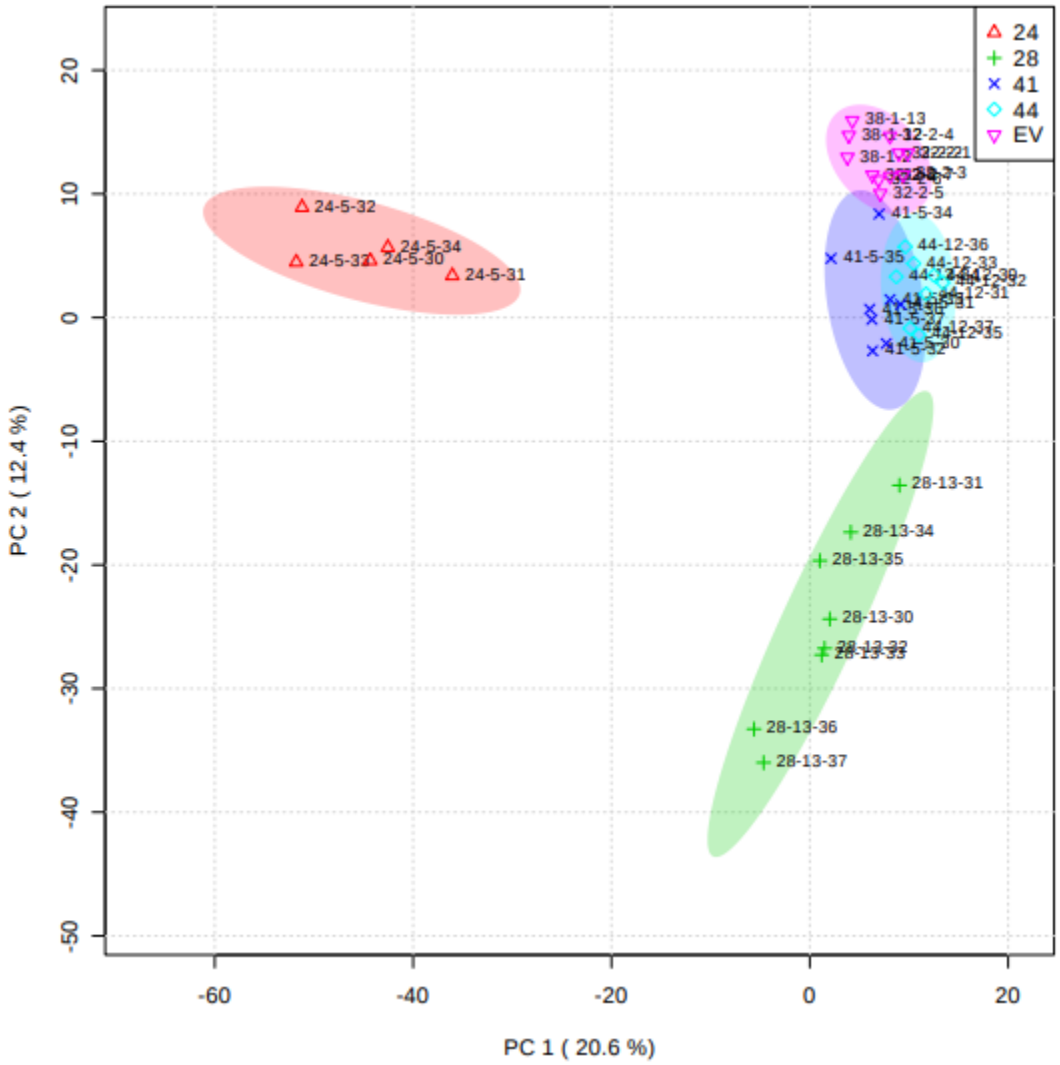


Figure 3.11. Principle component analysis comparing all detected VOC features from ground leaf tissue. All *AtCCD4* expressing lines are separated from EV to some degree along PC2 (12.4%), but line 28-13 was the most different. Line 24-5 is clearly different from EV controls and every other line along PC1 (20.6%).

