Molecular and Functional Analysis of Volatile Isoprenoids in Arabidopsis

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1. Introduction

1.1 Plants and their invisible volatile organic compounds (VOCs)

Plants are sessile for most of their life cycle, but seem to compensate for their immobility in plant-organism interactions by emitting a huge variety of volatile organic compounds (VOCs) into the environment from both above-ground (leaves and flowers) and below-ground (roots) tissues. The amount of plant volatile emission is several orders of magnitude higher than that of animals (Dicke and Loreto, 2010). About 1700 organic compounds, with enough vapor pressure at normal temperature and pressure to volatilize, have been found to be released from plants (Dicke and Loreto, 2010; Loreto and Schnitzler, 2010). The estimated annual global VOC emission is 1150 tera-gram per year (Guenther et al., 1995). Most VOCs can be assigned into following groups (see Table 1.1).

Table 1.1 Plant volatile organic compounds (VOCs)

Group	**Name of examples**
Simple gas	carbon dioxide (CO_2)
C1 and C2 oxygenated compounds	methanol (CH_3OH, CH_4O) formaldehyde (CH_2O) ethanol (CH_3CH_2OH, CH_6O) acetaldehyde (C_2H_4O)
Terpenes	hemiterpene (C5): e.g. isoprene and methylbutenol monoterpene (C10): e.g. limonene, myrcene, (*E*)-β-ocimene sesquiterpene (C15): e.g. (*E*)-β-caryophyllene
Fatty acid derivatives	e.g. methyl jasmonate, green leaf volatiles (C6 aldehydes, alcohols and esters)
Benzenoids	e.g. benzylacetate, methylsalicylate (Boatright et al., 2004)
Phenylpropanoids	e.g. ethyl cinnamate, sinapate derivatives (Dixon et al., 2002)
Amino acid derived metabolites	e.g. amines, nicotine and other simple alkaloids
Others	methane (CH_4) ethylene (C_2H_4)

Volatile terpenes (*i.e.* isoprenoids, terpenoids) represent more than 50% of the total volatiles released from plants with up to 10 times higher emissions than those of other VOCs (Pichersky and Gershenzon, 2002; Loreto and Schnitzler, 2010). For instance, isoprene (44%) and monoterpenes (11%) are major components of annual global VOC emissions (Guenther et al., 1995). Sesquiterpenes are estimated to make up to 28% of overall plant VOCs in certain forest sites (Helmig et al., 1999). These substances can comprise a significant fraction of fixed photosynthetic carbon (Vickers et al., 2009), which leads to the question why plants release volatile terpenes into the environment. To date, we still have a limited understanding of the biological role of plant volatile terpene emissions.

Plants release VOCs in different ways: Some plants constitutively emit terpenes either throughout their whole life cycle or at specific developmental stages, such as flowering, fruit ripening, leaf and needle maturation and senescence; other plants release terpenes in association with various biotic and abiotic stresses. Abiotic factors (high light, high temperature, atmospheric pollutant O_3, atmospheric CO_2 concentration, water, salt and other nutrients) significantly affect volatile terpene emissions (Vickers et al., 2009; Loreto and Schnitzler, 2010). Among biotic stresses, herbivore feeding and pathogen attack are triggering factors for inducing the release of VOCs (Mithofer et al., 2005; Holopainen and Gershenzon, 2010). Constitutively emitted volatiles may be stimulated or quenched by external stresses (Loreto and Schnitzler, 2010), while induced terpene volatiles can be released either from existing storage sites (glandular trichomes or resin ducts) or be synthesized *de novo* at wound sites or systemically in more distant tissues. In response to stress, it is sometimes observed that the emission of volatile terpenes is more sustained than that of other volatiles even when carbon uptake and photosynthesis are limited (Brilli et al., 2007; Vickers et al., 2009; Loreto and Schnitzler, 2010) suggesting that such emission has an

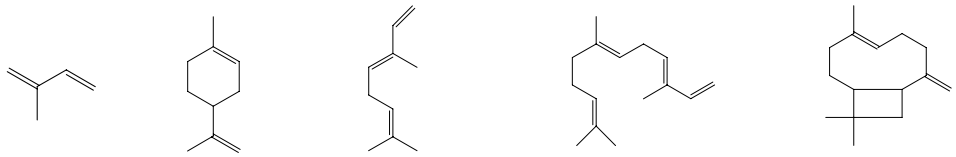
important purpose. A variety of different functions have been associated with the emission of volatile terpenes: Terpenes have been found to protect plants against herbivores either directly by repelling oviposition and feeding or indirectly by attracting enemies of herbivores (Pichersky and Gershenzon, 2002; Dicke et al., 2003; Koellner et al., 2008). Volatiles released upon herbivore attack also previously attacked plants to respond faster or to a greater degree to a second round of damage (Unsicker et al., 2009). Terpenes, such as isoprene and monoterpenes also appear to mitigate abiotic stresses, such as high temperature and oxidative stress (Holopainen and Gershenzon, 2010; Loreto and Schnitzler, 2010; Schnitzler et al., 2010).

Once plant volatiles are released into the atmosphere, they take part in many processes that influence the levels of ozone and aerosols (Atkinson and Arey, 2003; Loreto and Schnitzler, 2010). The resulting chemical and physical changes in the atmosphere may have indirect feedback on plants. Under ongoing global climate change, terpene emission patterns may be altered leading to dramatic changes in plant-plant, plant-herbivore, plant-atmosphere interactions that are beyond the current expectations of ecologists and biologists (Holopainen and Gershenzon, 2010). The obvious abundance of plant terpenes but a yet limited understanding of their biological roles and molecular regulation prompted us to investigate these aspects in greater detail by using *Arabidopsis thaliana* as a model system.

1.2 Volatile terpene biosyntheses and regulations

Volatile terpenes belong to the largest and most diverse class of plant secondary metabolites, with more than 30,000 structural variants (Degenhardt et al., 2009). The majority of hemiterpenes (C5), monoterpenes (C10), and sesquiterpenes (C15) are volatile (representative volatile terpene structures in Fig. 1.1). Due to their low molecular weights

(< 300 Da), these compounds can be released into the atmosphere or the soil at ordinary biological temperatures. Terpene volatiles are mostly lipophilic and can cross cell membranes easily (Dudareva et al., 2006; Vickers et al., 2009).



Isoprene Limonene (*E*)-β-Ocimene (*E,E*)-α-farnesene (*E*)-β-Caryophyllene

Figure 1.1. Chemical structures of representative terpene volatiles: isoprene is a hemiterpene; limonene and (*E*)-β-ocimene are monoterpenes; (*E,E*)-α-farnesene and (*E*)-β-caryophyllene are sesquiterpenes.

Generally, two alternative pathways are responsible for the formation of the basic units of volatile terpenes in plants, the plastid-localized MEP (2-*C*-methyl-D-erythritol 4-phosphate) pathway and the cytosol-localized MVA (mevalonic acid) pathway, which is involved in ER (endoplasmic reticulum) membrane systems and a partial peroxisomal localization according to the newest finding by Sapir-Mir et al. (Sapir-Mir et al., 2008) (Fig. 1.2). The two pathways are both responsible for the formation of the basic C5 units IPP (isopentenyl diphosphate) and DMAPP (dimethylallyl diphosphate) (Lange et al., 2000). The MEP pathway was discovered in many bacteria and plastids of plants (Rohmer, 2008), while the MVA pathway is common in animals, fungi and plants (Lange et al., 2000). Once the C5 units are formed, isoprenyl diphosphate synthases (part of the prenyltransferase enzyme family), catalyse condensations of IPP and DMAPP into GPP (geranyl diphosphate, C10), FPP (farnesyl diphosphate, C15) and GGPP (geranylgeranyl diphosphate, C20) (Liang et al., 2002). Finally, diverse terpene synthases (TPSs) convert DMAPP, GPP and GGPP into hemiterpenes (C5), monoterpenes (C10), diterpenes (C20) and tetraterpenes (C40) in plastids;

whereas other TPSs convert FPP to sesquiterpenes (C15) and triterpenes (C30) usually in the cytosol (Bohlmann et al., 1998; Lange et al., 2000) (Fig. 1.2).

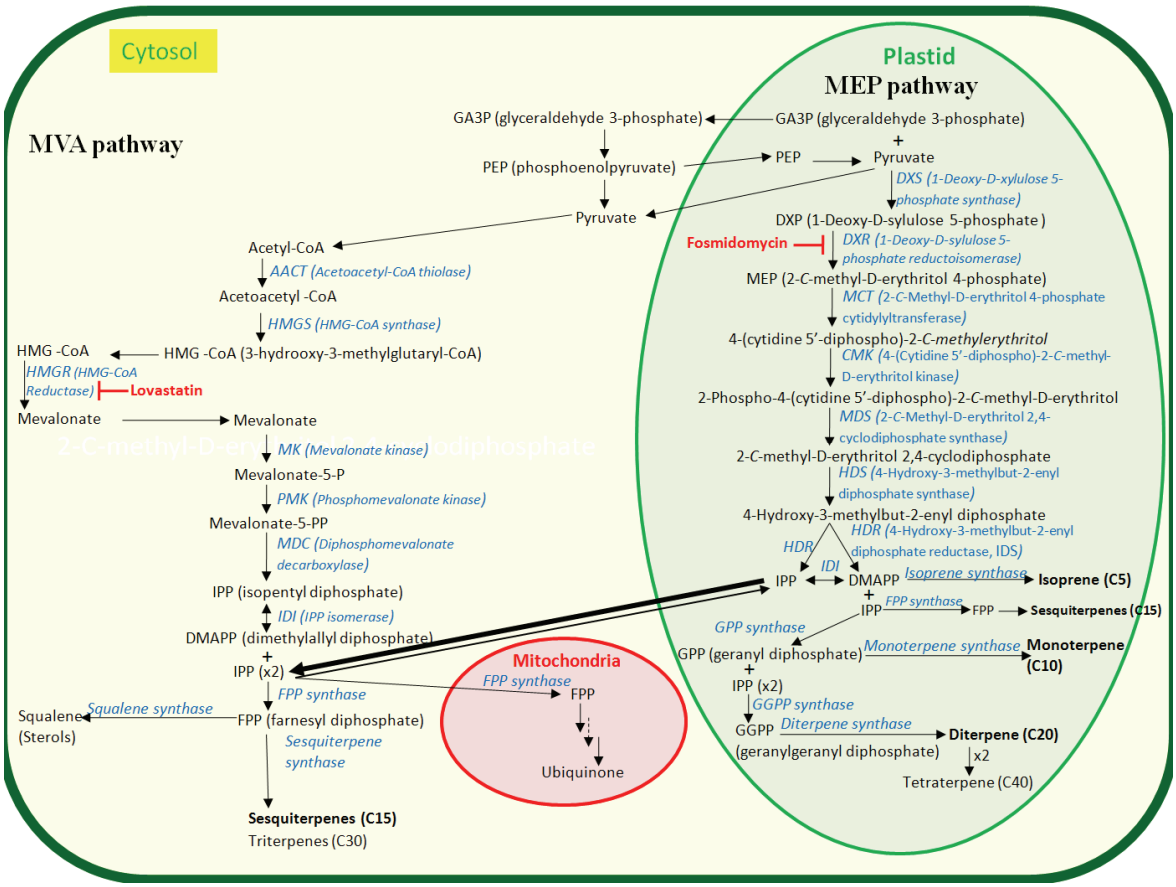


Figure 1.2. Scheme of the subcellular compartmentation of volatile terpene pathways, including the newly proposed crosstalk between the pathways and plastidial FPP biosynthesis (Cunillera et al., 1996; Lange et al., 2000; Phillips et al., 2008; Sapir-Mir et al., 2008; Sallaud et al., 2009).

Beyond the outline of the two pathways mentioned above, many complex aspects of volatile terpene biosynthesis have been described in recent years. For example, a flow of intermediates between the pathways occurs (Hemmerlin et al., 2003; Laule et al., 2003; Schuhr et al., 2003; Dudareva et al., 2005) especially from the chloroplasts towards the

cytosol (Laule et al., 2003). The intermediate FPP, once thought to be confined to the cytosol, is also present in the plastid (Aharoni et al., 2003; Schuhr et al., 2003). Sesquiterpene formation occurs not only in the cytosol, but also in plastids, ER systems, and mitochondria (Cunillera et al., 1996; Dudareva et al., 2005; Sapir-Mir et al., 2008; Sallaud et al., 2009). At the regulatory level, substrate availability, gene regulation, enzyme activity, subcellular compartmentation and other factors control the rate of terpene formation. However, it is still unclear how these different modes of regulation contribute to the diverse patterns of terpene production in nature, especially for volatile terpenes. In Chapter I, we have investigated the natural diversity of volatile terpenes from the model plant *Arabidopsis thaliana* and elucidated some of the molecular mechanisms that control it.

1.3 Caryophyllene: an important sesquiterpene

Sesquiterpenes are among the most studied volatile terpenes investigated. Compared to isoprene and monoterpenes, they have more complex chemical structures, a higher molecular weight and lower vapor pressure. Caryophyllene, a widespread volatile terpene, is a common constituent of essential oils or resins in plants, and thanks to modern quantification measurements (Komenda et al., 2001; Helmig et al., 2003; Helmig et al., 2004; Tholl et al., 2006), it is considered to be one of the most abundant sesquiterpenes in nature (Arey et al., 1995; Ciccioli et al., 1999). Actually, the earliest study of caryophyllene may date back more than 170 years, and the chemical structure of caryophyllene was established at the beginning of the 1950s (Tkachev, 1988). The chemical name of caryophyllene is (1R,4E,9S)-4,11,11-Trimethyl-8-methylenebicyclo[7.2.0]undec-4-ene (Fig. 1.3). Caryophyllene has *trans*-linked butane and nonane carbon rings and a *trans*-substituted double bond in the nine-membered ring. As an unusual hydrocarbon, it has attracted chemists'

attentions for many years. In nature, the most abundant isomer is (*E*)-β-caryophyllene (Fig. 1.3A). The *cis* isomer (Z)-β-caryophyllene (i.e. isocaryophyllene) (Fig. 1.3B) with the *cis*-substituted endocyclic double bond only rarely accompanies (*E*)-β-caryophyllene and is found in an insignificant amount (Tkachev, 1988). The ring-opened isomer, designated as α-caryophyllene or α-humulene (Fig. 1.3C) is found in low amounts as well. Nearly 40 years ago, at the beginning of the 1970s, the biosynthetic pathway of (*E*)-β-caryophyllene was discovered for the first time by using radioactive mevalonate-2-^{14}C in peppermint (*Mentha piperita* L.). Interestingly, the biosynthetic location of (*E*)-β-caryophyllene appeared to be different from that of the monoterpenes in this species, based on different incorporation patterns, a fact substantiated many years later when the biosynthetic genes were finally cloned (Croteau and Loomis, 1972).

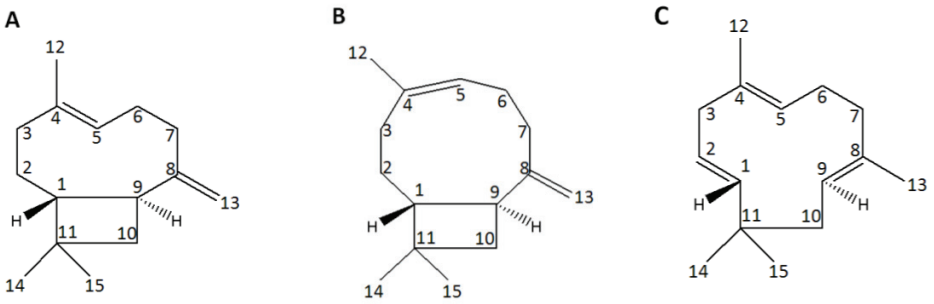


Figure 1.3. Chemical structures of (*E*)-β-caryophyllene (A), (Z)-β-caryophyllene (B) and α-humulene (C).

(*E*)-β-caryophyllene has a typical dry wood smell, although it is aromatically weak and not obviously distinctive to most human noses. The presence of (*E*)-β-caryophyllene in herbs and herbal extracts has been noticed for a long time. For instance, it is one of the most abundant metabolites in green tea flavors (Nose et al., 1971), and also a main contributor (up to 79%) to the spicy taste of the essential oil of pepper (*Piper nigrum* L.) (Orav et al., 2004). Interestingly, in the atmosphere, the reactive life time of (*E*)-β-caryophyllene is only 1-2 min

because of its rapid reaction with various oxygen species via its internal and external double bonds (Shu and Atkinson, 1994; Shu and Atkinson, 1995; Atkinson and Arey, 2003). The major oxidation products, as much as 70%, are main contributors to aerosol formation in secondary organic aerosol chamber experiments (Lee et al., 2006; Asa-Awuku et al., 2009). The special features of (*E*)-β-caryophyllene have made it one of the most popular sesquiterpenes for study in recent atmospheric, ecological and biochemical research projects.

From animal studies, (*E*)-β-caryophyllene in foods has been identified as a functional nonpsychoactive CB2 (cannabinoid receptor type 2) receptor ligand and a macrocyclic anti-inflammatory cannabinoid in *Cannabis sativa* L. (Gertsch et al., 2008). But, no corresponding receptor has been found in plants. From many reports, we know that (*E*)-β-caryophyllene is emitted with different emission rates from various plant species (Duhl et al., 2008); it can either be emitted constitutively or be induced by abiotic or biotic environmental factors as other volatile terpenes. An overview of (*E*)-β-caryophyllene emissions from different plant species under various environmental stresses is given in Tables 1.2 and 1.3.