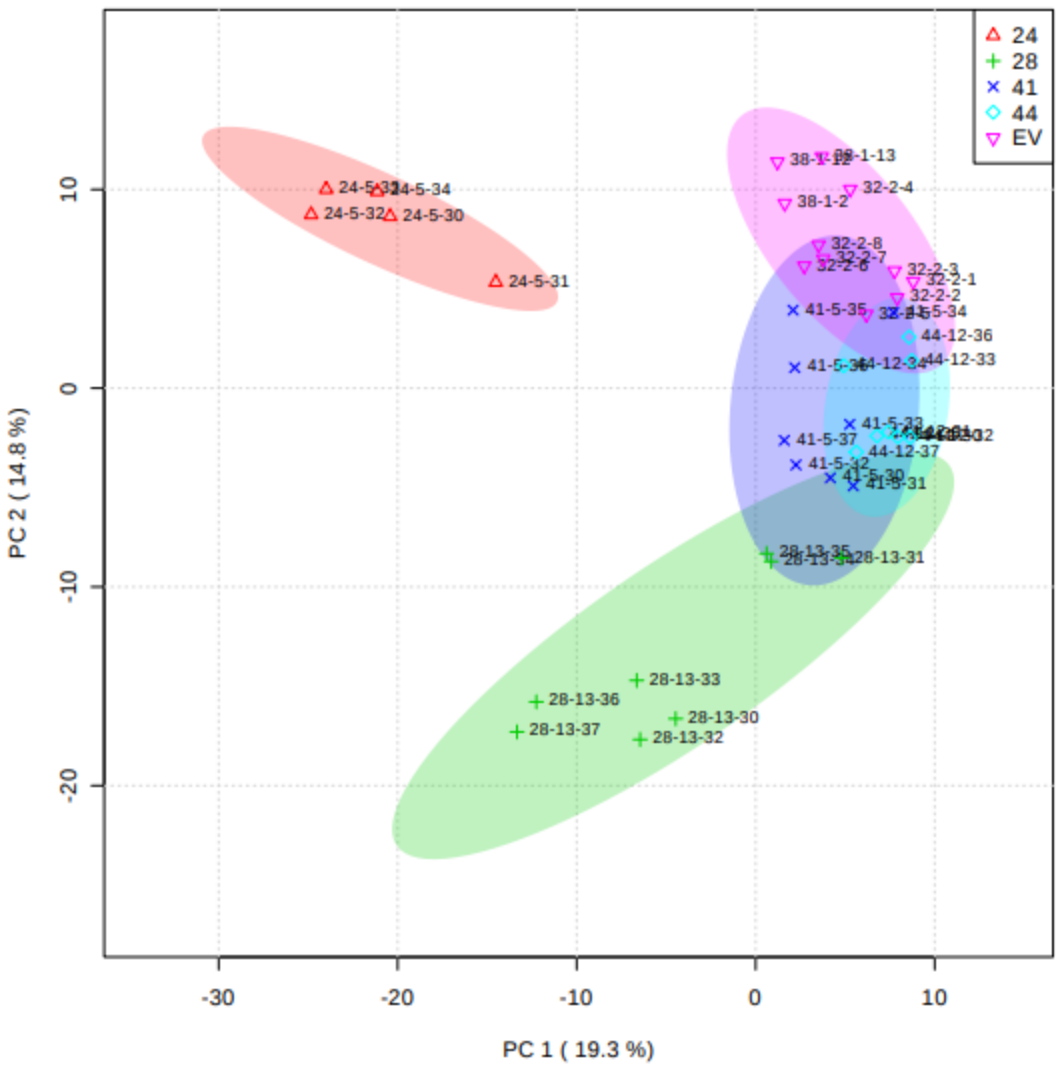
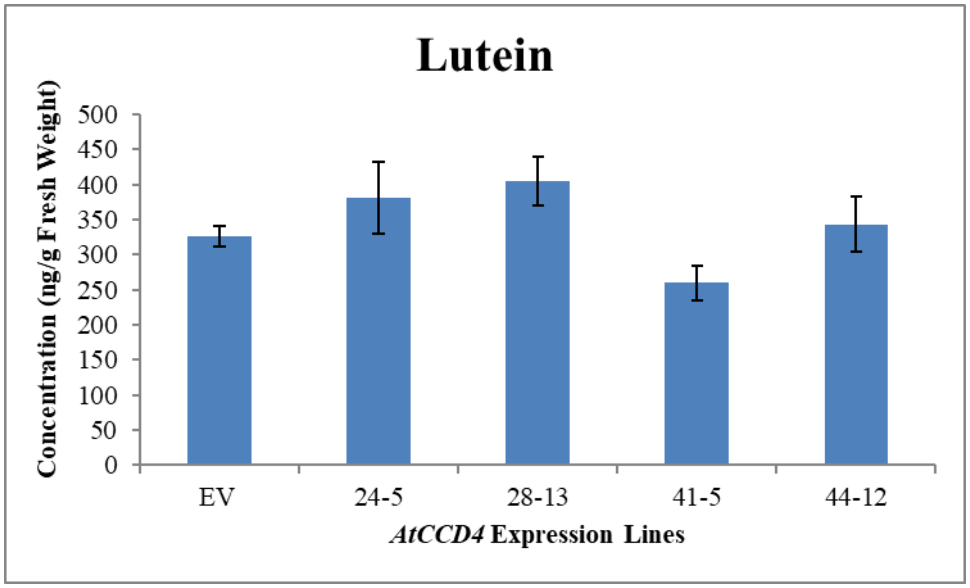


Figure 3.12. Principle component analysis plot comparing tentatively identified VOC features from ground leaf tissue. Similar to Figure 3.11, the AtCCD4 expressing lines 28-13 and 24-5 are the most different from EV, while in this case lines 41-5 and 44-12 partially overlap with EV.

3.3.4 Analysis of Tomato Leaf Carotenoids

Carotenoids were extracted and analyzed by HPLC-DAD to determine if the expression of *AtCCD4* in tomato plants had an effect on the concentrations of the main leaf carotenoid compounds. This includes β-carotene and lutein, which are both reported substrates for the CCD4 enzyme (Huang et al., 2009; Rottet et al., 2016). Plants from lines 24, 28, 41, and 44 were used for carotenoid analysis. Statistical analysis of both β-carotene and lutein concentrations using ANOVA revealed no significant differences between the lines tested (Figure 3.13).