Table 1.2. Emission of (*E*)-β-caryophyllene from selected plant species*

Plant species	% of total VOC emission	% of total sesquiterpene emission	Estimated emission rate	References
Rosa hybrida L. cv. Honesty (flowers)	**0.4%**	22%	100 ng h^{-1}	(Helsper et al., 1998)
Corn (*Zea mays* cv. Delprim)	**3%**		0.1125 ng g_{DW} h^{-1}	(Ruther and Kleier, 2005)
Marsh Elder (*Iva frutescens*)	**1-4%**	5-39%	1000 -11000 ng m^{-2} h^{-1}	(Degenhardt and Lincoln, 2006)
Silver Birch (*Betula pendula* Roth)	**1-4%**	10-74%	4-1184 ng g_{DW} h^{-1}	(Vuorinen et al., 2005)
Rock Rose (*Cistus albidus* L.)	**6.8%**	14%	150 ng g_{DW} h^{-1}	(Ormeno et al., 2007)
Tomato (*Lycopersicon esculentum*)	**7%**			(Buttery et al., 1987)
Hornbeam (*Carpinus betulus*)	**12.6%**	100%	20.3 ng g_{DW} h^{-1}	(Koenig et al., 1995)
Corn (*Zea mays*)	**0.7-15%**	1-17%	wide variation	(Gouinguene and Turlings, 2002; Ruther and Kleier, 2005)
Cinnamon (*Cinnamomum* spp.)	**22%**			(Jayaprakasha et al., 2003)

Carnation (*Dianthus caryophyllus*) (flowers)	**23%**			(Lavy et al., 2002)
Scots Pine (*Pinus sylvestris* L.)	**0-26%** [(1), (2)]	9-36% [(3)]	0-533 ng g_{DW} h^{-1} [(1)]	(1) (Tarvainen et al., 2005), (2) (Hakola et al., 2006), (3) (Holzke et al., 2006)
Sunflower (*Helianthus annuus* L. cv. giganteus)	**26%**	~91%	93-303 ng g_{DW} h^{-1}	(Schuh et al., 1997)
Sage (*Salvia* spp.)	**12-32%**			(Liang et al., 2009)
Nicotiana sylvestris(flowers)	**35%**			(Loughrin et al., 1990)
Ginger (*Zingiber* spp.)	**42%**			(Sabulal et al., 2006)
***Arabidopsis thaliana*,** Col-0 ecotype (Thale Cress) (flowers)	**44%**	~75%	11.5 ng h^{-1}	(Chen et al., 2003)
Orange (*Citrus sinensis* L.) OSBECK	**40-45%**		20-410 ng g_{DW} h^{-1}	(Hansen and Seufert, 1999)
Potato (*Solanum tuberosum* L. cv. Desireé)	**38-46%**	38-46%	609-1276 ng h^{-1}	(Agelopoulos et al., 2000)
Tobacco (*Nicotiana tabacum*)	**24-47%**		1227-3741 ng h^{-1}	(De Moraes et al., 2001)
Orange (*Citrus sinensis* and *Citrus clement,* sampled during July)	**50-70%**	>98%	180000 -360000 ng m^{-2} h^{-1}	(Ciccioli et al., 1999)
Downy Birch (*Betula pubescens*)		46-61%		(Zhang et al., 1999)
Pepper (*Piper nigrum* L.)	**79%**			(Orav et al., 2004)
Black Elder (*Sambucus nigra*)		0-5%		(Zhang et al., 1999)
Beach Pine (*Pinus contorta*)		6%		(Helmig et al., 2007)
White Pine (*Pinus strobes*)		7%		(Helmig et al., 2007)
Trembling Aspen (*Populus tremula)*		0-15%		(Zhang et al., 1999)
Ponderosa Pine (*Pinus ponderosa*)		22%		(Helmig et al., 2007)
Loblolly Pine (*Pinus taeda* L.)		26-67%	297 ng g_{DW} h^{-1}	(Helmig et al., 2006)
Norway spruce [Picea abies L. (Karst)]		49%		(Martin et al., 2003)

* Accurate quantifications of (*E*)-β-caryophyllene are listed here; studies reporting approximate amounts are not included.

Table 1.3. Changes of (*E*)-β-caryophyllene emission under various environmental influences

Environmental factors	Plant species	Emission changes	References
High temperature	Corn (*Zea mays*)	Increase ↗	(Gouinguene and Turlings, 2002)
High temperature	Orange (*Citrus sinensis* L., *Citrus clementi*)	Increase ↗	(Hansen and Seufert, 1999)
High temperature	Sunflower (*Helianthus annuus* L. cv. giganteus)	Increase ↗	(Schuh et al., 1997)
High light	Sunflower (*Helianthus annuus* L. cv. giganteus)	Increase ↗	(Schuh et al., 1997)
High light	Corn (*Zea mays*)	Increase ↗	(Gouinguene and Turlings, 2002)
High light	Scots Pine (*Pinus sylvestris* L.)	*No effect*	(Tarvainen et al., 2005)
Diurnal circadian rhythms	Black sage (*Salvia mellifera*)	High emission ↗ in earlier day, decrease ↘ later	(Arey et al., 1995)
Diurnal circadian rhythms	*Rosa hybrida* L. cv. Honesty (flowers)	Peak ↗ at 6-9h in photoperiod, never dropped off completely in dark period	(Helsper et al., 1998)

Diurnal circadian rhythms	Orange (*Citrus sinensis* L., *Citrus clementi*)	Increase ↗ in the morning, peak in early afternoon, decrease ↘ in the evening	(Ciccioli et al., 1999)
Diurnal circadian rhythms	Loblolly pine (*Pinus taeda* L.)	Increase ↗ in the morning, peak in early afternoon, decrease ↘ in evening	(Helmig et al., 2006)
Diurnal circadian rhythms	Corn (*Zea mays*)	*No effect*	(Gouinguene and Turlings, 2002)
Diurnal circadian rhythms	Potato (*Solanum tuberosum* L. cv. Desireé)	Increase ↗ in the morning, peak in the afternoon	(Agelopoulos et al., 2000)
Diurnal circadian rhythms	Tobacco (*Nicotiana tabacum*)	Higher ↗ emission at day time, during a 2 h interval after 48 h of feeding by *Heliothis. virescens*	(De Moraes et al., 2001)
Diurnal circadian rhythms	Norway spruce [*Picea abies* L. (Karst)]	Peak ↗ in light period	(Martin et al., 2003)
Drought	Corn (*Zea mays*)	*No effect*	(Gouinguene and Turlings, 2002)
Drought	Orange (*Citrus sinensis* L.)	Decrease ↘ after severe drought, no effect after mild drought	(Hansen and Seufert, 1999)
Nutrient deficiency	Corn (*Zea mays*)	Decrease ↘	(Gouinguene and Turlings, 2002)
Nutrient types in soil (siliceous and calcareous)	Rock Rose (*Cistus albidus* L.)	*No effect*	(Ormeno et al., 2007)
Nitrogen deficiency	Corn (*Zea mays*)	Increase ↗	(Schmelz et al., 2003)
Nitrogen deficiency	Cotton (*Gossypium hirsutum* L.)	Increase ↗	(Chen et al., 2008)
Elevated CO_2	Silver Birch (*Betula pendula* Roth)	*No effect*	(Vuorinen et al., 2005)
Elevated O_3	Tobacco (*Nicotiana tabacum* L.)	Induction ↗	(Heiden et al., 1999)
Elevated O_3	Silver Birch (*Betula pendula* Roth)	*No effect*	(Vuorinen et al., 2005)
MeJA	Norway spruce [*Picea abies* L. (Karst)]	Increase ↗	(Martin et al., 2003)
Wounding	Birch (*Betula pubescens*)	Increase ↗	(Hakola et al., 2001)
Wounding	Plantain (*Plantago lanceolata* L.)	Induction ↗	(Fontana et al., 2009)
Wounding	Blue berry (*Vaccinium corymbosum*)	Increase ↗	(Rodriguez-Saona et al., 2009)
Gypsy moth (*Lymantria dispar*)	Blue berry (*Vaccinium corymbosum*)	Increase ↗	(Rodriguez-Saona et al., 2009)
Spodoptera littoralis	Tomato (*Lycopersicon esculentum* Mill. 'Moneymaker')	Increase ↗	(Maes and Debergh, 2003)
Spodoptera littoralis	Plantain (*Plantago lanceolata* L.)	Induction ↗	(Fontana et al., 2009)
Heliothis Virescens	Tobacco (*Nicotiana tabacum*)	Increase ↗	(De Moraes et al., 2001)
Spodoptera littoralis	Corn (*Zea mays*)	Induction ↗ (leaves)	(Koellner et al., 2008)
Western corn rootworm (*Diabrotica virgifera virgife*)	Corn (*Zea mays*)	Induction ↗ (roots)	(Rasmann et al., 2005; Koellner et al., 2008)
Aphid	Silver Birch (*Betula pendula*)	Increase ↗	(Blande et al., 2010)

The emission of (*E*)-β-caryophyllene may be controlled by the expression of (*E*)-β-caryophyllene synthase genes, which have been characterized from the model plant *Arabidopsis thaliana* and various economically and/or ecologically important species (summarized in Table 1.4).

Table 1.4. Characterized caryophyllene synthases

Plant species	Caryophyllene synthases	References
Sweet wormwood (*Artemisia annua*)	(AAL79181)	(Cai et al., 2002)
Arabidopsis thaliana	(NP_197784; TPS21/At5g23960)	(Chen et al., 2003)
Cucumber (*Cucumis sativus*)	(AAU05952)	(Mercke et al., 2004)
Rice (*Oryza sativa* L.)	(ABJ16553, OsTPS3; Os08g04500)	(Cheng et al., 2007; Yuan et al., 2008)
Medicago truncatula	(AAV36464, MtTPS1)	(Arimura et al., 2008)
Corn (*Zea mays*)	(ABY79206, ABY79209~79214)	(Koellner et al., 2008)
Mikania micrantha	(ACN67535)	(Wang et al., 2009)

In the model plant *Arabidopsis thaliana*, there are 32 putative terpene synthase (TPS) genes in a large multigene family (Aubourg et al., 2002). In this species, the (*E*)-β-caryophyllene synthase enzyme (TPS21, At5g23960) converts farnesyl diphosphate (FPP) to (*E*)-β-caryophyllene and small amounts of α-humulene (Fig. 1.4) (Chen et al., 2003; Tholl et al., 2005).

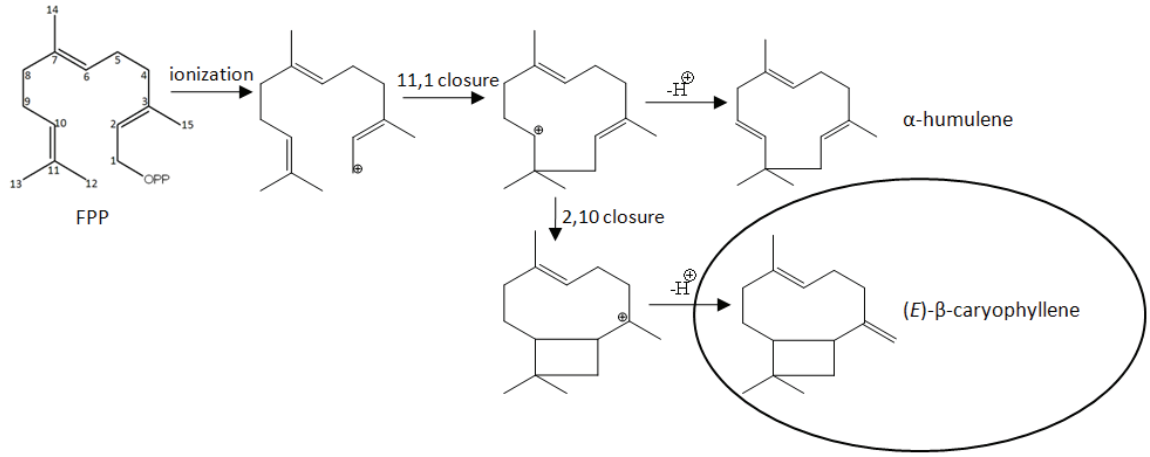


Figure 1.4. Proposed reaction mechanism for the formation of (*E*)-β-caryophyllene by *TPS21* (At5g23960) in *A. thaliana* (Tholl et al., 2005).

While advanced volatile collection and detection techniques have improved knowledge of the distribution of (*E*)-β-caryophyllene volatiles, and molecular characterization has helped us to understand the molecular and biochemical mechanisms of (*E*)-β-caryophyllene biosynthesis, still little is known about the biological significance of this compound. (*E*)-β-Caryophyllene has been reported to defend plants directly against herbivores (Langenheim, 1994) or indirectly by attracting herbivore enemies (Rasmann et al., 2005; Koellner et al., 2008), but whether this terpene is involved in resistance to pathogens or oxidative stresses is still unknown. To study the function of (*E*)-β-caryophyllene as a natural volatile, it seems important to investigate its emission from whole plants and to study plants with enhanced or abolished emission of (*E*)-β-caryophyllene. In this thesis, transgenic *Arabidopsis* over-expression and loss-of-function lines were employed to study the role of (*E*)-β-caryophyllene in response to biotic and abiotic stresses (described in Chapters II and III).

1.4 Objectives of this thesis

The objective of this work is 1) to explore the molecular mechanisms controlling induced volatile terpene emissions in *Arabidopsis thaliana* plants; 2) to understand how (*E*)-β-caryophyllene influences plant defense capabilities during pathogen (*Pseudomonas syringae* pv. *tomato* DC3000) infection and oxidative stress; and 3) to demonstrate the complexity of (*E*)-β-caryophyllene-induced transcriptome profile changes in *Arabidopsis thaliana* transgenic plants under various conditions.

Chapter I describes the qualitative and quantitative variation of herbivore-induced volatile terpenes occurring among different *Arabidopsis thaliana* ecotypes. We demonstrate that allelic variation of terpene synthase genes and subcellular segregation of homologous

bifunctional terpene synthase are controlling factors for some of these intraspecific differences.

Chapter II demonstrates how the sesquiterpene (*E*)-β-caryophyllene protects floral organs in *Arabidopsis thaliana* Col-0. Using transgenic plants with ectopic expression of (*E*)-β-caryophyllene synthase and loss-of-function mutants, the ecological significance of this sesquiterpene as an anti-microbial defense compound, specifically in flowers was revealed.

Chapter III shows the influence of (*E*)-β-caryophyllene on gene transcript profiles in transgenic (*E*)-β-caryophyllene synthase expressing *Arabidopsis* plants and how this compound affects the cellular redox network by adjusting concentrations of reactive oxygen species and expressions of antioxidative enzymes.

2. Chapter I. Variation of herbivore-induced volatile terpenes among Arabidopsis ecotypes depends on allelic differences and subcellular targeting of two terpene synthases, TPS02 and TPS03

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Chapter overview and authors' contributions

This manuscript describes the molecular mechanisms controlling the variation of herbivore-induced volatile terpenes, (*E*)-β-ocimene and (*E*,*E*)-α-farnesene, by using different *Arabidopsis* ecotypes. I was responsible for quantifying differential volatile variation between ecotypes Col-0 and Ws; sequencing analyses of Col-*TPS02*, Col-*TPS03*, Ws-*TPS03*; analyzing transcript differences of *TPS02* and *TPS03* in flowers and leaves under the induction of coronalon, wounding and *Plutella xylostella* feeding damage; characterizing induced volatiles and transcripts of RNAi and knock-out lines of *TPS02* and *TPS03*; cloning Col-*TPS03* and heterologously expressing *TPS02* and *TPS03* in the *E. coli* system; purifying recombinant proteins and performing enzymatic assays with differential substrates; quantifying the effects of inhibitors on induced volatile terpenes; characterizing induced GUS activity of Ws *ProTPS02:GUS*. Christian Abel was responsible for quantifying induced volatiles from 27 *A. thaliana* ecotypes; cloning Ws-*TPS02* and making GFP constructs; analyzing confocal microscopy images. Reza Sohrabi made Ws-*TPS02* RNAi lines. Jana Petri and Ina Haupt made GUS constructs of Col-0 *ProTPS03:GUS* and Ws *ProTPS02:GUS*. Jana Petri characterized induced GUS activity of Col-0 *ProTPS03:GUS*. Dorothea Tholl determined kinetic parameters of *TPS02* and *TPS03*. John Cosimano participated in sesquencing analysis of Ws-*TPS02* and RT-PCR analyses of Ws-*TPS03*. Under the supervision of Jonathan Gershenzon, Dorothea Tholl and I planned and designed all experiments with help from Christian Abel in some experiments. Dorothea Tholl and I wrote the manuscript with help from Jonathan Gershenzon.

2.1 Abstract

When attacked by insects and microbes, plants release mixtures of volatile compounds that are beneficial for direct or indirect defense. Natural variation of volatile emissions frequently occurs between and within plant species but knowledge of the underlying molecular mechanisms is limited. We investigated intraspecific differences of volatile emissions induced from rosette leaves of 27 accessions (ecotypes) of *Arabidopsis thaliana* upon treatment with the jasmonate-mimic coronalon, which elicits a response similar to that caused by insect feeding. Quantitative variation was found for the emission of the monoterpene (*E*)-β-ocimene, the sesquiterpene (*E*,*E*)-α-farnesene, the irregular homoterpene, 4,8,12-trimethyltridecatetra-1,3,7,11-ene (TMTT), and the benzenoid compound methyl salicylate (MeSA). Differences in the relative emissions of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene from accession Ws, a high-(*E*)-β-ocimene emitter, and accession Col-0, a trace-(*E*)-β-ocimene emitter, were attributed to allelic variation of two closely related, tandem-duplicated terpene synthase genes, *TPS02* and *TPS03*. The Ws genome contains a functional allele of *TPS02* but not of *TPS03*, while the opposite is the case for Col-0. Recombinant proteins of the functional Ws *TPS02* and Col-0 *TPS03* genes both showed (*E*)-β-ocimene and (*E*,*E*)-α-farnesene synthase activities. However, differential subcellular compartmentalization of the two enzymes in plastids and the cytosol was found to be responsible for the ecotype-specific differences in (*E*)-β-ocimene/(*E*,*E*)-α-farnesene emission. Expression of the functional *TPS02* and *TPS03* alleles is induced in leaves by elicitor and insect treatment and occurs constitutively in floral tissues suggesting multiple functions of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene in the plant. Our studies show that both pseudogenization in the *TPS* family and subcellular segregation of functional TPS enzymes control the variation and plasticity of induced volatile emissions in wild plant species.

2.2 Introduction

Plants emit a large variety of volatile organic compounds from their foliage and flowers. These volatiles serve a variety of functions ranging from the attraction of pollinating insects and fruit dispersers to direct and indirect defense against herbivores and microbial pathogens (Pichersky and Gershenzon, 2002; Dicke et al., 2003; Dudareva et al., 2006; Unsicker et al., 2009). Usually, plant volatiles are released as mixtures, which may be important in targeting a variety of different organisms. In this context, differences in the composition of volatile blends within and among species may be the result of adaptation to specific communities of organisms.