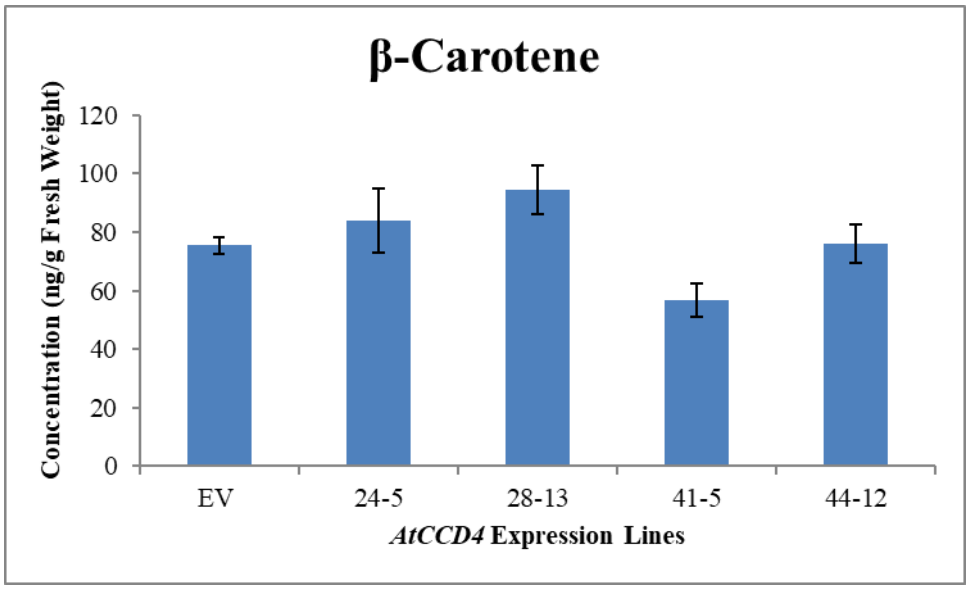


Figure 3.13. Concentrations of lutein and β-carotene in leaf tissue of plants expressing *AtCCD4*. EV controls and four *AtCCD4* expressing lines were analyzed. Leaves of four-week-old plants were weighted and frozen in liquid nitrogen prior carotenoid extraction. Each bar is an average of a minimum of five biological replicates (±SEM).

3.3.5 Effect of *AtCCD4* Expression on Greenhouse Whitefly Oviposition Preference

Whitefly oviposition preference assays were conducted to investigate whether a group of mixed sex, mixed age greenhouse whiteflies preferred to oviposit on either EV control plants or transgenic plants expressing *AtCCD4*. In each trial, eggs were counted on both leaves and flowers. Significantly fewer eggs were found on plants from line 41-5 compared to EV control plants (N=5, P-val=0.0008) (Figure 3.14). On average, the number of eggs laid on line 41-5 was 40% of those laid on EV control plants. No significant differences were found for the total egg counts of other lines. When egg counts were split between leaves and flowers for each line, the main differences were found on the leaves, where the eggs laid on line 41-5 leaves totaled 23% of those laid on EV control leaves (Figure 3.15A). Each transgenic line had fewer eggs laid on their leaves than EV controls, but only line 41-5 had a statistically significant difference (N=5, P-val=0.004) (Figure 3.15A). On the other hand, egg counts on flowers were variable across all lines with no distinct pattern evident (P-val>0.05) (Figure 3.15B).

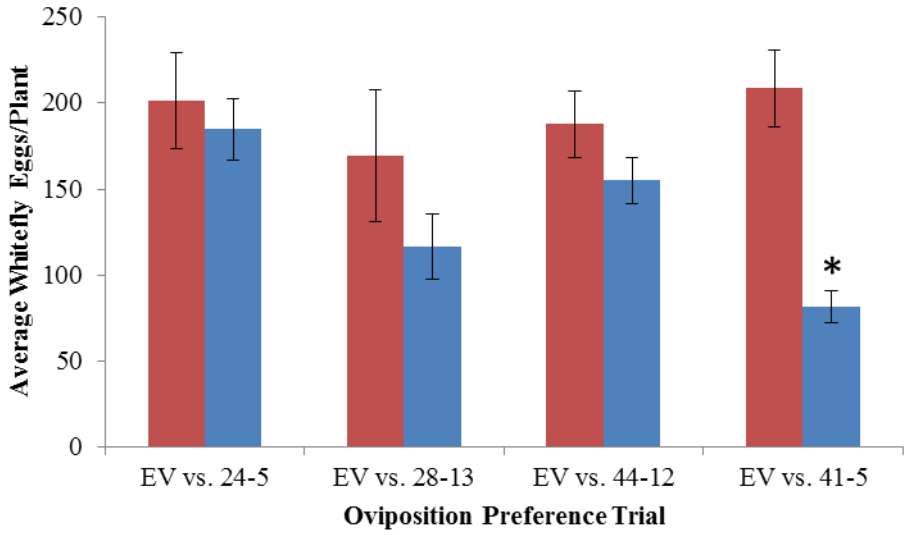


Figure 3.14. Oviposition preference of greenhouse whiteflies for each transgenic *AtCCD4* line relative to EV controls. One trial comparing one transgenic plant (blue) versus one EV control plant (red) was treated as one biological replicate. Each paired bar is representative of the average number of total eggs oviposited per plant calculated from a minimum of four trials (±SEM). Significantly less whitefly eggs were counted on plants from line 41-5, as indicated by an asterisk (P-val=<0.05).

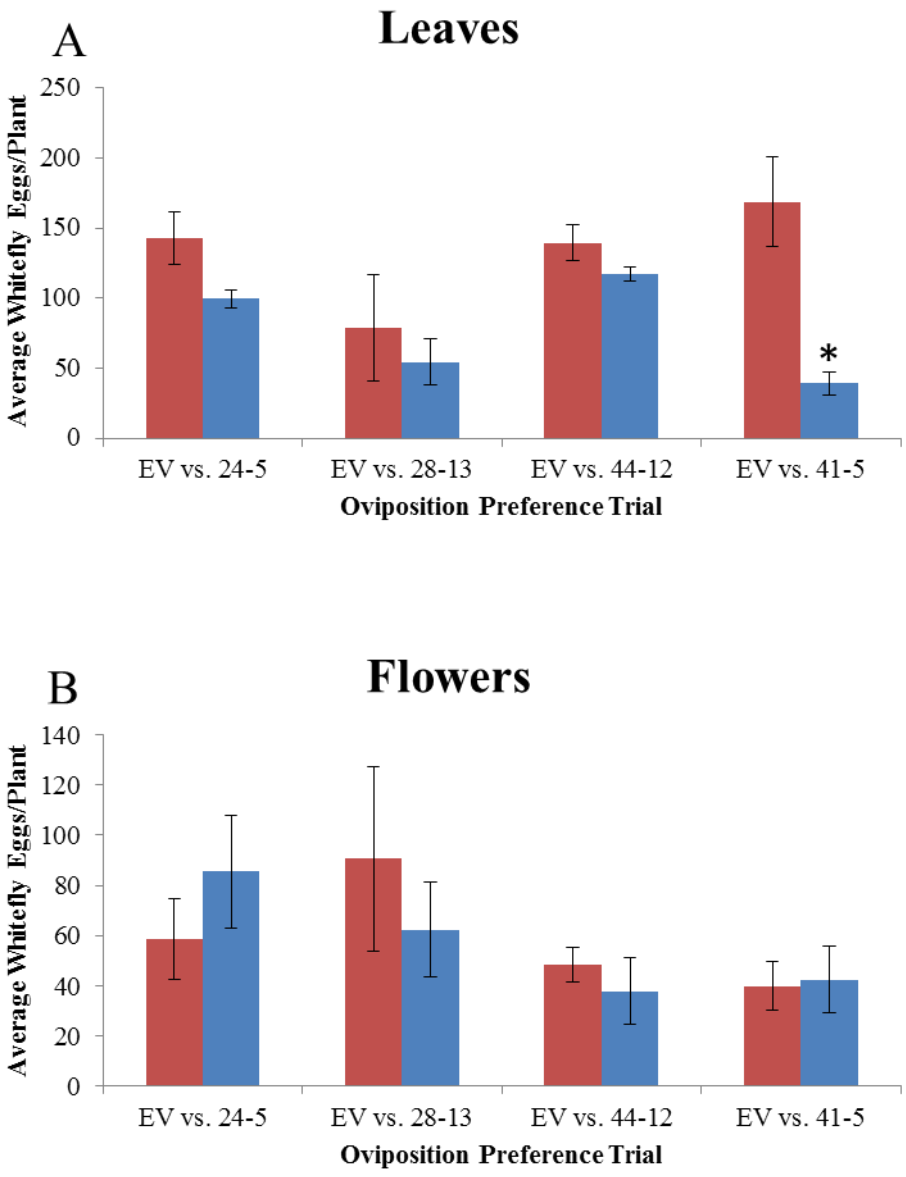


Figure 3.15. Separation of the number of eggs laid on tomato leaves and flowers of transgenic *AtCCD4* and EV controls. Each trial contained a single transgenic AtCCD4 plant (blue) and a single EV control plant (red). Each trial was treated as a single biological replicate, with each bar representing an average number of eggs per plant (±SEM) for a minimum of four replicates. Significantly less eggs were found on the leaves (A) of plants belonging to line 41-5, as indicated by an asterisk (P=<0.05). No trend could be discerned in the number of eggs laid on flowers (B).

3.4 Discussion and Conclusions

MicroTom tomato lines expressing *AtCCD4* were evaluated on multiple levels to determine the effect expression of the transgene had on leaf internal chemistry and headspace, as well as on interactions with greenhouse whiteflies. Within the leaf, increasing amounts of *AtCCD4* expression did not appear to affect the levels of β-carotene and lutein. Line 28-13 appeared to have slightly more β-carotene and lutein than EV, while line 41-5 had slightly less, but with the relatively small sample size used in this study there was no significant difference in levels of these two carotenoids. This was unexpected, as previous studies determined that both β-carotene and lutein were potential substrates for CCD4 (Huang et al., 2009; Rottet et al., 2016). This is also in contrast to previous data generated in our lab, where overexpression of *AtCCD4* in *Arabidopsis* increased levels of β-carotene and lutein in *Arabidopsis* leaves (Lakshminarayan, 2013). At the time, it was suggested that the increase could be due to a feedback loop caused by increased catabolism of leaf carotenoids. This would in turn cause the plant to compensate by increasing carotenoid biosynthesis overall, and it was found that the increased levels of β-carotene and lutein was accompanied by an increase in the expression of *phytoene synthase* (*PSY*) (Lakshminarayan, 2013). While no expression analysis of carotenoid biosynthesis genes was conducted, it is an interesting future direction to pursue. In addition, it may be worthwhile to investigate other minor carotenoids such as xanthophylls, as they may also be targets of CCD4 (Lätari et al., 2015). It may also be informative to investigate carotenoid content and biosynthesis in other tissues such as seeds, as CCD4 is known to be heavily involved in the breakdown of β-carotene in *Arabidopsis* seeds (Gonzalez-Jorge et al., 2013).

To complement the expected reduction in the leaf β-carotene and lutein content, it was assumed that *AtCCD4* expression would lead to a corresponding increase in the volatile apocarotenoids emitted into the plant headspace. The work in this study shows that that is not necessarily the case, as *in vivo* VOC collections only detected one apocarotenoid VOC, 6-methyl-5-hepten-2-one. This VOC was present as a minor peak in the VOC emissions from leaves of line 24-5 and 41-5 as well as the flowers of all transgenic EV lines. There was no real difference in the level of 6-methyl-5-hepten-2-one from flowers, and the detection of this VOC from leaves of some but not all of the transgenic plants suggests that it may be just near the level of detection in the other lines as well as potentially the EV controls. Since it is detected in the lowest expressing line and

the highest expressing line but not in the moderate expressing lines, it is likely that emission from leaves and flowers *in vivo* is not heavily influenced by CCD4 under these experimental conditions. Similar to 6-methyl-5-hepten-2-one, β-ionone and β-cyclocitral may be below the detection limit of our method when sampling *in vivo*, as neither were detected in any samples. It should be noted however that previous members of our lab have also been unable to detect β-ionone emissions from leaves or flowers using other sampling and GC-MS methods (Challa, 2015; Laur, 2018). It is possible that the lack of apocarotenoid VOC emissions from MicroTom is cultivar-specific, as they have been noted as constituents in VOC emissions from other tomato cultivars under normal growth conditions (López-Gresa et al., 2017; Silva et al., 2017).