Terpenoids represent the largest and most diverse class of plant volatile metabolites. Low molecular weight terpenes with a 10-carbon (monoterpenes) or 15-carbon (sesquiterpenes) skeleton are common constituents of floral and herbivore-induced leaf volatile blends (Dudareva et al., 2006). Monoterpenes and sesquiterpenes are produced from the central terpene precursors, geranyl diphosphate (GPP) and farnesyl diphosphate (FPP), respectively, by the enzymatic activity of terpene synthases (TPSs) (Tholl, 2006; Degenhardt et al., 2009). In the plant cell, terpene metabolism is compartmentalized with GPP and monoterpene formation occurring primarily in plastids and FPP and sesquiterpenes being synthesized predominantly in the cytosol (Aharoni et al., 2005).

Studies of cultivated plants have given the first insight into the cellular, molecular genetic and biochemical mechanisms controlling the variability of terpene volatile mixtures. For example, variation of monoterpene and sesquiterpene blends produced in the glandular trichomes of different basil cultivars was attributed to the differential expression of *TPS* genes in these cultivars (Iijima et al., 2004). In maize, allelic variation of two terpene synthases was found to be responsible for the quantitative compositional differences of sesquiterpene volatile blends released from mature leaves and husks of different varieties (Koellner et al., 2004). Furthermore, a characterization of linalool/nerolidol synthases in snapdragon flowers indicated that subcellular segregation of bifunctional terpene synthases in plastids and the cytosol can lead to a compartment-specific formation of monoterpenes and sesquiterpenes, respectively, thereby increasing the diversity of terpene volatile mixtures (Nagegowda et al., 2008).

While the variation of herbivore-induced volatiles has been studied in some wild species (Halitschke et al., 2000; Gouinguene et al., 2001; Delphia et al., 2009), knowledge of the molecular mechanisms governing natural diversity of volatile compounds is rather limited. We have been investigating the intra- and interspecific variation of volatile profiles in *Arabidopsis* species (Tholl et al., 2005; Abel et al., 2009) to explore the natural evolution of volatile mixtures in more detail. A previous survey of 37 accessions (ecotypes) revealed quantitative differences in floral sesquiterpene volatile compositions, which are controlled by differences in *TPS* gene transcription and putative posttranslational modifications (Tholl et al., 2005). Here we turn our attention to vegetative volatiles. Leaves of the *A. thaliana* accession Columbia-0 release a simple three-compound blend consisting of the benzenoid compound methyl salicylate (MeSA), the C_{16}-homoterpene, 4,8,12-trimethyltridecatetra-1,3,7,11-ene (TMTT), and the sesquiterpene (*E,E*)-α-farnesene in response to treatment with the fungal peptide elicitor alamethicin and feeding by the crucifer specialist insects, *Pieris rapae* and *Plutella xylostella* (Van Poecke et al., 2001; Herde et al., 2008). The induced volatile mixture is assumed to serve as an indirect defense signal by attracting parasitoids of *P. rapae* larvae. With the exception of No-0 (Faeldt et al., 2003), no other *A. thaliana* accession has been investigated for elicitor- or insect-induced volatiles and there is no information on the genetic and molecular mechanisms responsible for these differences.

Here we report the analysis of induced volatile terpene emissions from rosette leaves of 27 *A. thaliana* accessions. We show that several accessions such as Ws emit the monoterpene (*E*)-β-ocimene and the sesquiterpene (*E,E*)-α-farnesene while others such as Col-0 release (*E,E*)-α-farnesene without any or only traces of (*E*)-β-ocimene. We demonstrate that the difference in terpene volatile emission between Col-0 and Ws is caused by allelic variation leading to differential expression and subcellular targeting of two closely related bi-functional (*E*)-β-ocimene/(*E,E*)-α-farnesene synthases TPS02 and TPS03. Our work provides evidence that natural diversity of herbivore-induced terpene volatiles evolves at multiple levels of *TPS* gene function and regulation, including the organelle-specific compartmentation of TPS enzymes.

2.3 Results

2.3.1 Elicitor- and Insect-Induced Emission of the Terpene Volatiles (*E*)-β-Ocimene and (*E*,*E*)-α-Farnesene Vary among Different *A. thaliana* Accessions, including Col-0 and Ws

Since *A. thaliana* accessions have been shown to differ substantially in their composition of secondary metabolites such as glucosinolates (Kliebenstein et al., 2001) and floral volatiles (Tholl et al., 2005), we investigated the ecotype-specific variation of terpene emissions from leaves of 27 accessions in response to treatment with coronalon, a synthetic mimic of jasmonic acid and other octadecanoid plant hormones. As previously demonstrated in accession Col-0, coronalon induces the emission of MeSA, (*E,E*)-α-farnesene, the homoterpene TMTT, and its precursor geranyllinalool, a response similar to that observed upon feeding damage by larvae of *P. xylostella* (Herde et al., 2008). By comparing emissions of volatile terpenes from leaves of intact plants treated with coronalon for 22-30 h, we found that 20 accessions released the monoterpene (*E*)-β-ocimene as the predominant volatile (approximately 75 to 95% of the total amount of volatiles) at rates differing 80-fold between lowest and highest emitters (Table 2.1). Ws was among the accessions with highest emission of (*E*)-β-ocimene. The remaining accessions, including Col-0, emitted no or only very small amounts of this monoterpene (Table 2.1). The sesquiterpene (*E,E*)-α-farnesene was released from almost all accessions, including Col-0 and Ws, at emission rates approximately 10-100 fold lower than those of (*E*)-β-ocimene (Table 2.1). In addition to (*E*)-β-ocimene and (*E,E*)-α-farnesene, TMTT and MeSA were detected in the induced volatile blends of all investigated ecotypes, although some accessions released these compounds only in trace amounts (Table 2.1). No emission or only traces of terpene volatiles were found in untreated plants.

Previous feeding experiments with the specialist *P. xylostella* on Col-0 had shown that insect feeding induces a volatile response similar to that observed with coronalon (Herde et al., 2008). In this study, when *P. xylostella* larvae were applied to rosette leaves of accession Ws, a high (*E*)-β-ocimene emitter, emission of (*E*)-β-ocimene, TMTT, and MeSA was induced upon 21-30 h of feeding (Fig. 2.1) with a compound ratio similar to that obtained upon coronalon treatment. Emission rates were substantially lower for all compounds (30-fold lower for (*E*)-β-ocimene) than after application of coronalon but in the range of those observed for Col-0 upon *P. xylostella* damage (Herde et al., 2008) (Fig. 2.1).

Because of this overall lower response, no (*E,E*)-α-farnesene was detected in Ws in response to *P. xylostella* feeding.

We investigated possible correlations among coronalon-induced emissions of the different volatile compounds in all accessions. A high correlation coefficient was found between emission of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene (Fig. 2.2, Supplemental Table SI) indicating a common biosynthetic origin of both compounds. However, the formation of (*E*,*E*)-α-farnesene in Col-0 and other ecotypes that do not produce (*E*)-β-ocimene (Table 2.1) suggested the existence of more than one biosynthetic route to (*E*,*E*)-α-farnesene in the *A. thaliana* genome.

Table 2.1. Emission of the four major volatile compounds from leaves of 27 *A. thaliana* accessions in response to treatment with coronalon (coron). Volatiles were collected for 8 h from single intact plants (see "Material and Methods"). Emission was determined in ng g^{-1}FW h^{-1}. Mean values ± SE of n = 3 are shown. The order of accessions corresponds to increasing (*E*)-β-ocimene emission rates. 0.0 indicates values below 0.01 ng g^{-1}FW h^{-1}; n.d., not detected.

		Compound								
Ecotype		(*E*)-β-ocimene		(*E,E*)-α-farnesene		TMTT		MeSA		Total
		Coron	Control	Coron	Control	Coron	Control	Coron	Control	Coron
1	Bl-1	n.d.	n.d.	0.1±0.0	0.0±0.0	1.8±0.2	0.0±0.0	0.7±0.1	0.0±0.0	2.5±0.4
2	Lip-0	n.d.	n.d.	1.8±0.6	n.d.	3.1±1.0	0.2±0.0	4.1±1.8	n.d.	9.0±3.4
3	Pi-0	n.d.	n.d.	0.8±0.3	0.1±0.1	1.1±0.2	0.0±0.0	1.5±0.2	0.0±0.0	3.4±0.6
4	Tsu-1	n.d.	n.d.	n.d.	0.0±0.0	0.4±0.3	0.0±0.0	1.3±1.0	0.0±0.0	1.8±1.4
5	Can-0	0.2±0,1	n.d.	n.d.	n.d.	6.5±2.5	0.0±0.0	3.1±1.0	0.0±0.0	9.7±3.6
6	Bla-10	0.2±0,0	n.d.	n.d.	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	n.d.	0.3±0.1
7	Col-0	0.7±0.7	n.d.	2.4±0.6	n.d.	8.8±2.0	2.4±0.7	18±5.2	0.3±0.1	29.9±8.5
8	Di-g	2.6±0.8	n.d.	0.2±0.1	n.d.	0.4±0.1	0.0±0.0	1.8±1.0	n.d.	5.0±2.0
9	Ri-0	9.4±4.4	n.d.	0.7±0.4	n.d.	0.6±0.2	0.0±0.0	1.7±1.2	0.0±0.0	12.3±6.2
10	Stw-0	9.7±1.3	0.3±0.6	0.2±0.1	n.d.	1.2±0.1	0.0±0.0	0.0±0.0	n.d.	11.1±1.5
11	Lu-1	11.1±2.8	0.3±0.3	0.4±0.2	n.d.	1.9±0.7	0.1±0.0	1.2±0.5	0.0±0.0	14.6±4.1
12	Est-0	18.6±8.8	n.d.	1.3±0.6	n.d.	0.6±0.1	n.d.	2.6±1.0	n.d.	23.2±10.5
13	Sei-0	19.9±5.8	0.1±0.0	0.3±0.1	n.d.	0.2±0.0	n.d.	0.2±0.1	n.d.	20.5±6.0
14	Chi-0	20.4±9.1	0.3±0.1	0.4±0.2	n.d.	0.5±0.2	0.0±0.0	0.6±0.2	n.d.	21.9±9.7
15	Hodja	20.8±6.7	0.2±0.2	0.6±0.2	0.0±0.0	0.6±0.2	0.0±0.0	1.5±0.7	n.d.	23.5±7.8
16	Bla-1	29.9±4.9	0.1±0.1	1.2±0.3	0.0±0.0	0.1±0.0	0.0±0.0	0.2±0.0	0.0±0.0	31.3±5.3
17	Pog-0	36.2±10.9	n.d.	2.9±0.9	0.3±0.2	0.1±0.0	0.0±0.0	0.7±0.2	0.2±0.1	39.8±12.0
18	An-1	37.5± 4.5	0.3±0.2	1.2±0.0	0.1±0.1	0.4±0.1	0.0±0.0	1.2±0.2	0.1±0.1	40.3±4.8
19	Tul-0	39.6±11.6	n.d.	3.9±1.1	n.d.	0.0±0.0	n.d.	3.4±1.1	n.d.	46.8±13.9
20	Mt-0	45.9±19.5	0.1±0,0	5.9±2.5	0.1±0.1	2.2±0.1	0.2±0.1	1.4±0.5	0.0±0.0	55.4±22.6
21	Kil-0	46.9±17.4	0.0±0.0	1.1±0.4	n.d.	0.3±0.1	0.0±0.0	1.2±0.5	n.d.	49.5±18.4
22	JI-3	51.2±20.1	n.d.	2.8±1.2	0.1±0.1	1.3±0.3	0.0±0.0	1.2±0.4	n.d.	56.4±22.1
23	Ang-0	64.4±21.9	0.8±0.1	2.1±0.8	0.1±0.1	5.1±1.4	0.2±0.1	1.9±0.9	0.0±0.0	73.5±25.0
24	Ws	64.6±16.5	0.5±0.3	5.4±1.8	0.0±0.0	7.2±1.7	0.2±0.1	2.2±0.7	0.1±0.1	79.3±20.8
25	Condara	80.4±19.4	0.3±0.1	3.6±0.8	0.3±0.3	3.5±0.8	0.1±0.1	1.1±0.3	0.0±0.0	88.6±21.4
26	Ty-0	127.3±19.4	0.1±0.1	9.6±1.8	0.1±0.0	0.4±0.2	0.1±0.0	5.7±3.3	0.0±0.0	143.0±24.6
27	Kas-1	160.6±12.2	0.2±0.1	2.7±0.2	0.0±0.0	5.4±0.9	n.d.	3.1±0.4	0.1±0.0	171.8±13.6

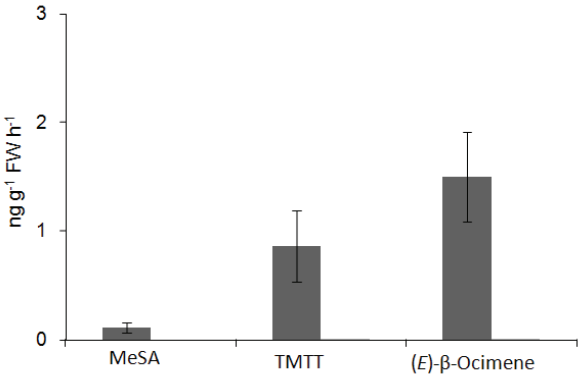


Figure 2.1. Induced emission of (*E*)-β-ocimene, MeSA, and TMTT from leaves of accession Ws in response to insect feeding. Volatiles were collected for 9 h from intact individual plants upon application of *P. xylostella* larvae as described under "Materials and Methods". Results from the second day of treatment (21 to 30 h) are shown. Error bars are SE of n = 3.

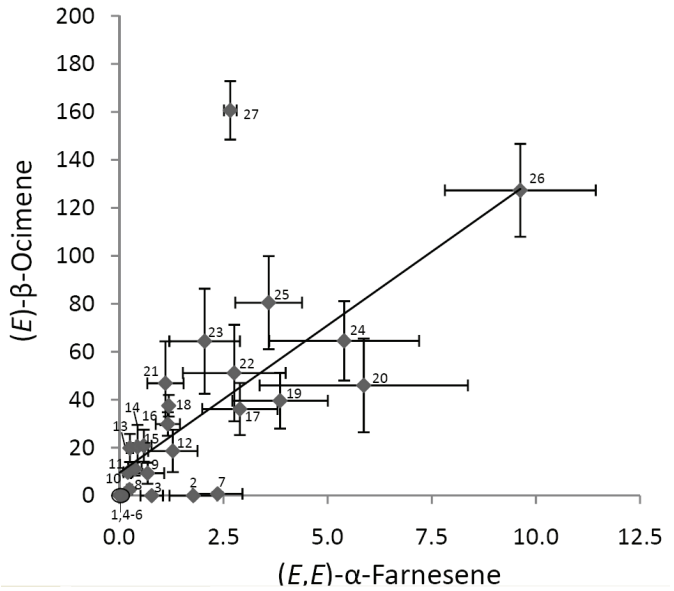


Figure 2.2. Correlation of (*E*)-β-ocimene and (*E,E*)-α-farnesene emission from coronalon-treated leaves of 27 *A. thaliana* accessions. Treatment with coronalon and volatile collection were conducted as described under "Materials and Methods". Emissions are in ng g^{-1} FW h^{-1}. Numbers indicate individual accessions according to Table 2.1. Each value represents the mean ± SE of three replicates.

2.3.2 The Tandem Terpene Synthase Genes *TPS02* and *TPS03* Differ in Expression between the Col-0 and Ws Accessions

From the analyzed accessions, we selected Ws and Col-0 for further investigation since these two accessions showed clearly different, elicitor-induced terpene volatile profiles and therefore seemed suitable for determining the molecular mechanisms underlying these differences. We reasoned that the variation of (*E*)-β-ocimene emission between the two accessions could be due to the differential expression or function of terpene synthase 3 (*TPS03*/At4g16740) or a monoterpene synthase closely related to *TPS03*. A *TPS03*-encoded recombinant enzyme from *A. thaliana* accession C24 was previously shown to catalyze the conversion of the ubiquitous precursor GPP to (*E*)-β-ocimene (Bohlmann et al., 2000; Faeldt et al., 2003). Moreover, transcription of *TPS03* was shown to be induced in leaves of the (*E*)-β-ocimene emitting ecotype No-0 by treatment with jasmonic acid and wounding. Besides *TPS03*, only one other monoterpene synthase (*TPS10*) has been found to be induced in *A. thaliana* leaves (Bohlmann et al., 2000). However, this enzyme produces myrcene as the primary product with minor amounts of (*E*)-β-ocimene *in vitro* and seems to have negligible activity in the investigated ecotypes since no emission of myrcene was detected. In the *A. thaliana* genome, *TPS03* is positioned on chromosome 4 in close proximity to terpene synthase 2 (*TPS02*/At4g16730) (Fig. 2.3A). Both genes are 51.5% identical at the nucleotide sequence level and share similar structures with seven exons and six introns indicating they likely emerged by gene duplication. We therefore analyzed both *TPS02* and *TPS03* alleles and their expression in the Col-0 and Ws accessions.

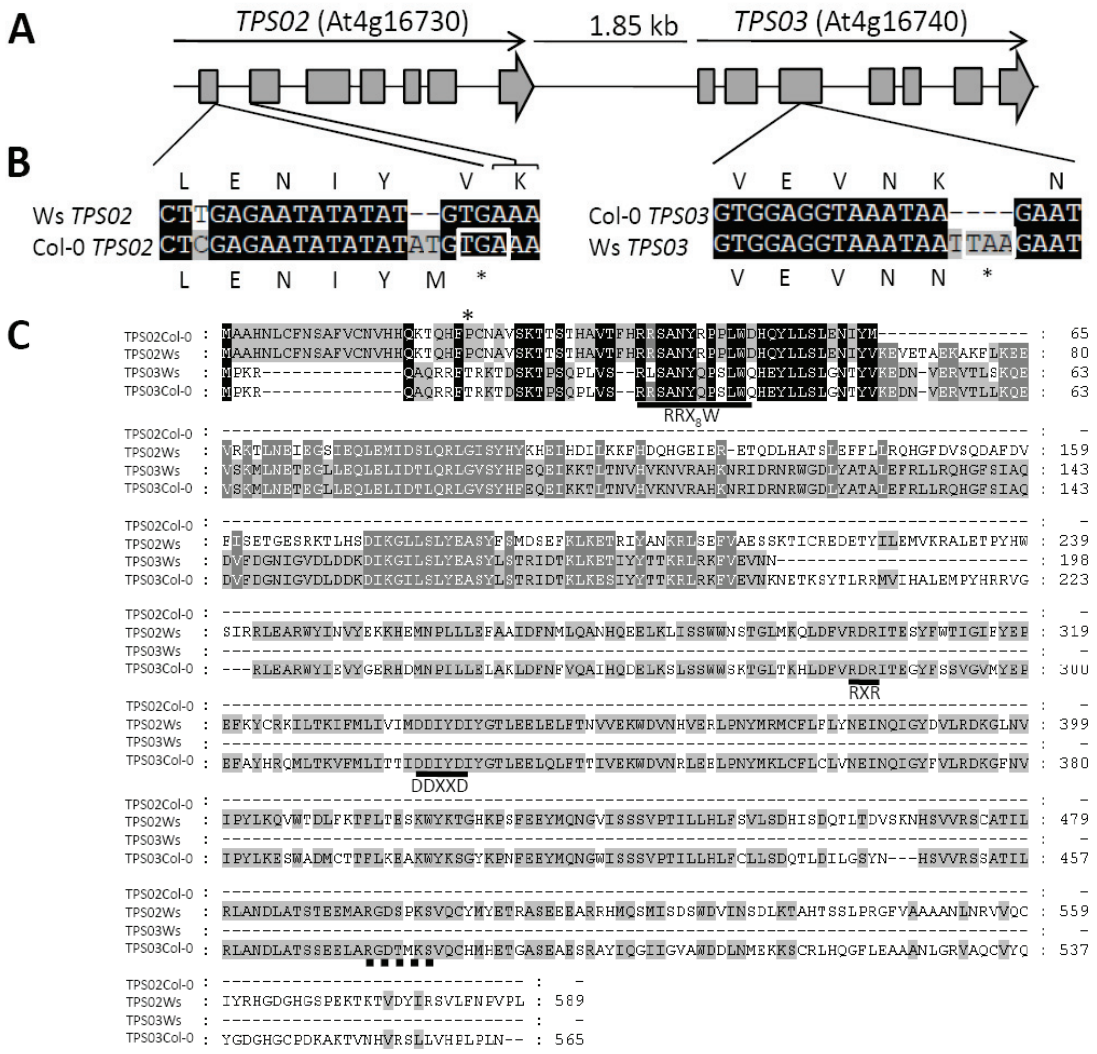


Figure 2.3. Molecular nature of the *TPS02* and *TPS03* alleles in accessions Col-0 and Ws.
A, Schematic representation of the structures of *TPS02* and *TPS03*. Exons are represented by the gray boxes, and flanking regions and introns are represented by the line between boxes. **B**, Alignment of nucleotide and amino acid sequence regions of the *TPS02* (left) and *TPS03* (right) alleles from accessions Col-0 and Ws indicating frame shift mutations caused by base pair insertions in the Col-0 *TPS02* and Ws *TPS03* genes. White boxes mark premature stop codons. The gene-specific position of the sequences is indicated. **C**, Amino acid sequence alignment of the full-length and truncated proteins of the Col-0 and Ws *TPS02* and *TPS03* alleles. Amino acids shaded in black are conserved in all sequences, and gray shades indicate amino acids conserved in two or three sequences. Dashes indicate gaps inserted for optimal alignment. Horizontal lines mark the highly conserved DDXXD, RXR, and RRX_8W motifs. A motif similar to the H-α1 loop region of apple MDAFS1 is marked by a thick dashed line. The asterisk indicates the putative cleavage site for a 25 amino acid plastidial transit peptide of the TPS02 protein.

In the Col-0 ecotype, which does not emit (*E*)-β-ocimene, TPS02 does not encode a full length TPS protein because of a two-base (AT) insertion 184 nucleotides downstream of the start codon leading to a frame shift and premature translational termination (Fig. 2.3B, C). Despite the apparent loss of function of this allele, transcription of TPS02 was induced upon treatment with coronalon (Fig. 2.4A). A splice variant of the TPS02 transcript was found, which lacks a part of the first exon and the entire second exon (Supplemental Fig. S1) and does not code for a functional protein. In contrast to TPS02, the Col-0 TPS03 gene encodes a full length terpene synthase protein of 565 amino acids (Fig. 2.3C). Transcription of TPS03 was induced by coronalon treatment as well as in response to 24 h of *P. xylostella* feeding while only low levels of TPS03 transcript were observed upon mechanical wounding (Fig. 2.4A). However, the induced expression of TPS03 (reported as an (*E*)-β-ocimene synthase in ecotype C24 (Faldt et al., 2003)) was hard to reconcile with the lack of (*E*)-β-ocimene emission in the Col-0 ecotype, indicating possible differences in the regulation or function of the Col-0 TPS03 protein compared to that in ecotype C24.

We further analyzed the expression of the *TPS02* and *TPS03* alleles in the (*E*)-β-ocimene-emitting accession Ws. Semiquantitative RT-PCR analysis demonstrated that *TPS02* was transcribed upon coronalon treatment (Fig. 2.4B). Transcription of Ws *TPS02* was also induced by *P. xylostella* feeding while no transcript was detected upon mechanical wounding (Fig. 2.4B). A full length 1770 bp *TPS02* cDNA was amplified from coronalon-treated Ws leaves encoding a 589 amino acid protein (Fig. 2.3C). The *TPS02* coding sequence differs from the Col allele in the position of 18 nucleotides corresponding to 11 amino acid differences and does not show the frame shift mutation due to the AT-insertion (Fig. 2.3B). The Ws TPS02 protein shares 62 % amino acid sequence identity with the TPS03 (*E*)-β-ocimene synthase protein from C24. Thus, it seemed possible that a functional TPS02 enzyme could produce (*E*)-β-ocimene or very similar monoterpene compounds.

No transcript was found for *TPS03* in response to any of the treatments of Ws leaves. To investigate the absence of the *TPS03* transcript in Ws in more detail, we amplified the *TPS03* gene including the 5’ and 3’-UTRs from genomic DNA of Ws. The nucleotide sequences of the Ws *TPS03* gene and the Col *TPS03* gene were 99.5% identical. An insertion of four nucleotides (TTAA) was found in the third exon causing a frame shift mutation and premature translational termination (Fig. 2.3B). We then performed RT-PCR with gene

specific primers designed to amplify small fragments of a putative *TPS03* transcript. These experiments resulted in only two amplicons, 240 bp and 170 bp, situated consecutively at the 5'-end of the gene, indicating the instability and posttranscriptional degradation of the *TPS03* mRNA (Supplemental Fig. S2).

We also analyzed the transcription of *TPS02* and *TPS03* in flowers of the Col-0 and Ws accessions to determine a possible correlation between the expression of one or the other gene and the floral emission of (*E*)-β-ocimene. Previous analysis of terpene volatiles from flowers of different *A. thaliana* accessions demonstrated that inflorescences of Ws but not Col-0 emit (*E*)-β-ocimene, which is similar to the difference observed for elicitor-induced (*E*)-β-ocimene emission from both accessions. Inflorescences of both accessions also emit small amounts of (*E,E*)-α-farnesene. In Ws flowers, *TPS02* but not *TPS03* was found to be expressed (Fig. 2.4C). In flowers of the Col-0 ecotype, transcripts of *TPS03* were detected, but, in contrast to elicitor-treated leaves, none or only traces of full-length *TPS02* mRNA were amplified (Fig. 2.4C).

Based on the transcriptional differences of *TPS02* and *TPS03* between the two accessions, we hypothesized that *TPS02* is responsible for the formation of (*E*)-β-ocimene in Ws, while the *TPS02* allele is inactive in the Col-0 ecotype. We further presumed that the actively transcribed *TPS03* gene in Col-0 might encode a protein that produced (*E,E*)-α-farnesene, the C_{15} analog of (*E*)-β-ocimene from FPP, instead of (*E*)-β-ocimene itself derived from GPP. Several enzymes with (*E*)-β-ocimene synthase activity from GPP have been shown to also catalyze the formation of (*E,E*)-α-farnesene *in vitro* when supplied with FPP (Pechous and Whitaker, 2004; Nieuwenhuizen et al., 2009) (see below). As an intermediate of terpene biosynthesis, GPP is thought to be largely restricted to the plastids, while FPP is restricted mainly to the cytosol (Aharoni et al., 2005). Analysis of putative protein targeting sequences via computer algorithms indicated that the Col-0 TPS03 protein does not carry a plastidial transit peptide while the TPS02 protein does.

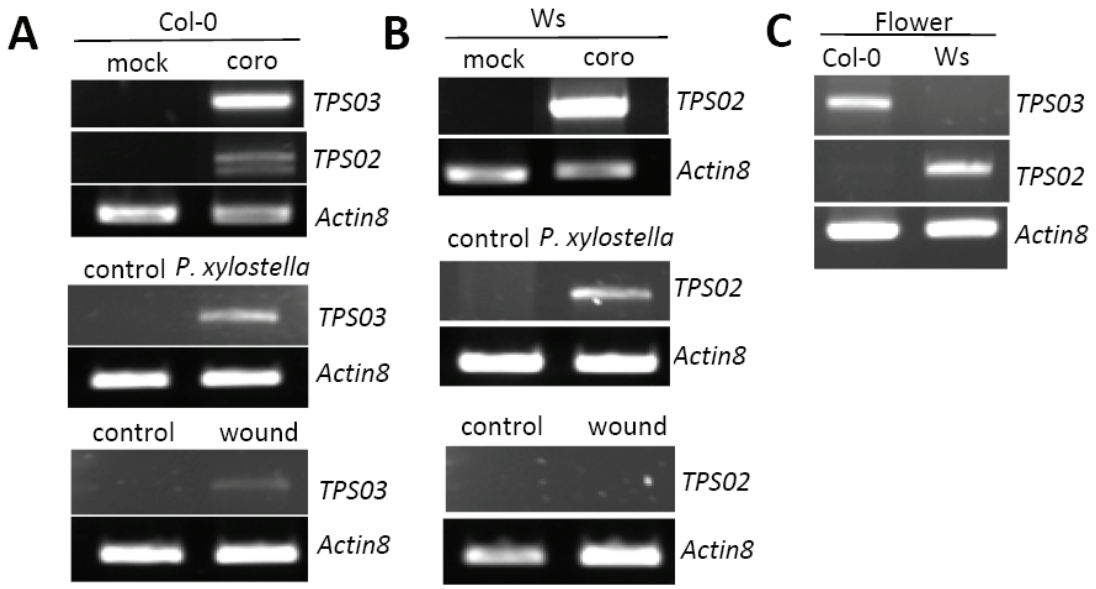


Figure 2.4. Semi-quantitative RT-PCR analysis of *TPS02* and *TPS03* transcript levels in Col-0 and Ws tissues. *Actin 8* transcripts were analyzed as a control. Results are representative for at least three independent experiments. **A**, *TPS02* and *TPS03* transcript analysis from rosette leaves of accession Col-0 treated with coronalon (coro) (upper panel), *P. xylostella* larvae (middle panel), and after mechanical wounding (lower panel). The two amplicons obtained for *TPS02* represent splice variants (Supplemental Figure S1). No *TPS02* transcript was detected upon insect feeding and wounding. Treatments were conducted as described under "Materials and Methods". **B**, Transcript levels of *TPS02* in leaves of accession Ws in response to treatments as described under (A). No mRNA of *TPS03* was detected. **C**, Analysis of *TPS02* and *TPS03* transcripts in flowers of Col-0 and Ws.

2.3.3 *TPS2* and *TPS3* Loss-of-Function Plants Lack Induced Emission of (*E*)-β-Ocimene and/or (*E,E*)-α-Farnesene

To investigate the in planta function of the Ws TPS02 and Col-0 TPS03 genes, we analyzed elicitor-induced volatile profiles of the respective gene knockout lines. In line *Salk_ 132694*, a T-DNA is located in the 5th exon of Col-0 TPS03 (Fig. 2.5A). In contrast to Col-0 wild type plants, the mutant line did not accumulate any TPS03 mRNA after coronalon treatment as determined by semi-quantitative RT-PCR (Fig. 2.5B). No (*E,E*)-α-farnesene was found to be emitted from *Salk_ 132694* upon treatment with coronalon (Fig. 2.5C). Although MeSA and TMTT were released at rates somewhat lower than those of the Col-0 wild type, emission of these two compounds indicated that the elicitor had been successfully administered (Fig. 2.5C). Moreover, no changes were observed for the induced transcription of the TPS02 pseudogene and the myrcene/(*E*)-β-ocimene synthase TPS10 in comparison to

wild type plants. Since no other TPSs with putative (*E,E*)-α-farnesene or (*E*)-β-ocimene synthase activities are expressed in Col-0 leaves upon coronalon treatment (Herde et al., 2008), the results strongly suggested that the TPS03 gene is responsible for the induced formation of (*E,E*)-α-farnesene in the Col-0 accession.

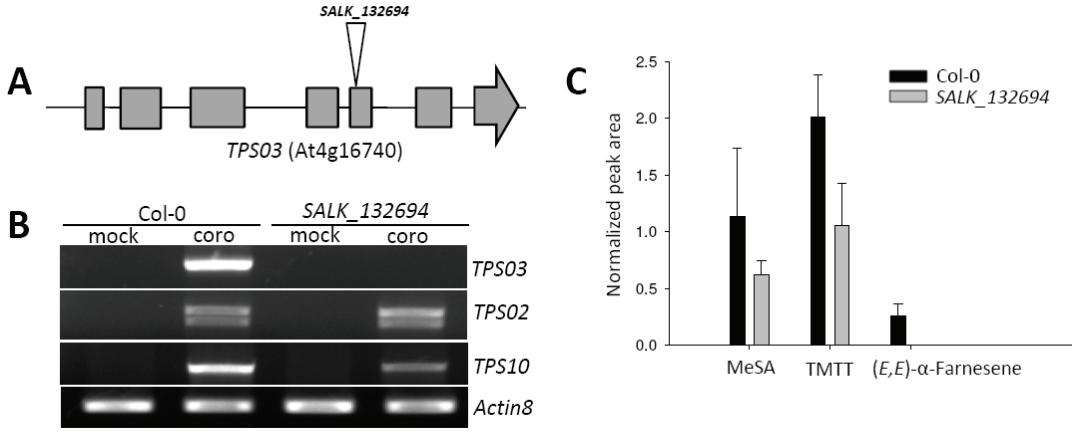


Figure 2.5. Coronalon-induced expression of TPS03 and volatile emission in detached leaves of Col-0 wild type plants and the TPS03 T-DNA insertion line *SALK_132694*. A, Position of the T-DNA insertion in the TPS03 gene. Gray boxes represent exons, and flanking regions and introns are shown by the black line. B, Semi-quantitative RT-PCR analysis of transcripts of TPS03 in comparison to genes TPS02 and TPS10. Transcript levels of Actin 8 were analyzed as a control. Coro, coronalon. C, Emission of MeSA, TMTT, and (*E,E*)-α-farnesene as measured between 21 and 30 h of coronalon treatment of Col-0 wild type plants and the T-DNA insertion line. Normalized peak areas are shown for each compound as analyzed by GC-MS (see "Materials and Methods"). No (*E*)-β-ocimene could be detected from wild type or mutant plants under these conditions. Results are average values ± SE (n = 3). None of the volatiles was detected in mock controls.

Next, we analyzed line *FLAG_406A04*, which carries a T-DNA in the first intron of the Ws *TPS02* allele (Fig. 2.6A).When leaves of this mutant were treated with coronalon, no induced *TPS02* transcript could be detected in comparison to wild type Ws plants (Fig. 2.6B). The absence of the *TPS02* transcript correlated with the loss of (*E*)-β-ocimene and (*E,E*)-α-farnesene emission in the *FLAG_406A04* mutant (Fig. 2.6C). We also investigated two transgenic Ws lines, in which transcription of *TPS02* was severely reduced or completely abolished by RNA interference in response to application of the fungal elicitor alamethicin (Supplemental Fig. S3). As expected, traces or no emission of (*E*)-β-ocimene and (*E,E*)-α-farnesene were found in the RNAi lines confirming the result obtained for the *FLAG_406A04* mutant (Supplemental Fig. S3A, B). MeSA and TMTT were released from all lines at rates similar to those of wild type Ws showing that elicitor treatment was effective (Supplemental

Fig. S3B). The results demonstrated that formation of (*E*)-β-ocimene and (*E,E*)-α-farnesene is dependent on the expression of *TPS02* in the Ws ecotype.

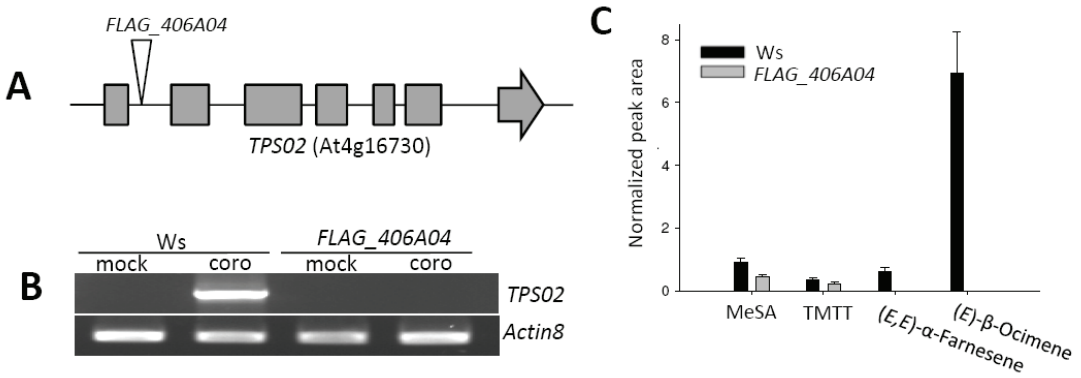


Figure 2.6. Coronalon-induced expression of *TPS02* and volatile emission in detached leaves of Ws wild type plants and the *TPS02* T-DNA insertion line *FLAG_406A04*. **A**, Schematic presentation of the position of the T-DNA insertion in the *TPS02* gene. Exons are represented by gray boxes, and flanking regions and introns are shown by the black line. **B**, Semi-quantitative RT-PCR analysis of transcripts of *TPS02*. Transcript levels of *Actin 8* were analyzed as a control. No full-length transcripts were found for *TPS03* and *TPS10* in wild type and mutant plants. Coro, coronalon. **C**, Emission of MeSA, TMTT, (*E,E*)-α-farnesene, and (*E*)-β-ocimene between 21 and 30 h of coronalon treatment of Ws wild type plants and the T-DNA insertion line. Normalized peak areas are shown for each compound as analyzed by GC-MS (see "Materials and Methods"). Results represent mean values ± SE (n = 3). None of the volatiles was detected from mock control leaves.