After failing to detect the apocarotenoids *in vivo*, it was considered that *AtCCD4* expression could still be influencing apocarotenoid production but that the products were non-volatile. Apocarotenoids are known to be stored in *Arabidopsis* glycoside conjugates, and AtCCD4 has been proposed as the main enzyme responsible for their formation (Lätari et al., 2015). The release of aglycones after tissue disruption is a common defense strategy against herbivorous insects and can also contribute to the aroma of plant tissues (Mithofer and Boland, 2012; Tikunov et al., 2010). To determine if this was perhaps also the case for apocarotenoids in tomato leaves, leaf tissue was ground and headspace VOCs were collected. This was also done in a reduced headspace of 2 mL in order to concentrate VOC emissions further and increase the chances of detection of trace VOCs. While this collection method did allow for the detection of both β-ionone and β-cyclocitral, the peak areas of these two compounds were not significantly different between lines. Since an *Arabidopsis CCD4* knockdown mutant had reduced levels of apocarotenoid glycosides in leaves, the lack of an increase in volatile apocarotenoids in tomato leaves upon *AtCCD4* expression was unexpected (Lätari et al., 2015). There was however some variability in the apocarotenoid peak areas, for example the error bars for β-ionone emitted by individuals of line 28-13 ranged from 3.5×10^6 and 6×10^6. This variability was mirrored in the peak areas of the internal standard 2-octanone from the *in vivo* VOC analysis, suggesting that the variability could have more to do with the SPME collection system than the plants themselves. In order to obtain more accurate quantitative measures of VOC emissions, the SPME collection method could be altered to allow VOCs to reach equilibrium in the headspace. Otherwise, a dynamic headspace collection method or direct solvent extraction from the tissue in question may be better suited.

Regardless, the detection of these two compounds after tissue disruption suggests that glycosylation of apocarotenoid VOCs does occur in tomato leaves. As has been noted in previous work on phenylpropanoid VOCs in tomato fruit, differential glycoconjugation can result in molecules that are unable to be cleaved by native glycosidases even when they are readily accessible (Tikunov et al., 2010). This may be also occurring in the tomato leaves to an extent, resulting in a potentially incomplete picture of the effect of *AtCCD4* on apocarotenoid production. In future experiments, ground tissue could be treated with commercially available glycosidases to help liberate volatile aglycones for headspace analysis. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of tomato leaf carotenoids and apocarotenoid as well as their glycosides could also be conducted to shed light on the complexities of carotenoid breakdown and storage in tomato leaves.

In oviposition preference assays with *T. vaporariorum* line 41-5, which expressed *AtCCD4* at the highest level of all lines tested, proved to be less preferable than EV control plants for whitefly oviposition. As noted above, only 6-methyl-5-hepten-2-one was detected from *in vivo* collections, suggesting that the volatile apocarotenoids had little role to play in this effect. The difference seen in preference could potentially be due to heightened production of non-volatile apocarotenoids causing a repellent effect upon feeding. It could also be due to changes in the volatile emission profile as a result of feeding. It is well documented that plant VOC emissions can change rapidly upon herbivory, so it is possible that expression of *AtCCD4* could cause an increased response by the plant. This could be investigated by measuring VOC emissions post-feeding. Y-tube olfactometer assays could also be conducted to determine if the whitefly preference is truly due to volatile differences between transgenic and non-transgenic plants and if those cues are present prior to whitefly feeding and oviposition. Y-tube assays would also indicate whether the effect seen is truly due to a volatile cue or if it is something non-volatile acting on the whiteflies on contact with the plant. In addition, the oviposition preference assays should also be repeated again as the statistical significance seen in the small sample size of four could be due to sampling error. As a final additional assay, no-choice oviposition preferences assays must be conducted to rule out any positional or other off-target preferences of whitefly oviposition.

Overall, this chapter outlines the feasibility of manipulating plant VOCs through the use of transgenic plants as well as the hurdles that remain to confirm their efficacy. Line 41-5 was less attractive than EV plants to greenhouse whiteflies during oviposition preference cage trials, however more replicates as well as no-choice assays are required in order to make a stronger statistical case... This plant-insect interaction can also be investigated further by using similar methods of VOC analysis on plants post-feeding to determine if VOC changes caused by herbivory are at work, as well as Y-tube olfactometery to confirm the effect is due to VOC emissions. It may also be useful to conduct more detailed analysis of other leaf carotenoids such as xanthophylls, as well as non-volatile apocarotenoids and their glycosides to determine how the AtCCD4 enzyme is truly functioning. Regardless, it is worthwhile to note that *CCD4* is just one of perhaps hundreds of genes that are involved in the biosynthesis of VOCs. The possibilities for tweaking the VOC emission profiles of crops are quite numerous, and could theoretically be done to specifically target certain types of pests that are a particular problem for any given crop. While manipulating plant VOCs may not be an absolutely effective solution to all pest problems, it may certainly be one effective tool within an IPM toolbox. In the future, plants like those produced here could have an important role to play in reducing pest populations as farmers move away from their traditional reliance on chemical pesticides.

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Chapter 4. General Discussion and Conclusion

In this study, attempts were made to manipulate VOC biosynthesis at the genetic level to produce transgenic tomato plants with altered VOC emission profiles. In the first chapter the *Arabidopsis* gene *CYP82G1* was cloned and expressed successfully, but the two compounds that were expected to be emitted as a result of transgene insertion could not be detected. However, whole volatile profile analysis through PCA revealed that differences between *AtCYP82G1* expression lines generated and EV control may exist within the portion of VOCs that were unidentified. While no direct evidence of AtCYP82G1 function was found, it suggests that perhaps there is an indirect effect on the plant VOC profile as a result of *AtCYP82G1* expression.

In the second chapter, a more in depth study of *AtCCD4* expression plants was conducted to determine if AtCCD4 could improve the emissions of volatile apocarotenoids involved in insect repellence (Caceres et al., 2016; Wei et al., 2011). *In vivo*, 6-methyl-5-hepten-2-one was the only apocarotenoid detected, and its peak area was not altered by the expression level of *AtCCD4*. When tissue was ground and the headspace was reduced, β-ionone and β-cyclocitral were detected but were not significantly different in the tested plants. This was coupled with no changes in lutein or β-carotene in tomato leaves, both of which are potential cleavage targets of AtCCD4 (Huang et al., 2009; Rottet et al., 2016). In spite of all this, choice oviposition preference bioassays revealed that the *AtCCD4* line with the highest expression was significantly less attractive to *T. vaporariorum* for oviposition. This was unexpected given the lack of direct effect on volatile apocarotenoids, but much like the *AtCYP82G1* the PCA plots illustrated that differences could be found between the transgenic and EV lines. This suggests either an unexpected shift in VOC biosynthesis due to the transgene insertion, or some other volatile or non-volatile actor within the plant that was not detected using the methods used in this study was influencing *T. vaporariorum*.

Taken together, these two parallel studies provide useful insight into the challenges faced when seeking to manipulate plant specialized metabolism. While advances in biotechnology have made crop improvement more attainable than in the past, the path to achieving the desired improvements is generally less than straightforward. This is especially true in plant VOCs. Hundreds of compounds have been identified as constituents of plant volatile emissions, each

with the potential to influence plant-insect interactions (Kaplan, 2012). The two genes studied here were chosen based on their role in the biosynthesis of plant VOCs that either attracted herbivore enemies or repelled herbivores directly (Caceres et al., 2016; Lee et al., 2010; Wei et al., 2011). While these were tested in previous studies on specific plant-insect interactions, it is entirely possible that manipulating the VOC profile to benefit one interaction could have unintended negative consequences for other plant-insect or plant-microbe interactions. Hypothetically, the VOC changes in the *AtCCD4* tomatoes generated here could be unattractive to predatory insects that feed on tomato pests other than *T. vaporariorum.* This could result in a reduction of the *T. vaporariorum* population but a potential increase in a secondary pest. This sort of problem is difficult to address in species such as tomato which are fed on by numerous pests. This is especially true in the case of this study, where none of the predicted changes to the plant VOC profiles were realized and the differences in insect behaviour seen could not be attributed to any obvious reason. Additionally, the results found in this study also highlight the need to follow up any choice oviposition assays with similar no-choice assays in order to definitively say that the differences seen are due to the insertion of the transgene.

While the work done in this study highlights some of the difficulties faced when seeking to influence plant VOCs, there are cases where specific interactions with pests and predators have been targeted for manipulation through plant VOC biosynthesis to great success. Some of these have been straightforward, such as the introduction of a caryophyllene synthase gene in maize that increased emission of caryophyllene from roots to attract protective entomopathogenic nematodes (Degenhardt et al., 2009). Others however have taken a more indirect approach, such as targeting the *nerolidol synthase* (*NES*) gene from strawberry to the mitochondria of *Arabidopsis* in order to improve nerolidol and DMNT production (Kappers et al., 2005). A lack of precursors has been suggested as a problem in the past for *CYP82G1* overexpression plants specifically, so shifting flux down this specific pathway by expressing the rate-limiting *NES* gene along with increased *AtCYP82G1* expression may be a more effective strategy to enhance DMNT and TMTT emissions (Kappers et al., 2005; Lee et al., 2010).

It is also important to mention some other limitations within the above study with regards to the molecular and chemical analysis methods. As mentioned in both above chapters, none of the results above prove without a doubt that the transgenes in question are truly functioning. In order

to determine if the transgene is functioning at a protein level, substrate for the target VOCs could be added to a crude protein extract followed by headspace VOC collection. Alternatively, more transgenic lines could be generated using an expression vector that couples the transgene protein product to a protein with a commercially available antibody such as GFP, which would allow the detection of the transgene product at the protein level by western blotting.

As for the VOC analysis methods, in order to determine which VOCs may be acting to influence the insect of interest, proper collection and analysis of VOCs is of critical importance. One of the limitations of the VOC analyses done in this study was the manual *in vivo* collection method using PDMS/DVB SPME fibres. The manual nature of this method coupled with the differences in individual plants allows for the potential for error when attempting to maintain consistency in the collection chamber. In any given collection period, the timing of accumulation and collection of VOCs could be slightly off, as well as the distance between leaf and fibre. Another limitation is the challenging normalization of the SPME collected VOC data. The method used in this study was selected based on previous work conducted in our lab where fibre coating types were tested, but this was more of a qualitative than quantitative study (Caceres et al., 2015). As a result of this previous study, PDMS/DVB was selected as a good all-purpose fibre coating and was the only fibre type used in the current work (Caceres et al., 2015). It is well documented that SPME benefits greatly from method standardization and calibration (Ouyang et al., 2011). The variability of the SPME system is evident when looking at the differences in 2-octanone peak area between individual fibres. 2-Octanone was originally added in an attempt to provide a stable peak within each chromatogram to normalize other peaks to, but given the variability this was not carried out. The easiest way to achieve accurate quantitative measurements with SPME is equilibrium extraction, where the concentration of analyte in the sample and on the fibre is directly proportional based on the analyte's distribution coefficient between the matrix and the fibre (Ouyang et al., 2011). This type of calibration was not possible in the present study, as the *in vivo* and ground samples would continue to release VOCs over the one hour collection time period that was used, thus the headspace was never truly a static system. Due to this, the fibres were unable to reach an equilibrium point. External calibration could be used to produce a calibration curve for specific targeted analytes, but this is less accurate than equilibrium calibration and not suited for untargeted analyses (Ouyang et al., 2011). In the future, it may be worthwhile to conduct external calibrations for the apocarotenoids β-ionone, β-cyclocitral and 6-

methyl-5-hepten-2-one in order to more accurately assess their production in transgenic *AtCCD4* lines. Other calibration methods exist for *in vivo*, non-equilibrium analysis using SPME, but to my knowledge none have been published for headspace GC-MS without the use of isotopically labeled standards which were not available for this work (Zhang et al., 2009; Zhao et al., 2007).