2.3.4 Recombinant TPS02 and TPS03 Proteins both Produce (*E*)-β-Ocimene and (*E,E*)-α-Farnesene *in vitro*

To further confirm the catalytic activities of the TPS02- and TPS03-encoded enzymes in Ws and Col-0, respectively, cDNAs of both genes were cloned into the *E. coli* expression vector pET101 in fusion with a C-terminal histidine-tag and the resulting proteins were partially purified by affinity chromatography and assayed for terpene synthase activity. For TPS02, a truncated 1647 bp cDNA was used that was amplified from the isolated full length Ws sequence. This truncation removed 41 amino acids containing a predicted plastidial transit peptide upstream of the conserved RR motif at the N-terminus of the TPS02 protein (Fig. 2.3C) in an effort to enhance the activity of the recombinant enzyme. It has previously been shown that monoterpene synthases often have higher specific activity when expressed in *E. coli* as mature proteins rather than as full-length preproteins (Williams et.al., 1998). The affinity-purified, recombinant Ws TPS02 protein converted the substrate GPP into (*E*)-β-ocimene as the major product, with (*Z*)-β-ocimene and myrcene as minor products (Fig.

2.7A). In assays with FPP as the substrate, the TPS02 enzyme produced primarily (*E,E*)-α-farnesene and small amounts (*Z,E*)-α-farnesene and (*E,E*)-β-farnesene (Fig. 2.7A). No activity was observed with GGPP as the substrate, and none of the terpene products was found in purified extracts of *E. coli* carrying the empty pET101 vector (not shown).

We also tested whether a truncated 539 amino acid protein resulting from possible alternative translation at nucleotide 153 of the *TPS02* gene from Col-0 had enzyme activity. However, this protein was shown to be inactive when expressed in *E. coli*. In addition, we investigated if removal of the frame shift mutation in the Col *TPS02* gene restored a functional TPS02 protein. A 1772 bp cDNA of Col-0 TPS02 was amplified from RNA isolated from coronalon-treated Col-0 leaves and site directed mutagenesis was applied to remove the AT-two base nucleotide insertion (Fig. 2.3B). When the resulting cDNA clone was expressed in *E. coli* as described above, no terpene synthase activity was detected indicating that additional mutations in the Col-0 *TPS02* gene contribute to the loss of enzyme activity.

To analyze the activity of the Col-0 *TPS03* encoded protein, a 1695 bp *TPS03* cDNA corresponding to the full-length TPS03 protein was isolated from RNA of Col-0 leaves treated with coronalon. When enzyme assays were performed with GPP and FPP as substrates, the recombinant, partially purified TPS03 enzyme produced the same monoterpenes and sesquiterpenes as those synthesized by the TPS02 protein from Ws (Fig. 2.7B).

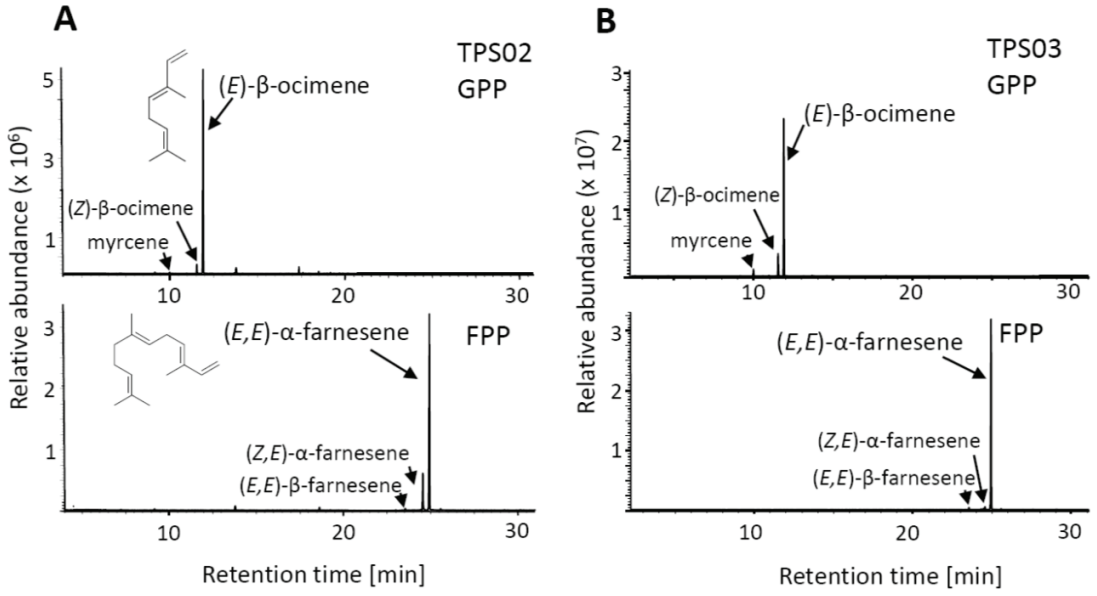


Figure 2.7. GC-MS analysis of monoterpene and sesquiterpene products of recombinant Ws TPS02 and Col-0 TPS03 enzymes. Recombinant proteins were expressed in *E. coli*, extracted, partially purified and applied for terpene synthase assays using the substrates GPP and FPP. **A**, Total ion GC-MS chromatograms of monoterpenes (upper panel) and sesquiterpenes (lower panel) produced by recombinant Ws TPS02 protein from GPP and FPP, respectively. The molecular structures of (*E*)-β-ocimene and (*E,E*)-α-farnesene are shown. **B**, Monoterpene products (upper panel) and sesquiterpene products (lower panel) of recombinant Col-0 TPS03 enzyme detected in assays with GPP and FPP, respectively. Terpene products were identified by comparison to authentic standards or by library suggestion (for (*Z,E*)-α-farnesene). No products were found in purified extracts from *E. coli* carrying the empty expression vector.

To analyze possible differences of the recombinant TPS proteins in conversion rates of GPP and FPP, we determined the catalytic properties of both enzymes for these substrates. Both enzymes had similar V_{max} and k_{cat} values for GPP and FPP with a 7 to 8-fold higher catalytic activity for GPP than for FPP (Table 2.2). The apparent K_m values for GPP and FPP (1.7 – 6.7 µM) were low for both enzymes (Table 2.2) and in the range of K_m values reported previously for other plant monoterpene and sesquiterpene synthases (Cane, 1999). Despite these similarities, the Ws TPS02 protein had an approximately 4-fold lower K_m for GPP and an approximately 1.5-fold higher K_m for FPP in comparison to the Col-0 TPS03 enzyme resulting in 4-fold higher and 1.5-fold lower corresponding k_{cat}/K_m values for GPP and FPP, respectively (Table 2.2). Catalysis of both enzymes was dependent on Mg^{2+} for maximum activity. We also tested the dependency of both enzymes on K^+ ions. Neither Ws TPS02 nor Col-0 TPS03 showed any change in activity in the presence of 40 mM K^+ (data not shown).

Taken together, our analysis of the recombinant TPS02 and TPS03 proteins from Ws and Col-0, respectively, showed that both enzymes can produce (*E*)-β-ocimene and (*E,E*)-α-farnesene *in vitro* without major kinetic differences. However, analyses of the loss-of-function plants clearly indicated different product profiles of the encoded enzymes *in vivo* with Ws TPS02 making both compounds (Fig. 2.6C) and Col-0 TPS03 producing predominantly (*E,E*)-α-farnesene (Fig. 2.5C). Thus, these findings supported the notion that TPS02 and TPS03 might be present in separate subcellular compartments with differential access to the substrates GPP and FPP.

Table 2.2. Kinetic parameters of Ws TPS02 and Col-0 TPS03 recombinant enzymes. Each value represents the average ± SE of three replicates. K_m, Michaelis-Menten constant; V_{max}, maximal velocity; k_{cat}, turnover number.

Enzyme	Substrate	K_m (μM)	V_{max} (pkat/mg)	k_{cat} (sec^{-1})	k_{cat}/K_m (sec^{-1} mM^{-1})
Ws TPS02	GPP[a]	1.7±0.3	539.0±40.0	$3.5x10^{-2}$±$2.6x10^{-3}$	21.4±2.35
	FPP[a]	4.0±0.4	69.0±3.1	$4.5x10^{-3}$±$2x10^{-4}$	1.12±0.05
Col-0 TPS03	GPP[a]	6.7±0.9	514.8±49.8	$3.4x10^{-2}$±$3.3x10^{-3}$	5.12±0.18
	FPP[a]	2.6±0.2	72.4±1.2	$4.8x10^{-3}$±$8.0x10^{-5}$	1.82±0.09

2.3.5 Subcellular Localization of TPS02 in Plastids and TPS03 in the Cytosol

Analysis of putative targeting sequences using different algorithms (CHLOROP, http://www.cbs.dtu.dk/services/-ChloroP; TARGETP, http://www.cbs.dtu.dk/services/TargetP;

PWOLF PSORT, http://wolfpsort.seq.cbrc.jp; Predator, http://urgi.versailles.inra.fr/predotar/predotar.html) suggested that the TPS02 protein from Ws carries a plastidial transit peptide of approximately 25 amino acids and is therefore targeted to chloroplasts. To experimentally determine the subcellular localization of the Ws TPS02 protein, a 105 bp cDNA fragment beginning with the start codon of the *TPS02* gene was inserted into the vector pCAMBIA 1302 under the control of the CaMV 35S promoter generating a 35 amino acid TPS02 peptide with a C-terminal fusion to GFP (green-fluorescent protein). GFP analysis of hypocotyl cells of several independent plant lines transformed with the TPS02-GFP construct showed green fluorescence located in plastids, which clearly indicated that the TPS02 protein is targeted to chloroplasts (Fig. 2.8C, D).

In contrast to TPS02, no consistent prediction for the subcellular localization of TPS03 was obtained with different algorithms with suggestions of either a cytosolic/nuclear

localization or the presence of a 9-28 amino acid mitochondrial targeting sequence. Longer transit peptides beginning at earlier start codons were not in frame with the TPS03 protein. We generated a Col TPS03-GFP fusion construct as described for TPS02 by cloning of a 150 bp *TPS03* cDNA fragment encoding a 50 amino acid N-terminal TPS03 peptide. In plants transformed with the TPS03-GFP construct, GFP fluorescence was not observed in plastids but appeared to reside in the cytosol with diffusion into nuclei (Fig. 2.8G, H).

The GFP constructs clearly support the localization of TPS02 in plastids and TPS03 in the cytosol. Given the preponderance of GPP in the plastids, TPS02 thus seems responsible for synthesizing (*E*)-β-ocimene from GPP in the Ws accession. On the other hand, the cytosolic TPS03 is in a compartment thought to be supplied with FPP rather than GPP, and thus is likely to form (*E,E*)-α-farnesene from FPP in Col-0. The formation of small amounts of (*E,E*)-α-farnesene in Ws is probably also attributable to TPS02, and demonstrates the existence of low levels of FPP in the chloroplast or low levels of TPS02 in a subcellular compartment provided with FPP.

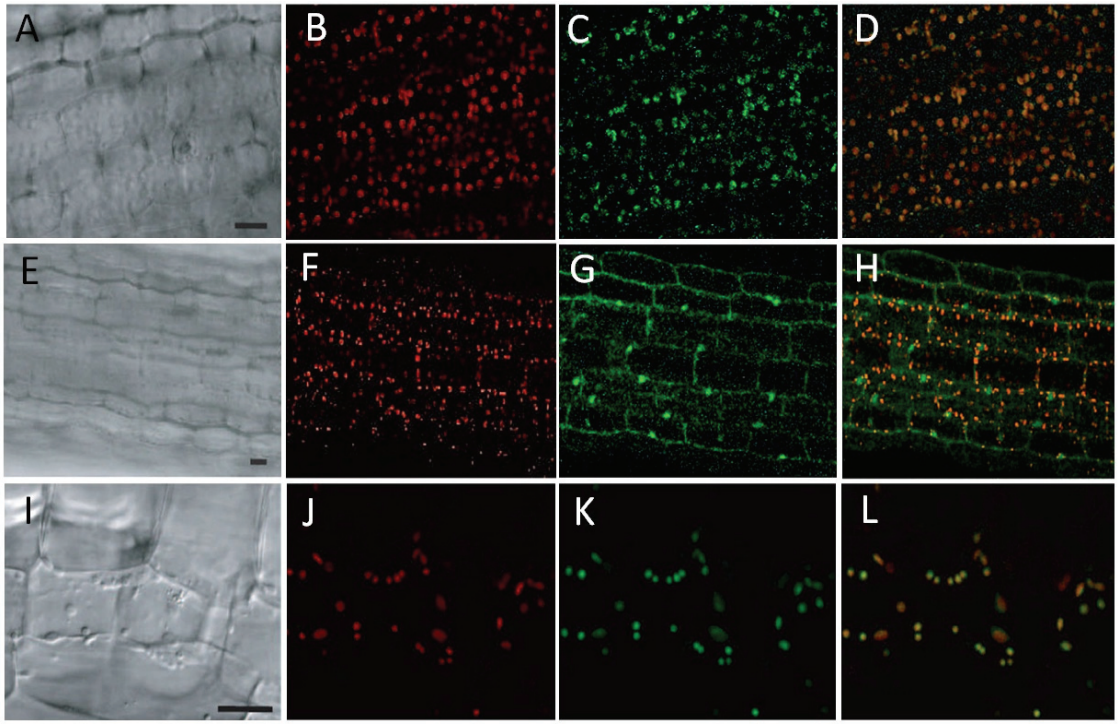


Figure 2.8. Confocal laser scanning microscopy of stably expressed Ws TPS02 and Col-0 TPS03 peptide-GFP fusion proteins. Microscopic images were taken from the hypocotyl of two-week old seedlings. The first column (A, E, and I) shows light microscopic images of hypocotyl cells. Chlorophyll autofluorescence, detected in the red channel, is shown in the second column (B, F, and J). The third column (C, G, K) shows GFP fluorescence, detected in the green channel, and the fourth column (D, H, L) shows merged green and red channel images. A 35 amino acid N-terminal TPS02 peptide (containing a putative 25 amino acid plastidial transit peptide) fused to GFP localizes to chloroplasts (A-D). No plastidial localization was detected for a fusion protein containing a 50 amino acid N-terminal peptide of Col-0 TPS03 (E-H). Ferredoxin N-reductase (FNR)-eGFP carrying a plastidial target peptide was used as a chloroplast marker (I-L). Scale bars: 20 μM.

2.3.6 Inhibitor Studies of the MEP and the Mevalonate Pathways Support the Subcellular Localization of TPS02 and TPS03

To further investigate the subcellular compartmentation of the Ws TPS02 and Col-0 TPS03 enzymes, inhibitors were employed that are specific for one of the two pathways of IPP/DMAPP formation in plants, either the plastid-localized MEP pathway (Lichtenthaler, 1999) or the cytosol-localized mevalonate pathway. Inhibitors were applied together with coronalon to leaves of both ecotypes. Administration of fosmidomycin, which inhibits the DXR enzyme in the MEP pathway, to Ws leaves, caused an almost complete loss of coronalon-induced (*E*)-β-ocimene emission (Fig. 2.9A). By contrast, inhibition of the mevalonate pathway enzyme HMGR by lovastatin led to only a 28% reduction in (*E*)-β-

ocimene formation (Fig. 2.9A). The emission of (*E,E*)-α-farnesene was reduced by both inhibitors in a similar way as (*E*)-β-ocimene indicating both products are likely produced in the same compartment (Fig. 2.9B). Treatment of Col leaves with one or the other inhibitor caused effects opposite to those observed for the Ws ecotype. While application of lovastatin severely reduced the release of (*E,E*)-α-farnesene in response to coronalon treatment, emission of (*E,E*)-α-farnesene was inhibited by only 37% upon administration of fosmidomycin (Fig. 2.9C). The fact that some reduction was observed in the emission of (*E,E*)-α-farnesene by treatment of Col with fosmidomycin and in the emission of (*E*)-β-ocimene upon treatment of Ws with lovastatin can be attributed to an exchange of terpenoid precursors between the cytosol and plastid as previously described (Hemmerlin et al., 2003; Laule et al., 2003; Schuhr et al., 2003; Dudareva et al., 2005). Overall, our results are in agreement with the biosynthesis of (*E*)-β-ocimene and (*E,E*)-α-farnesene by Ws TPS02 in plastids and the formation of (*E,E*)-α-farnesene by the Col-0 TPS03 enzyme in the cytosol.

2.3.7 Expression Profile of *TPS02* and *TPS03* Promoters

To gain a better understanding of the organ- and tissue-specific formation of (*E*)-β-ocimene and (*E,E*)-α-farnesene in leaves and flowers of the Col-0 and Ws ecotypes, a 2.1 and 1.8 kb intergenic fragment upstream of the start codon of the functionally active Ws *TPS02* and Col-0 *TPS03* genes, respectively, was cloned 5' to the *β-glucuronidase* (*GUS*) reporter gene of the pDW137 vector, and the *TPS* promoter-*GUS* constructs were stably transformed into the respective Ws and Col-0 backgrounds. GUS activity driven by the Col-0 *TPS03* promoter was detected in sepals, anthers and the stigma of immature and mature flowers (Fig. 2.10A-1). In anthers, GUS activity was found particularly in pollen (Fig. 2.10A-2). Moreover, GUS staining was observed in the abscission zone of developing siliques (Fig. 2.10A-3). In leaves, *TPS03*-GUS activity was induced locally around sites of feeding damage by *P. xylostella* (Fig. 2.10A-4). No activity was found in undamaged leaves. GUS staining was also detected at mechanical wound sites (not shown) in agreement with the transcription of *TPS03* observed upon mechanical wounding (Fig. 2.4A). Analysis of transgenic Ws plants expressing GUS under control of a *TPS02* promoter fragment showed GUS staining only in response to treatment of leaves with the strong elicitor coronalon (Fig. 2.10B). GUS activity was strongest around the petiole after submersion in coronalon solution. No GUS activity

was observed in Ws flowers or leaves upon *P. xylostella* feeding in contrast to the detection of *TPS02* transcripts in these organs, which suggested that the cloned promoter fragment lacked regulatory elements responsible for full activity in the intact plant.

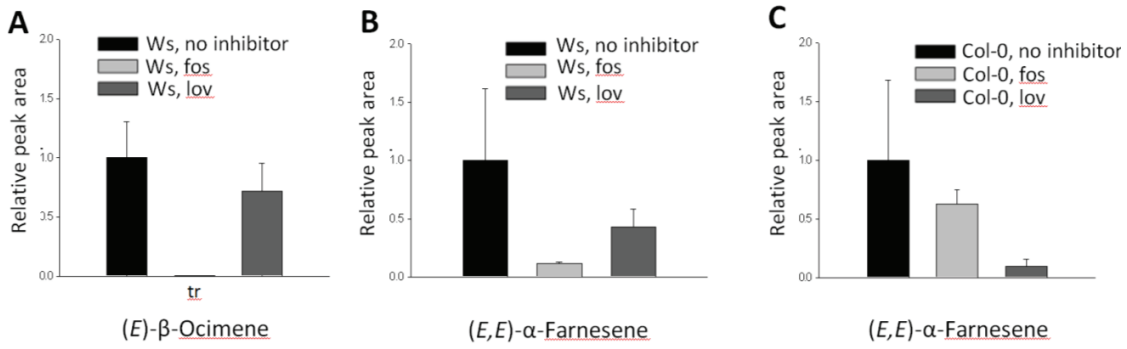


Figure 2.9. Effect of the MEP pathway inhibitor fosmidomycin (fos) and the mevalonate pathway inhibitor lovastatin (lov) on emission of (*E*)-β-ocimene and (*E,E*)-α-farnesene from leaves of accessions Ws and Col-0. Volatiles were collected for 8 h from detached rosette leaves treated with coronalon in the presence of a single inhibitor. Relative peak areas of compounds are shown. Peak areas from controls without the addition of inhibitors were arbitrarily set to 1.0. Results represent mean values ± SE (n = 3).

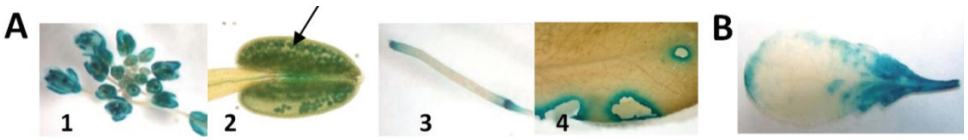


Figure 2.10. GUS activity in Col-0 *ProTPS03*:*GUS* and Ws *ProTPS02*:*GUS* plants.
A, Histochemical GUS staining of an inflorescence (1), pollen grains (2, arrow), a silique (3), and a *P. xylostella*-damaged mature leaf from a Col-0 *ProTPS03*:*GUS* plant. In A-4, GUS activity is induced locally around the sites of feeding damage. **B**, Induced GUS activity in a rosette leaf of a Ws *ProTPS02*:*GUS* plant treated for 24 h with coronalon through the petiole. The results are representative for at least three independent lines.

2.4 Discussion

2.4.1 Elicitor- and Insect-Induced Volatile Emissions Vary among *A. thaliana* Ecotypes

Intraspecific variation of herbivore-induced volatile emissions has been reported primarily from varieties of cultivated plants (Loughrin et al., 1995; Geervliet et al., 1997; Gouinguene et al., 2001; Degen et al., 2004; Lou et al., 2006). By contrast, variation of induced volatiles among non-domesticated species has been the subject of only few investigations of wild solanaceous species (Halitschke et al., 2000; Glawe et al., 2003; Hare, 2007; Delphia et al., 2009) and teosinte (Gouinguene et al., 2001). In this study, we conducted a survey of 27 *A. thaliana* accessions for volatiles released upon treatment with coronalon, a synthetic mimic of octadecanoid plant hormones. As demonstrated for accessions Ws and Col-0 (Herde et al., 2008) and for lima bean (Schueler et al., 2001), coronalon induces volatile blends similar to those emitted upon insect feeding. Most *A. thaliana* accessions emitted volatile blends of the same composition consisting of the monoterpene (*E*)-β-ocimene, the sesquiterpene (*E*,*E*)-α-farnesene, the benzenoid volatile MeSA, and the irregular C_{16}-homoterpene TMTT, all of which are common constituents of herbivore-induced volatile mixtures (Turlings et al., 1990; McCall et al., 1994; Takabayashi and Dicke, 1996; Pichersky and Gershenzon, 2002; Ament et al., 2004).

Large ecotype-specific quantitative differences were observed for the emission of (*E*)-β-ocimene with approximately one third of the accessions, including Col-0, producing none or only traces of this monoterpene. Quantitative variation was also apparent in the emission of the other volatile compounds (MeSA, TMTT) resulting in ecotype-specific differences of compound ratio. Our results correspond to differences in herbivore-induced volatile blends observed among genotypes of other wild species (Halitschke et al., 2000; Gouinguene et al., 2001; Hare, 2007; Delphia et al., 2009). The ecological significance of intraspecific variation of insect-induced volatile production is still not well understood because the roles of these compounds in plants are not completely elucidated. Among the investigated *A. thaliana* accessions, no definite correlation could be found between the geographical distribution and the total amount of volatiles emitted or their profiles. However, variability of herbivore-induced volatile emissions may reflect habitat-dependent, selective adaptations of ecotypes to specific populations of herbivores and their natural enemies. Natural selection is also assumed to be responsible for significant variation of other specialized metabolites among *A.*

thaliana accessions, such as glucosinolates (Kliebenstein et al., 2001). The insect-induced volatile blend emitted from *A. thaliana* Col-0 has been shown to attract parasitic wasps such as *Cotesia rubecula* (Van Poecke et al., 2001) and preferences of parasitoid wasps for particular volatile mixtures have been demonstrated (Hoballah et al., 2002). Moreover, associative learning of parasitoids (De Boer and Dicke, 2006; Smid and Vet, 2006) may facilitate optimal use of the volatile cues emitted by a specific plant population. Since volatile terpenes exhibit antimicrobial activities, ecotype-specific variation of induced volatiles might also emerge under selective pressure by different microbial pathogens. Bacterial and fungal pathogens can elicit emission of volatile mixtures in *A. thaliana*, which are very similar to those induced by insects, as shown for infections by *P. syringae* (Attaran et al., 2008) and treatment with the fungal elicitor alamethicin (Herde et al., 2008). Interestingly, two accessions, Bla-1 and Bla-10, originating from the same region (Blanes/Genora, Spain) differed more than 100-fold in the emission of (*E*)-β-ocimene indicating intraspecific variability even among populations occurring close together.

A significant correlation was apparent between emissions of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene (Fig. 2.2, Suppl. Table I), which suggested that in most accessions both terpene volatiles are produced either simultaneously by a single bi-functional (*E*)-β-ocimene/(*E*,*E*)-α-farnesene synthase or by co-expressed enzyme activities. Significant correlations were also found between emissions of MeSA and TMTT, on the one hand, and MeSA and (*E*,*E*)-α-farnesene, on the other (Suppl. Table I), which reflect overlapping, coronalon-induced responses in the expression of biosynthetic enzymes in the formation of MeSA (benzoic acid/SA carboxyl methyltransferase 1, AtBSMT1) (Chen et al., 2003a), TMTT, and (*E*,*E*)-α-farnesene.

2.4.2 Col-0 and Ws Accessions Show Allelic Differences for the Duplicated (*E*)-β-Ocimene/(*E*,*E*)-α-Farnesene Synthase Genes *TPS02* and *TPS03* that lead to Variability in Terpene Biosynthesis

Within the *A. thaliana* terpene synthase (TPS) family, the proteins encoded by genes *TPS02* and *TPS03* cluster together with five other monoterpene synthases (TPS10, TPS14, TPS23, TPS27, TPS24), all of which belong to the plant TPS-b subfamily (Aubourg et al., 2002; Chen et al., 2003) (Fig. 2.11). Based on their close proximity and similarity in gene

structure and sequence, *TPS02* and *TPS03* are most likely the result of tandem gene duplication. Several other terpene synthase genes are arranged as tandem pairs in the *A. thaliana* genome such as identical gene copies of the root-expressed 1,8-cineole synthase AtTPS-Cin (*TPS23* and *27*) (Chen et al., 2004) and the (*Z*)-γ-bisabolene synthases *TPS12* and *TPS13* (Ro et al., 2006). Rapid radiation of genes by duplication and sequence divergence is common within the plant *TPS* superfamily and other gene families of plant specialized metabolism (Pichersky and Gang, 2000; Kliebenstein et al., 2001; Koellner et al., 2004) and is thought to contribute to generating diversity of metabolites that function in plant-organism interactions (van der Hoeven et al., 2000; Aubourg et al., 2002).

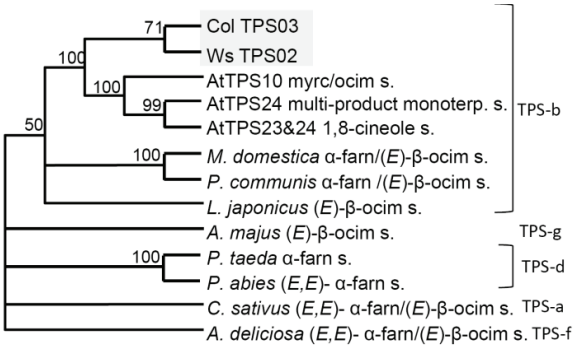


Figure 2.11. Phylogenetic tree illustrating the relationship of *Arabidopsis* Ws TPS02 and Col-0 TPS03 (shaded grey) to other selected *Arabidopsis* monoterpene synthases and to other plant α-farnesene and (*E*)-β-ocimene synthases of different TPS subfamilies. The tree was generated from 13 TPS proteins using ClustalW and phylogenetidc analysis was conducted using maximum parsimony in PAUP. Accession numbers of all genes are listed under "Materials and Methods". Numbers above nodes are bootstrap values based on searches with 1000 replicates. Myrc, myrcene; ocim, ocimene; farn, farnesene.

The Ws and Col-0 accessions each maintain only one functional allele at the *TPS02* and *TPS03* locus, whose recombinant proteins exhibit both (*E*)-β-ocimene and (*E*,*E*)-α-farnesene synthase activities. The *TPS03* allele in Ws and the *TPS02* allele in Col-0 are inactive because of frame-shift mutations, which cause the introduction of premature stop codons (Fig. 2.3B). Both alleles are still transcribed under induced conditions; however, in the case of the Ws *TPS03* gene, most of the mRNA is degraded except of a short fragment upstream of the nonsense codon (Supplemental Fig. S2). Premature nonsense codons, particularly those occurring in early exons, can decrease mRNA stability by activating nonsense-mediated decay pathways (vanHoof and Green, 1996; Gutierrez et al., 1999; Hori and Watanabe, 2007). For the TPS02 transcript in Col-0, where the nonsense codon is positioned at the junction between the first and second exon, full-length mRNA transcripts

were observed in elicitor-treated leaves. By contrast, none or only traces of full length mRNA of TPS02 were found in Col-0 flowers and only partial transcripts of this gene were previously amplified successfully from floral tissue (Chen et al., 2003), which indicates a possible higher mRNA instability of the TPS02 pseudogene in Col-0 flowers than under induced conditions in leaves.

In some cases, transcripts of pseudogenes have been shown to regulate the stability of their homologous coding gene transcripts (Hirotsune et al., 2003). By analyzing the Ws *FLAG_406A04* mutant and *TPS02* RNAi lines, we did not find evidence for this regulatory mechanism, since the absence of the *TPS02* transcript did not positively affect the mRNA stability of the *TPS03* allele. In addition, no micro RNAs have been identified for both *TPS02* and *TPS03* genes.

Examples of pseudogene transcripts have been described in other defense metabolic pathways in *A. thaliana* (Kliebenstein et al., 2001). In particular, stress-responsive gene families such as the *TPS* family have elevated levels of pseudogenization, which can be interpreted as a rapid turnover of genes under varying selection pressures (Thibaud-Nissen et al., 2009; Zou et al., 2009). Analysis of the *TPS02 – TPS03* gene pair in Col-0 and Ws demonstrated that either one of the duplicated genes can lose function. However, since most of the investigated accessions emit (*E*)-β-ocimene, selection pressure seems to support expression of an active (*E*)-β-ocimene synthase. Moreover, the presence of a full-length though inactive *TPS02* transcript in elicitor-treated leaves of the Col-0 ecotype indicates a more recent loss of function of the *TPS02* allele than the *TPS03* allele.

Allelic variation of *TPS* genes contributing to terpene diversity has also been described in varieties of different crop plants such as basil, tomato and maize (van der Hoeven et al., 2000; Iijima et al., 2004; Koellner et al., 2004). For example, in maize inactivation of alleles of the insect-induced, duplicated sesquiterpene synthase genes *tps4* and *tps5*, caused by frame shift mutation, was observed in a comparison of two different cultivars (Koellner et al., 2004). While extrapolating from these results to wild species is possible, the findings presented here provide a direct demonstration that allelic diversification of *TPS* genes in wild gene pools contributes to the natural variation in terpene formation.

2.4.3 Subcellular Compartmentalization of the TPS02 and TPS03 Proteins Contributes to Ecotype Variation of Induced (*E*)-β-Ocimene and (*E*,*E*)-α-Farnesene Emission

Subcellular localization experiments demonstrated that the Ws TPS02 protein is targeted to plastids (Fig. 2.8C, D), where it converts GPP to (*E*)-β-ocimene. Inactivation of *TPS02* in Ws caused the loss of emission of both (*E*)-β-ocimene and (*E*,*E*)-α-farnesene (Fig. 2.6, Supplemental Fig. S3), which clearly showed that the TPS02 enzyme not only converts GPP to (*E*)-β-ocimene in plastids, but also catalyzes FPP to (*E*,*E*)-α-farnesene conversion in the same organelle. The presence of plastidial FPP was also inferred when a strawberry linalool/nerolidol synthase carrying a plastidial-targeting peptide was expressed in *A. thaliana*, causing the emission of a small amount of the sesquiterpene nerolidol together with linalool (Aharoni et al., 2003). A plastidial FPP pool might be the result of an import of FPP from the cytosol as a result of metabolic cross-talk between both compartments (Schuhr et al., 2003) and/or the biosynthesis of FPP inside the organelle. The latter explanation is supported by the lower emission of (*E*,*E*)-α-farnesene in the presence of fosmidomycin, an inhibitor of the plastid-localized MEP pathway, than after treatment with lovastatin, an inhibitor of the cytosol-localized mevalonate pathway (Fig. 2.9A, B). Although an isoform of the *A. thaliana* FPP synthase 1 (FPPS1L) has been shown to be targeted to mitochondria (Cunillera et al., 1996), a specific FPP synthase activity in plastids has not been demonstrated. Instead, FPP might be produced as a side product of plastidial GPP synthase activity.

In comparison to TPS02, the TPS03 protein lacks 14 amino acids at its N-terminus (Fig. 2.3C) and is not targeted to plastids, but resides instead in the cytosol (Fig. 2.8G, H). The TPS03 enzyme produces (*E*,*E*)-α-farnesene mostly from cytosolic FPP, which is evident from a severe reduction of (*E*,*E*)-α-farnesene emission by treatment with lovastatin in comparison to the application of fosmidomycin (Fig. 2.9C). Formation of only trace amounts of (*E*)-β-ocimene in the Col-0 ecotype indicates the absence of a high GPP level in the cytosol and rules out an efficient export of GPP from plastids to the cytosol. Experimental evidence for small cytosolic levels of GPP has recently been provided from tobacco and kiwifruit (Wu et al., 2006; Nieuwenhuizen et al., 2009).

A previous analysis of TPS03 from accession C24 assumed that the TPS03 enzyme is responsible for the *in planta* formation of (*E*)-β-ocimene based on the characterization of the over-expressed protein *in vitro* (Faeldt et al., 2003). However, since the N-terminal region of

the C24 TPS03 protein is identical to that of the Col enzyme and because of negligible GPP pools in the cytosol of *A. thaliana* leaf cells, we can now hypothesize that (*E*)-β-ocimene, if emitted from this ecotype, is probably produced by a functional TPS02 protein rather than a TPS03 enzyme activity. No (*E,E*)-α-farnesene synthase activity was found for the TPS03 recombinant enzyme from C24 (Faldt et al., 2003) despite only a single amino acid difference at position 267 (S in Col, F in C24). It will be interesting to determine to what extent this amino acid change is indeed responsible for the loss of (*E*,*E*)-α-farnesene synthase activity of the TPS03 protein.

Subcellular segregation of homologous bifunctional terpene synthases has also been documented in cultivated plants. In snapdragon flowers, the subcellular location of two nearly identical linalool/nerolidol synthases determines whether each enzyme produces linalool (in plastids) or nerolidol (in the cytosol) (Nagegowda et al., 2008). In cultivated strawberry, cytosolic and plastidial linalool/nerolidol synthases (FaNES1 and FaNES2) were identified, of which only the FaNES1 enzyme is expressed and seems to be responsible for the formation of nerolidol but also linalool from FPP and GPP pools in the cytosol (Aharoni et al., 2004). Our results on enzymes from a non-cultivated species expand these findings by showing that differential subcellular targeting of dual-function terpene synthases is a molecular mechanism of general importance in the natural evolution of intraspecific volatile terpene diversity.

2.4.4 *TPS02* and *TPS03* Expression is under Constitutive Control in *A. thaliana* Flowers and Stress-Induced in Leaves

Transcript analysis of the Ws *TPS02* and Col-0 *TPS03* genes showed that expression of both genes is induced in leaves upon treatment with coronalon and in response to feeding by *P. xylostella* (Fig. 2.4A, B). Moreover, histochemical assays of Col-0 *TPS03* promoter activity demonstrated local expression of this gene at the site of *P. xylostella* feeding damage (Fig. 2.10A-4). The fact that no expression of Col-0 *TPS03* was found in undamaged leaves of *P. xylostella*-treated plants supports the notion of the lack of a systemic response in herbivore-induced terpene volatile emission in *A. thaliana*. Induction of the *TPS03* transcript was also demonstrated in leaves of *A. thaliana* ecotype No-0 in response to jasmonate

treatment and mechanical wounding, but not to feeding by the specialist herbivore *Pieris rapae* (Faeldt et al., 2003) suggesting ecotype- and/or insect-specific differences in herbivore-induced responses of the *TPS03* gene.