In conclusion, the work presented in this thesis outlines the great potential that plant VOCs present for future IPM programs. By exploiting one of the main lines of communication between plants and their surroundings, transgenic plants with enhanced VOC emissions could stand alongside other IPM tools as important players in maintaining pest populations below economic damage thresholds. I have also highlighted some of the challenges that remain to be solved in order to reliably produce these VOCs from transgenic plants, as well as the hurdles that exist in the analysis of VOCs and their effects on insects. Future analysis of the plants generated here will focus on additional chemical characterizations as well as further examination of their interactions with pests, predators, parasitoids and pollinators.

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Appendices

Appendix A. Tissue Culture Media Recipes

Germination Media

2.17g/L Murashige and Skoog (MS) Basal Salt
0.5 ml/L 1000X MS Vitamins
15g/L Sucrose
8g/L Agar
pH 5.8

Pre-Culture Media and Co-Culture Media

4.33g/L MS Basal Salt
1 ml/L 1000X MS Vitamins
30g/L Sucrose
8g/L Agar
2mg/L Zeatin
0.1mg/L Indole-3-Acetic Acid
100µM Acetosyringone
pH 5.8

Infection Media

4.33 g/L MS Basal Salt
30g/L Sucrose
100µM Acetosyringone
pH 5.8

Shoot Induction Media (SIM-I)

4.33g/L MS Basal Salt
1 ml/L 1000X MS Vitamins
30g/L Sucrose
8g/L Agar
2mg/L Zeatin
0.1mg/L Indole-3-Acetic Acid
300 mg/L Timentin
pH 5.8

SIM-II

SIM-I + 5 mg/L Hygromycin

SIM-III

SIM-I + 10 mg/L Hygromycin

Shoot Elongation Media (SEM)

2.17g/L Murashige and Skoog (MS) Basal Salt
0.5 ml/L 1000X MS Vitamins
15g/L Sucrose
8g/L Agar
300 mg/L Timentin
20 mg/L Hygromycin
pH 5.8

Root Induction Media

2.17g/L Murashige and Skoog (MS) Basal Salt
0.5 ml/L 1000X MS Vitamins
15g/L Sucrose
8g/L Agar
300 mg/L Timentin
5 mg/L Hygromycin
pH 5.8

Appendix B. Nucleotide Sequences of *AtCYP82G1* and *AtCCD4*

Nucleotide Sequence for the *AtCYP82G1* gene used in this study:

ATGACTTTTCTCTTTAGTACTCTCCAGTTGTCTCTCTTCTCTTTGGCCCTTGTAATCTT
TGGTTACATTTTCCTCAGAAAACAATTGAGTAGATGTGAAGTTGATAGCTCCACGAT
TCCTGAGCCATTGGGAGCTTTGCCTCTATTCGGACACCTCCATCTTTTGCGTGGCAAA
AAACTCCTTTGCAAGAAATTAGCTGCCATGTCCCAAAAACATGGTCCTATCTTCTCC
CTCAAGTTAGGGTTTTATAGGCTGGTTGTAGCCAGCGACCCAAAAACGGTGAAAGA
TTGTTTCACCACCAACGACTTGGCTACAGCAACCAGACCCAATATAGCCTTTGGTCG
GTACGTAGGCTACAACAATGCAAGTCTGACTCTGGCTCCCTATGGAGACTATTGGCG
TGAGTTACGTAAGATTGTCACCGTCCATCTATTCTCAAACCACAGTATAGAGATGCT
TGGTCACATTCGTTCTTCAGAAGTAAACACGTTGATCAAACACCTATACAAAGGGAA
TGGTGGAACTTCTATAGTGAAGATTGACATGTTATTTGAGTTTTTGACCTTCAATATA
ATCCTTAGGAAGATGGTGGGGAAGAGGATTGGTTTCGGTGAAGTGAATAGCGATGA
ATGGCGTTATAAGGAGGCCCTGAAGCATTGCGAGTACTTGGCTGTGATTCCTATGAT
AGGCGACGTTATTCCATGGTTGGGATGGTTGGATTTTGCAAAAAATTCTCAAATGAA
GAGACTATTTAAGGAGCTTGACTCAGTCAACACCAAGTGGCTCCACGAACATCTCAA
GAAGAGATCAAGAAATGAGAAGGATCAAGAAAGAACAATCATGGATCTACTGCTA
GACATCTTACCAGAGGATATTGTGATAAGTGGACACGTACGCGATGTCATTGTGAAG
GCAACAATTTTGGTATGCACATATAATTTTCACCTCTCGTTAATTTGGATATATATGT
TAGAAAATCTCACAAACAAATAATTTATGAACTAATGTAGTGTTTATATGAGAAATA
TATATGACTTAATTCAATCATGGTTTGAAATGAATGTATACCAACATATATGTTCTGT
TTCTTGTTGTAGGCTCTCACATTAACAGGATCAGACAGCACATCCATCACTTTGACA
TGGGCGGTATCGCTGCTACTCAACAATCCAGCTGCTTTAGAAGCAGCACAAGAAGA
GATTGATAATAGTGTCGGCAAAGGTAGATGGATTGAAGAATCCGATATACAAAACC
TCAAGTACCTACAAGCTATTGTTAAGGAAACGCACCGACTTTACCCGCCGGCTCCTC
TAACAGGTCACAAATCAAAGAATCTTTATTGTCATTATAATTCTTATTTCATGACCTT
ATTAATCTTGTAAATGTTCAACCTGAATTGTTTATACAAAATGATGAATGATTAGGA
ATCCGCGAAGCACGTGAAGATTGTTTCGTGGGAGGATACCGTGTTGAGAAAGGCAC
ACGCTTGCTCGTAAACATATGGAAACTTCATAGGGATCCCAAGATCTGGCCTGACCC
CAAAACCTTTAAGCCTGAGAGGTTCATGGAGGATAAATCACAATGTGAAAAGAGCA
ACTTTGAATACATTCCTTTCGGTTCGGGAAGGAGGTCGTGTCCGGGAGTCAATCTTG
GTCTAAGAGTTGTACACTTTGTATTGGCTAGATTGCTTCAAGGGTTTGAGTTACACA
AAGTGTCTGATGAACCACTGGATATGGCTGAAGGGCCTGGTTTAGCCTTGCCAAAGA
TTAACCCGGTCGAAGTAGTTGTAATGCCTCGGCTCGACCCGAAGTTGTATAGTTTAC
TCTAAACCATTGTGTACTCGATAATTATTGGGAGCTTCTTGTGA