Both Ws *TPS02* and Col-0 *TPS03* are also expressed constitutively in flowers (Fig. 2.4C), which is consistent with the formation of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene as common constituents of floral volatile blends in plants (Dudareva et al., 2003; Nieuwenhuizen et al., 2009). However, for the *TPS03* gene, the strong promoter-GUS activity and transcript abundance in Col-0 flowers (Fig. 2.10A-1, 2) do not correlate with the low emissions of its enzymatic product (*E*,*E*)-α-farnesene (app. 0.3 ng h^{-1} per 70 inflorescences) reported from Col-0 floral tissues (Tholl et al., 2005). This discrepancy might be caused by differences in TPS03 enzyme activity and /or substrate availability in floral tissue in comparison to elicitor-treated leaves, where (*E*,*E*)-α-farnesene is readily detected. The specific expression profile of *TPS03* in the stigma and sepals of Col-0 flowers may reflect the functions of (*E*,*E*)-α-farnesene in pollinator attraction or florivore/antimicrobial defense as discussed for the flower-specific genes *TPS21* (encoding a (*E*)-β-caryophyllene synthase) (Tholl et al., 2005) and *TPS24* (multi-product monoterpene synthase), which exhibit similar expression patterns in *A. thaliana* flowers (Chen et al., 2003). Interestingly, *TPS03* promoter activity was observed in pollen grains and resembles the recently reported expression of a valencene sesquiterpene synthase in the pollen of grape flowers (Martin et al., 2009). A pattern of constitutive expression in reproductive organs and induced expression in vegetative tissues has also been observed for *A. thaliana* geranyllinalool synthase (TPS04/GES) (Herde et al., 2008) in the formation of the volatile homoterpene TMTT and has been demonstrated for other genes involved in plant defense (Pollak et al., 1993; Hoegen et al., 2002; Stotz et al., 2009).

In contrast to (*E*,*E*)-α-farnesene, (*E*)-β-ocimene is the predominant volatile in Ws flowers (Tholl et al., 2005) consistent with the expression of an active *TPS02* gene (Fig. 2.3C). All of the accessions that emit (*E*)-β-ocimene from their foliage under induced conditions also release (*E*)-β-ocimene from floral tissue (Tholl et al., 2005), while non- or trace emitters of (*E*)-β-ocimene from leaves also lack emissions from flowers. We therefore assume that (*E*)-β-ocimene is produced primarily by the TPS02 enzyme in both leaves and flowers of most of the investigated ecotypes. Despite amplification of a 2.1 kb intergenic

promoter region, we were unable to detect *TPS02* promoter activity in flowers and in response to insect feeding. An expanded analysis of the *TPS02* promoter region may reveal regulatory elements necessary for full promoter activity and allow a more detailed comparison of organ-specific expression and function for the *TPS02* and *TPS03* genes.

2.4.5 Formation of (*E*)-β-Ocimene and (*E*,*E*)-α-Farnesene by Bifunctional Terpene Synthases Evolved Several Times in Flower, Fruit and Herbivore-Induced Volatile Biosynthesis

A phylogenetic comparison of the *A. thaliana* Ws TPS02 and Col-0 TPS03 proteins with other plant TPSs shows that both enzymes cluster in the TPS-b subfamily, which also includes the recently characterized bifunctional (*E*,*E*)-α-farnesene/(*E*)-β-ocimene synthase MdAFS1 from apple (Pechous and Whitaker, 2004) (Fig. 2.11). However, TPS02 and TPS03 are more closely related to other *A. thaliana* monoterpene synthases of the TPS-b subgroup (52-59% aa sequence identity) than to MdAFS1 (35- 37 % aa sequence identity) (Fig. 2.11) confirming the independent convergence of genes in the *A. thaliana TPS* family. Besides the (*E*,*E*)-α-farnesene/(*E*)-β-ocimene synthases of the TPS-b family, bifunctional enzymes with similar activities were identified from cucumber within the TPS-a subgroup (Mercke et al., 2004), and from flowers of kiwifruit (AdAFS1) in the TPS-f subfamily (Nieuwenhuizen et al., 2009). In contrast to the enzymes from apple and kiwifruit that preferably produce (*E*,*E*)-α-farnesene from FPP (Green et al., 2009; Nieuwenhuizen et al., 2009), the recombinant TPS02 and TPS03 enzymes exhibit 19 and 3-fold higher catalytic efficiency for GPP than FPP, respectively, (Table 2.2) suggesting their original function as monoterpene synthases. The apparent K_m values of Ws TPS02 and Col-0 TPS03 for GPP and FPP are in the range of those reported from other (*E*,*E*)-α-farnesene/(*E*)-β-ocimene synthases (Nieuwenhuizen et al., 2009). While the V_{max} and k_{cat} values of both enzymes for GPP and FPP, respectively, are very similar, differences between the apparent K_m values for both substrates make Col-0 TPS03 slightly more catalytically efficient in converting FPP to (*E*,*E*)-α-farnesene than Ws TPS02 and less efficient in producing (*E*)-β-ocimene from GPP (Table 2.2). These differences may reflect substrate preferences arising from the predominant substrate present in the environment of the enzyme, GPP for the plastid-localized TPS02 and FPP for the cytosol-localized TPS03.

The activity of the MdAFS1 enzyme was shown to be dependent on K^+ and the protein contains an H-α1 loop motif for optimal binding of K^+ ions (Green et al., 2009). Substitution of a serine residue (S487) in this motif with lysine rendered the enzyme K^+-independent. The activity of the TPS02 and TPS03 proteins was also not K^+-dependent, and these, as well as other TPS-b monoterpene synthases, have a Lys residue at the corresponding position (Fig. 2.3C).

In addition to bifunctional (*E*,*E*)-α-farnesene/(*E*)-β-ocimene synthases, several other TPSs producing one or both of these terpenes have been identified from gymnosperms (TPS-d) (e.g. (*E*,*E*)-α-farnesene synthase from *Picea abies*, (Martin et al., 2004)) and angiosperms (TPS-b, g subgroups) (e.g. (*E*)-β-ocimene synthases from *Lotus japonicas* (Arimura et al., 2004), or snapdragon (Dudareva et al., 2003). The sequence variation among these enzymes demonstrates that the formation of (*E*,*E*)-α-farnesene and (*E*)-β-ocimene has arisen independently several times in the evolution of higher plants suggesting repeated selection for their roles in pollinator attraction, fruit dispersal and plant defense.

2.5 Materials and Methods

2.5.1 Plant Materials and Growth Conditions

A. thaliana thaliana seeds of all accessions with the exception of Col-0 (Columbia) and Ws (Wassilewskija) were obtained from Tom Mitchell-Olds, Duke University, Durham, NC. Seeds of *A. thaliana* T-DNA insertion mutant lines (*TPS3KO SALK_132694*, *TPS2KO* FLAG_406A04) were from the ABRC stock center and INRA, France, respectively. Wild type and transgenic/mutant plants were cultivated on soil (Sunshine Growing Mix No.1 : sand, 8:1) for 5 to 6 weeks under controlled growth conditions (10 h-light/14 h-dark photoperiod with 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, 23 °C, 55% relative humidity). Kanamycin or hygromycin resistant transgenic plants were pre-selected on 1 × Murashige and Skoog (Duchefa, Haarlem, The Netherlands) plates with 1% sucrose and 100 $\mu g\ mL^{-1}$ kanamycin or 30 $\mu g\ mL^{-1}$ hygromycin prior to being transferred to soil. Hydroponic plants of various accessions were grown from seeds on rockwool support (Gibeaut et al., 1997) under the same

light and temperature conditions as described above. All plants were used in the prebolting rosette stage.

2.5.2 Reagents and Radiochemicals

Unlabeled GPP (geranyl diphosphate) and FPP (farnesyl diphosphate) were purchased from Echelon Biosciences Incorporated (Salt Lake City, UT, USA). Tritium labeled GPP and FPP ([1-^{3}H]-GPP, [1-^{3}H]-FPP, both ~ 0.74 TBq mmol^{-1}) were purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA). All other reagents or solvents were obtained from Fisher Scientific, Sigma-Aldrich, Invitrogen and Fluka, unless otherwise stated.

2.5.3 Plant Treatments

For treatment of various accessions with coronalon, hydroponically grown plants were placed with their roots in glass beakers containing 30 ml of hydroponic medium with 33 μg mL^{-1} (100 μM) coronalon, 0.1% ethanol (Schuler et al., 2004). Treatment was performed for 30 h. For treatments of detached *A. thaliana* leaves with coronalon or alamethicin, 16 leaves were cut off from soil grown plants and transferred with their petioles into glass beakers filled with 10 ml aqueous solutions of 16.5 μg mL^{-1} (50 μM) coronalon, 0.1% ethanol or 10 μg mL^{-1} alamethicin, 0.1% ethanol. Mock solutions for all treatments contained 0.1% ethanol. Treatments were performed for 24 h (coronalon) and 30 h (alamethicin). For inhibitor treatments of detached leaves, leaves were placed in aqueous solutions of fosmidomycin (50 μM) and lovastatin (50 μM) and incubated for 24 h prior to the addition of coronalon (50 μM) and an additional 24 h treatment. Mock treatments were done with 0.2% ethanol. For wounding experiments, fully expanded rosette leaves of intact soil-grown plants were evenly penetrated 20 times by needles and harvested after 30 h. Insect feeding experiments were performed by placing an average of six third- to fourth-instar *P. xylostella* larvae on each fully expanded rosette leaf of soil-grown plants and allowing them to feed for 24 h (for RNA extraction and GUS staining) or 30 h (for volatile collection). *P. xylostella larvae* were from a G88 colony and reared on artificial diet with a wheat germ base at 27 °C with an 18h light/6h dark cycle.

2.5.4 Volatile Collection and Analysis

Volatile collection from detached leaves or intact hydroponic plants was performed in 1 L bell jars by using the closed-loop stripping method (Donath and Boland, 1995) under controlled growth conditions as described previously (Chen et al., 2003). If not indicated otherwise in the figure legend, volatiles were collected in the light for 8 h during 22 h to 30 h from the beginning of the treatment. Collections were performed during this time period because of higher volatile emissions in comparison to earlier time points of treatment. Volatiles emitted from inhibitor-treated rosette leaves were collected between 40 to 48 h of incubation with the inhibitor (i.e. between 16 to 24 h of coronalon treatment). For the *P. xylostella* treatment, plants were placed with their root balls wrapped in aluminum foil in 3 L bell jars and volatiles were collected during 21 to 30 h from the start of larval feeding. Volatiles were trapped on 25 mg Super-Q (hydroponic plants, insect feeding) (Tholl, 2006) or 5 mg activated charcoal (detached leaves) and eluted with 100 µL (Super-Q) or 40 µL (charcoal) of CH_2Cl_2 containing 120 ng of nonyl acetate or 80 ng of 1-bromodecane, respectively, as an internal standard. No major differences in the volatile profiles were observed between the different trapping materials.

The eluted samples (1 µL) were injected in a splitless mode into a GC-2010 gas chromatograph (Shimadzu, Japan) coupled with a QP2010S mass spectrometer (Shimadzu). Separation was performed on an Rxi-XLB column (Restek, Bellefonte, PA, USA) of 30 m × 0.25 mm i.d. × 0.25 µm film thickness. Helium was used as the carrier gas (1.4 mL min^{-1} flow rate), and a temperature gradient of 5 °C/min from 40 °C (hold for 2 min) to 220 °C was applied. Samples collected from hydroponically grown accessions were analyzed in 2 µL volumes on a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 quadrupole mass detector. Compounds were separated at a flow rate of 2 mL min^{-1} on a 5% phenyl-methyl-polysiloxane (DB5) column (J&W Scientific) of the same dimensions as described above. Qualitative analysis of volatile products of the TPS02 and TPS03 recombinant enzymes was performed using an AOC-5000 Shimadzu autosampler with automated solid phase microextraction (SPME). Compounds were thermally desorbed in a 2:1 split for 5 min at 240 °C in the GC injector.

The identities of volatile compounds were confirmed by comparison of their retention times and mass spectra with those of authentic standards (individual compounds or components of essential oils) and with mass spectra in the National Institute of Standards and Technology and Wiley libraries. For absolute quantification of MeSA and TMTT, the primary ion peaks of each compound were integrated (single ion method) and the amounts were calculated in relation to the response of nonyl acetate at m/z 69. Response curves for the quantified compounds relative to the internal standard were generated by injecting a mixture of equal amounts of authentic standards and internal standard. Absolute quantification of (*E*)-β-ocimene and (*E,E*)-α-farnesene was performed using an FID detector. For relative quantification of volatiles, primary ion peaks (*m/z* 93 for MeSA, (*E*)-β-ocimene and (*E,E*)-α-farnesene; *m/z* 69 for TMTT) were integrated and normalized against 1-bromodecane (at *m/z* 135).

2.5.5 Genotyping of Plant Material

Homozygous mutants in the Col and Ws background carrying a T-DNA insertion in the *TPS03* and the *TPS02* gene, respectively, were identified in the insertional mutant population (Sessions et al., 2002). Genomic DNA was isolated using the method by Edwards et al. (Edwards et al., 1991). The T-DNA insertion in Ws *TPS02* and Col *TPS03* was confirmed by PCR, using primers P1 to 3 and P4 to 6, respectively (see Supplemental Table SII).

2.5.6 Generation of Ws *TPS02* RNAi Lines

To prepare an RNAi construct for targeting Ws *TPS02*, first, a DNA fragment spanning a portion of the last exon of *TPS02*, the downstream intergenic region, and a portion of the first exon of *TPS03* was amplified by PCR using primers P7 and P8 and Ws genomic DNA. The PCR product was cloned into the pCR2.1® TOPO vector (Invitrogen) and used as a template for a second PCR to amplify a 106-bp fragment spanning 29-bp of the last exon and 77-bp of the 3'-UTR of WS *TPS02* with primers P9 and P10. The amplicon was cloned into pENTR-TOPO-D (Invitrogen) and transferred to pHELLSGATE8 (Wesley et al., 2001) using the LR recombination reaction (Invitrogen). The resulting construct was transformed into *Agrobacterium tumefaciens* (strain GV3101) using chemical transformation

(An, 1987) and WS plants were transformed with the vacuum infiltration method (Bechtold et al., 1993). Transgenic plants were selected on kanamycin resistance as described above. Experiments were performed with plants in the T3 generation.

2.5.7 Determination of Terpene Synthase Gene Expression by Semi-Quantitative RT-PCR

Total RNA was isolated from treated and untreated leaves of Col-0 and Ws wild type and mutant lines as well as from roots and flowers of both accessions using the Trizol reagent (Invitrogen, Carlsbad, CA, USA). One µg of total RNA was treated with RQ1 RNase-free DNase (Promega, Madison, WI, USA). The DNA-free total RNA was reverse transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen) in a total volume of 20 µl following the manufacturer's instructions. PCRs were performed with primers P11 and P12 for *TPS02* (At4g16730), P13 and P14 for *TPS03* (At4g16740), P15 and P16 for *TPS10* (At2g24210), and P17 and P18 for *Actin8* (At1g49240) as listed in Supplemental Table SII. PCRs were conducted with 0.5 µM of each primer, 0.2 mM of each deoxynucleotide triphosphate (dNTP), 0.5 U of Taq DNA polymerase (New England Biolabs, Ipswich, MA, USA) and 30 cycles for each reaction. Reactions for *Actin8* were applied to judge the equality of cDNA template concentrations. RT-PCRs were performed in three replicates with similar results using RNA extracted from three individual plants. Expression of the *TPS03* gene in Ws was probed with seven pairs of gene specific primers (P19 to P32) (see Supplemental Table SII) designed to amplify small fragments of a putative *TPS03* transcript.

2.5.8 Genomic and cDNA Cloning of *TPS02* and *TPS03* by RT-PCR

To obtain genomic sequence information of the *TPS03* gene from accession Ws, PCR primers P19 and P33 were used to amplify a *TPS03* genomic fragment, and 5'- and 3'-UTRs were amplified using primer pairs P34/P35 and P36/P37, respectively. The *TPS03* amplicon was cloned into the pGEM-T easy vector (Promega) and sequenced.

For cloning of *TPS02* and *TPS03* cDNAs from accessions Ws and Col-0, respectively, total RNA was extracted from alamethicin-treated leaves and reverse transcribed as described above. Primer P38, which binds 123 nucleotides downstream of the start codon of *TPS02* and P39, which corresponds to the end of the *TPS02* coding region, were used for RT-PCR

amplification of the *TPS02* cDNA from Ws. For amplification of a truncated version of *TPS02* from Col-0, primer P40, binding 153 bp downstream of the start codon, and P39 were employed. To remove the AT insertion of the Col-0 *TPS02* pseudogene, a site-directed mutagenesis was performed with primer P41 and the Col-0 *TPS02* full-length cDNA cloned in the pCR-T7/CT vector (Invitrogen) using the Stratagene QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA).

The entire open reading frame of *TPS03* from Col-0 was amplified using primers P42 and P43. Both reverse primers (P39, P43) allowed a translational fusion to a carboxyl-terminal His_6-tag. The amplified *TPS02* and *TPS03* cDNA products were inserted by directional cloning into the protein expression vector pET101-TOPO (Invitrogen). Correctness of cDNA clones was confirmed by sequencing.