Nucleotide sequence for the *AtCCD4* gene used in this study:

ATGGACTCTGTTTCTTCTTCTTCCTTCCTCTCCTCCACATTCTCTCTTCATCACTCTCTT
CTTCGCCGCCGATCTTCCTCTCCTACTCTCCTCCGTATCAACTCCGCCGTCGTCGAAG
AACGTTCTCCAATCACAAACCCAAGCGACAACAATGATCGTCGTAACAAACCCAAA
ACACTCCACAACCGAACCAATCACACCTTAGTCTCATCACCACCGAAACTCCGACCA
GAAATGACTCTCGCAACAGCTCTCTTCACCACCGTCGAAGATGTAATCAACACGTTC
ATCGATCCACCTTCACGTCCTTCCGTTGATCCAAAACATGTCCTCTCTGATAACTTCG
CTCCTGTCCTCGACGAGCTTCCTCCAACAGACTGTGAAATCATCCACGGCACTCTTC
CACTGTCACTTAACGGCGCTTACATCCGTAACGGTCCAAATCCACAGTTTCTCCCTC
GTGGTCCTTACCATCTCTTCGACGGCGACGGTATGCTTCACGCCATAAAAATCCACA
ACGGTAAAGCCACTCTCTGTAGCAGATACGTCAAGACTTATAAATACAACGTCGAA
AAACAAACCGGAGCTCCGGTTATGCCTAACGTGTTTTCCGGATTCAACGGTGTAACG
GCGTCAGTAGCTCGTGGAGCTTTAACGGCAGCTAGGGTTTTAACCGGACAGTATAAT
CCGGTTAACGGCATAGGTTTAGCTAATACAAGTCTAGCTTTCTTCAGTAACCGTCTCT
TTGCTTTAGGTGAATCTGATTTACCCTACGCCGTCCGATTAACCGAATCAGGAGATA
TTGAAACGATCGGACGGTACGATTTCGACGGGAAATTAGCGATGAGTATGACAGCT
CATCCTAAAACCGATCCAATAACCGGAGAAACTTTCGCTTTCCGGTACGGTCCGGTT
CCACCGTTTTTAACATATTTCCGGTTTGATTCCGCCGGGAAAAAACAAAGAGACGTT
CCGATATTCTCGATGACGTCTCCGTCGTTTCTCCATGACTTCGCGATCACGAAACGTC
ACGCGATTTTCGCAGAGATTCAGCTTGGCATGAGGATGAACATGTTGGATTTGGTTC
TCGAAGGTGGTTCTCCGGTTGGTACTGATAACGGAAAAACTCCAAGGCTTGGAGTG
ATTCCTAAGTACGCCGGAGATGAGTCGGAGATGAAATGGTTCGAAGTTCCTGGATTC
AATATCATTCACGCTATTAATGCTTGGGATGAAGATGATGGAAACAGCGTCGTTTTG
ATTGCACCGAATATTATGTCGATTGAACATACTTTAGAGAGGATGGATCTGGTTCAT
GCTTTGGTGGAGAAGGTGAAGATCGATCTCGTCACCGGGATTGTGAGACGTCATCCG
ATCTCAGCGAGGAATCTCGATTTCGCTGTGATTAATCCGGCGTTTCTCGGGAGATGT
AGCAGGTACGTTTACGCGGCGATTGGAGATCCGATGCCGAAGATCTCCGGTGTGGT
GAAGCTTGATGTGTCTAAAGGAGATCGGGATGATTGTACGGTGGCCCGTAGAATGT
ACGGTTCAGGTTGTTACGGCGGAGAACCGTTTTTCGTAGCTAGGGATCCTGGTAATC
CGGAGGCGGAGGAGGATGATGGTTATGTGGTGACGTATGTTCACGATGAAGTGACT
GGAGAATCGAAGTTTCTGGTGATGGACGCTAAATCGCCGGAGCTTGAAATCGTCGC
CGCCGTGAGGTTGCCGCGAAGGGTTCCGTACGGATTCCATGGGTTATTTGTCAAGGA
AAGTGACCTTAATAAGCTTTAA

Curriculum Vitae

Name: Scott R. Hughes

Post-Secondary Education and Degrees:

B.Sc. – The University of Guelph
Guelph, Ontario, Canada
2011 – 2015

M.Sc. – The University of Western Ontario
London, Ontario, Canada
2016 – 2018

Related Work Experience:

Research Assistant – Vineland Research and Innovation Centre
Vineland, Ontario, Canada
2015 – 2016

Teaching Assistant – The University of Western Ontario
London, Ontario, Canada
2016-2018

Publication and Presentations:

- Laur, W., Hughes, S., Caceres, L., Challa, S., Hannoufa, A., & Scott, I. (2017) Modifying the expression of plant volatiles to affect the behaviour of greenhouse insect pests. International Organization for Biological Control and Integrated Control West Palaearctic Regional Section Bulletin 124: 31-36. [Short Communication]

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