2.5.9 Heterologous Protein Expression and Purification of Ws TPS02 and Col TPS03 in *E. coli*

The pET101-TOPO plasmids containing Ws *TPS02* or Col *TPS03* cDNA were transformed into *E.coli* BL21 DE3 competent cells (Invitrogen) for recombinant protein expression. Transformed cells were incubated in 500 ml cultures, started with an overnight culture, at 18 °C until an OD_{600} of 0.6 was reached. Protein expression was induced by adding 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) followed by additional incubation for 16 h at 18 °C. Cells were extracted and the TPS proteins were partially purified on 0.5 ml Ni-NTA-agarose columns (Quiagen, Valencia, CA, USA) as described by Tholl et al. (2004) and according to the manufacturers protocol. Following desalting into assaybuffer (10 mM MOPSO (3-N-morpholino-2-hydroxypropanesulfonic acid), pH 7.0, 1 mM DTT and 10% [v/v] glycerol), fractions with the highest enzyme activity were used for determination of enzyme kinetic parameters. Protein concentrations were determined by the Bradford method (Bradford, 1976) using reagents obtained from Bio-Rad (Richmond, CA, USA) and bovine serum albumin (BSA) as calibration standard. The specific amount of TPS protein in the active fraction was determined by SDS-PAGE separation of the partially purified protein and quantitative comparison of the intensity of the Coomassie-stained TPS protein band to those of BSA standards.

2.5.10 Terpene Synthase Enzyme Assay and Kinetic Characterization

For qualitative analysis of TPS enzyme products via SPME, enzyme assays were performed in screw-capped 10 ml glass vials in a total volume of 250 µl containing 10 µl (TPS02) or 150 µl (TPS03) of partially purified enzyme, 20 mM $MgCl_2$, 0.2 mM $NaWO_4$, 0.1 mM NaF, 1 mM DTT, and 60 µM GPP or FPP. The assay was incubated in the presence of a PDMS fiber at 30 °C for 10 min prior to volatile analysis by GC-MS as described above. For enzyme characterization, assays were carried out for 5 min (TPS03 with FPP) or 20 min in 50 µl reaction volumes with 0.35 to 4.5µg partially purified Ws TPS02 or Col TPS03 enzyme under the same buffer conditions described above. Reaction products were extracted with 300 µl hexane and total radioactivity was determined by scintillation counting. For evaluation of the K_m values for GPP and FPP, five different concentrations of [1-^{3}H]-GPP (35.2 MBq µmol^{-1}) and [1-^{3}H]-FPP (35.2 MBq µmol^{-1}) were applied. Assays were conducted in three replicates and apparent K_m values were determined by Hanes-Plot analysis using the Hyper 1.01 program (J.S. Easterby, University of Liverpool). To determine a possible activation of the TPS enzymes by K^+ ions, assays were performed with 5 µM [1-^{3}H]-GPP or [1-^{3}H]-FPP in the presence or absence of 50 mM KCl.

2.5.11 Construction and Analysis of *TPS02* and *TPS03* Promoter-GUS Reporter Gene Fusions

A 2.1 kb promoter fragment of Ws *TPS02* was isolated from genomic DNA of Ws via PCR using the forward primer P44, containing a *Hind*III site, and the reverse primer P45, containing the *TPS02* start codon and a *Bam*HI site. The PCR product was inserted into the *Hind*III and *Bam*HI cloning sites of the *uidA* (*GUS*) gene-containing binary vector pDW137 (Blazquez et al., 1997) according to Chen et al. (2004). A 1.8 kb Col-0 *TPS03* promoter-*GUS* fusion construct was obtained accordingly using primers P46 and P47. Transformation of the Ws *TPS02* and Col-0 *TPS03* promoter-fusion constructs into Ws and Col-0 plants, respectively, selection of transformed lines and GUS enzyme assays were conducted as described previously (Chen et al., 2003, 2004). At least four independent transformed lines were analyzed. For histochemical analysis of insect-induced GUS activity, *P. xylostella* larvae were allowed to feed for 24 h. Coronalon-induced GUS activity was determined after 24 h of treatment.

2.5.12 Construction of Ws TPS02 and Col-0 TPS03-GFP Reporter Fusions and Subcellular Localization

A 105 bp fragment encoding a 35 amino acid N-terminal peptide of Ws TPS02 was amplified by PCR with primers P48, carrying a *Nco*I site, and P49, which contains a *Spe*I site and cloned into pCR-TOPO4 (Invitrogen). The fragment was cut out of the vector with *Nco*I and *Spe*I and subsequently cloned downstream of a 35S-CaMV promoter in the vector pCAMBIA1302 (Hajdukiewicz et al., 1994) to generate a C-terminal fusion to mGFP5 (Siemering et al., 1996). The same procedure was applied to clone a 150 bp fragment encoding a 50 amino acid N-terminal peptide of the Col TPS03 protein using primers P50 and P51 for initial PCR amplification. Following confirmation of error free constructs by sequencing, constructs were transformed in *A. tumefaciens*, and Ws and Col-0 plants were transformed as described above. Transgenic plants were screened on agar plates for hygromycin resistance and genomic insertion of the corresponding fusion constructs was confirmed by genomic PCR. Two-week-old plantlets of at least four independent lines were used for observation by confocal laser scanning microscopy using a LSM 510 microscope (Carl Zeiss, Jena, Germany) equipped with a HeNe laser. Tissue autofluorescence was excited at 458 nm and GFP fluorescence at 488 nm, respectively. Band pass was set to 500-550 nm and long pass was set to 560 nm. Bright field images were acquired with the differential interference contrast channel. Images were processed from optical sections taken along the optical axis and projected into one image using the Zeiss LSM Image Browser 3.2.0.

2.5.13 Phylogenetic Analysis

Amino acid sequence alignment of plant TPS proteins was produced with ClustalW (Lasergene 8) and exported as a Nexus file. Phylogenetic analysis of the data set was conducted using maximum parsimony in PAUP* (D.L. Swofford, 2002, Florida State University). Maximum Parsimony analyses were conducted using heuristic tree searches with tree bisection-reconnection (TBR) branch-swapping and 1000 random addition sequence replicates. Support for the clades was obtained by performing bootstrap (BS) (Felsenstein,

1985) searches with 1000 replicates and 10 random sequence replicates. Trees were compiled using TreeGraph2 (Stover and Muller, 2010).

2.5.14 Statistical Analysis

Correlations of induced volatile emissions were determined with a Pearson correlation hypothesis test using the SAS program.

2.5.15 Accession Numbers

The deduced amino acid sequences of cited terpene synthase genes from gymnosperms and angiosperms can be found in the GenBank NCBI database with the following accession numbers: *A. thaliana thaliana* multi-product monoterpene synthase, (AtTPS24, At3g25810), NP_001031651; *A. thaliana thaliana* 1,8-cineole synthase, (AtTPS23&24, At3g25820& At3g25830), NP_189212; *A. thaliana thaliana* myrcene/(*E*)-β-ocimene synthase, (AtTPS10, At2g24210), NP_179998; *Malus domestica* α-farnesene synthase, AAS01424; *Pyrus communis* α-farnesene synthase, ABC25002; *Lotus japonicus* (*E*)-β-ocimene synthase, AAT86042; *Cucumis sativus* (*E,E*)-α-farnesene synthase, AAU05951; *Antirrhinum majus* (*E*)-β-ocimene synthase, AAO42614; *Pinus taeda* α-farnesene synthase, AAO61226; *Picea abies* (*E,E*)-α-farnesene synthase, AAS47697; *Actinidia deliciosa* (*E,E*)-α-farnesene/(*E*)-β-ocimene synthase, ACO40485.

2.6 Supplemental Material

Supplemental Table SI. Pearson correlation coefficients determined for emission of the four main volatile compounds induced in leaves of 27 *A. thaliana* accessions (Table 2.2.) in response to treatment with coronalon. The correlation hypothesis test rejected the null hypothesis of no linear relation between (*E*)-β-ocimene and (*E*,*E*)-α-farnesene as well as between (*E*,*E*)-α-farnesene and MeSA and TMTT and MeSA.

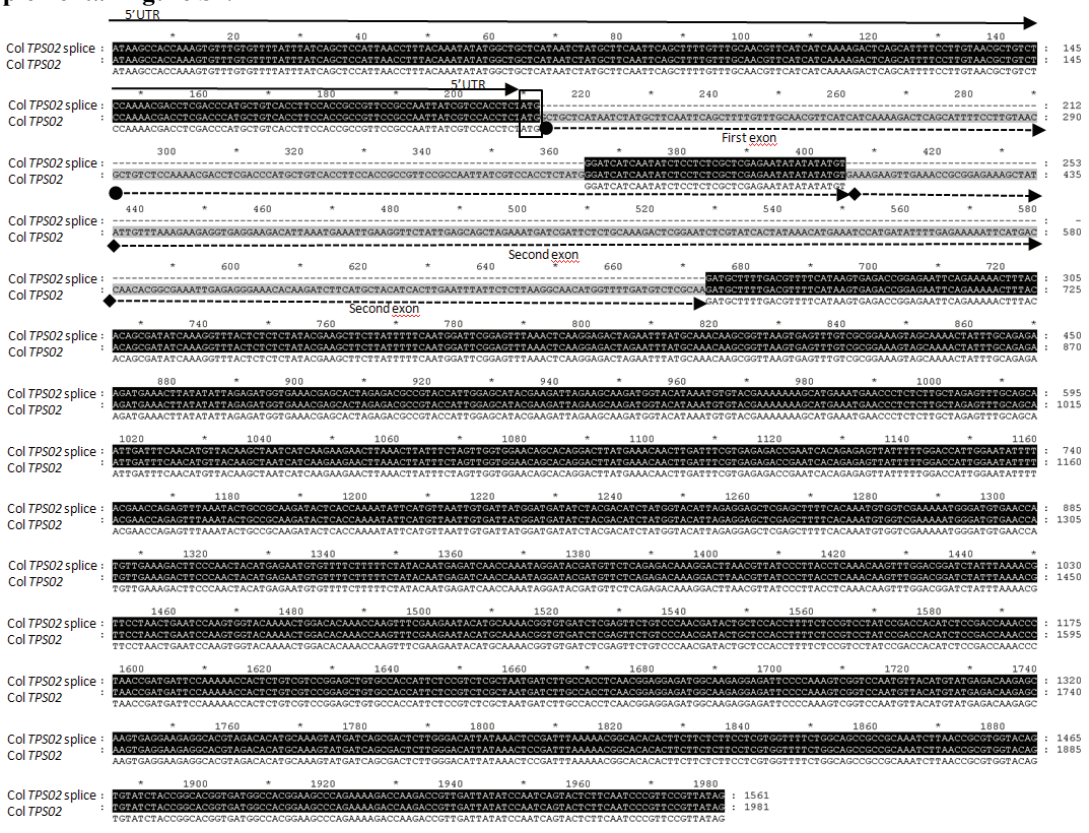
Pearson Correlation Coefficients, N = 81 Prob > |r| under H0: Rho=0				
Compound	MeSA	(*E*)-β-Ocimene	(*E*,*E*)-α-Farnesene	TMTT
MeSA	1.0	0.09 p=0.42	**0.33** **p=0.002**	**0.58** **p <0.0001**
(*E*)-β-Ocimene	0.09 p=0.42	1.0	**0.71** **p<0.0001**	0.22 p=0.05
(*E*,*E*)-α-Farnesene	0.33 p=0.002	0.71 p<0.0001	1.0	0.21 p=0.05
TMTT	0.58 p <0.0001	0.22 p=0.05	0.21 p=0.05	1.0

Supplemental Table SII. Primer sequences

Primer	Sequence (5' to 3')	Primer	Sequence (5' to 3')
P1	GAAAACGTCAAAAGCATCTGC	P37	TCGACTCCTCTATGAATAGCC
P2	TAAGCCACCAAAGTGTTTGTG	P38	CACCATGCGCCGTTCCGCCAATT
P3	CTACAAATTGCCTTTTCTTATCGAC	P39	TCTTCAATCCCGTTCCGTTA
P4	GAGCAAGACGGGATTAAC	P40	CACCATGGGATCATCAATATCTC
P5	GTCGCCTCTCGCTAATTC	P41	TCCTCTCGCTCGAGAATATATATGTGAAAGAAGTTG
P6	TGGTTCACGTAGTGGGCCATCG	P42	CACCATGCCTAAACGACAG
P7	AAGCCCAGAAAAGACCAAGACC	P43	ATTGAGTGGAAGAGGGTGG
P8	CGCCGTTGAGCCTGTCGTTTAG	P44	ATATTAAGCTTAGTAAGAGTTGACAACTCTGAG
P9	CACCCAGTACTCTTCAATCCCGTTCC	P45	ATATTGGATCCATATATTTGTAAAGGTTAATGGAGC
P10	GATCATTTTTCCGGCAAACCAAATACC	P46	ATATTAAGCTTTTTACATAATTAAAAGGTATTTGGTTTGC
P11	ATAAGCCACCAAAGTGTTTGTG	P47	ATATTGGATCCATTTCTTTGCGTTGCTAATGATTAC
P12	CTATAACGGAACGGGATTGAAG	P48	TAACCATGGCTGCTCATAATCTATGCTTC
P13	CACCATGCCTAAACGACAGGC	P49	TAACTAGTGGTCGAGGTCGTTTTGGAGACAGC
P14	ATTGAGTGGAAGAGGGTGGACG	P50	TAACCATGGCTAAACGACAGGCTCAACGGCG
P15	ATGGCCACTCTCCTGC	P51	TAACTAGTTTTCACATATGTATTACCGAGCG
P16	TCAATCTAAAGGAATCGGATTG		
P17	ATGAAGATTAAGGTCGTGGCAC		
P18	GTTTTTATCCGAGTTTGAAGAGGC		
P19	ATGCCTAAACGACAGGCTCAAC		
P20	CGATGAGCTCTAGCTGTTC		
P21	CGAACAGCTAGAGCTCATCG		
P22	CCATGTTGCCTTAGGAGTCG		
P23	CGAAGCTTCATATCTCTCGACC		
P24	GCATCTCTAACGCATGTATAACC		

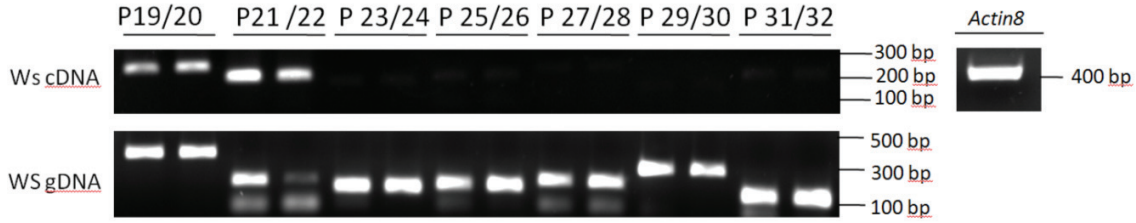
P25	GGTTATACATGCGTTAGAGATGC
P26	CTAGAGAGGGATTTGAGCTC
P27	GGAGCAAGACGGGATTAAC
P28	CTATGGTCGTGAATAGTTGGAGC
P29	AACCTTAGACATTCTTGGCTC
P30	GTGTCGCCTCTCGCTAATTCC
P31	GGAATTAGCGAGAGGCGACAC
P32	GGAAACCTTGATGTAGCCTACAAC
P33	TTAATTGAGTGGAAGAGGGTGGAC
P34	GGTGATGGCCACGGAAGC
P35	CGTTGAGCCTGTCGTTTAGG
P36	GCTCGTCCACCCTCTTCC

Supplemental Figure S1.



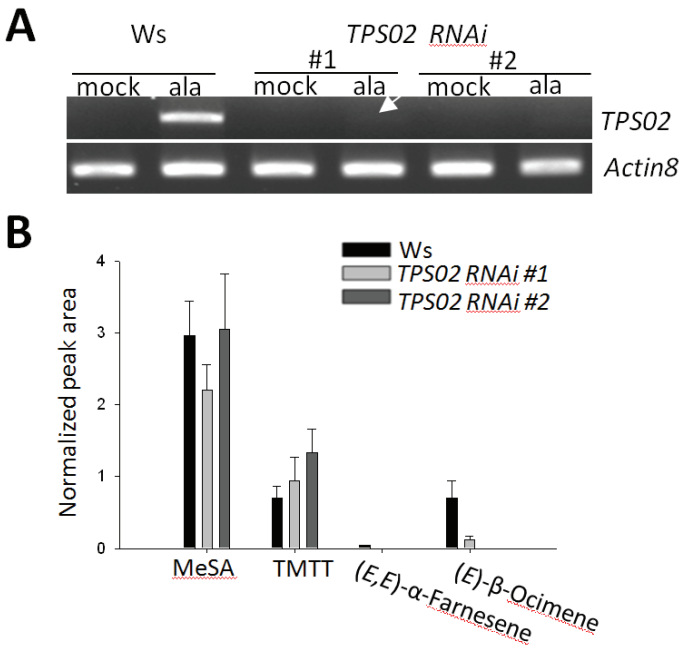
Supplemental Figure S1. Nucleotide sequence alignment of RT-PCR products of Col-0 *TPS02* amplified from RNA isolated from coronalon-treated Col-0 leaves. PCR primers P11 and P12 were used for amplification (see Supplemental Table SII). Identical nucleotides are marked in black. Horizontal arrows indicate the 5'-UTR (solid) and the first and second exons (dashed). The start codon is marked by a box. The upper sequence is a splice variant lacking 153 bp of the first exon and the entire second exon (267 bp).

Supplemental Figure S2.



Supplemental Figure S2. RT-PCR analysis of partial transcripts of the *TPS03* gene in coronalon-treated leaves of accession Ws. Results are shown for mRNA isolated from leaves of two different plants. Seven primer pairs (P19 to P32, see Supplemental Table SII) designed to amplify overlapping regions spanning the entire *TPS03* coding sequence were applied (upper row). Amplicons were only found for primer pairs P19/20 and P21/22. Transcript analysis of *Actin 8* was used as a control. The validity of all primer pairs was tested by PCR using genomic DNA of two separate plants (lower row). Low intensity bands represent unspecific PCR products.

Supplemental Figure S3.



Supplemental Figure S3. Expression of *TPS02* and volatile emission in detached leaves of Ws wild type plants and two *TPS02* RNA_i lines treated with the fungal elicitor alamethicin (ala). Alamethicin was used as an alternative elicitor because of limited availability of synthetic coronalon. A, RT-PCR analysis of transcript levels of *TPS02*. Transcripts of *Actin 8* were analyzed as a control. Trace levels of *TPS02* transcript were found in leaves of *TPS02* RNA_i line #1 (arrow), while no TPS02 transcripts were detected in *TPS02* RNA_i line #2. No transcripts were found for *TPS03* and *TPS10* in wild type and transgenic plants. C, Emission of MeSA, TMTT, (*E,E*)-α-farnesene, and (*E*)-β-ocimene monitored between 21 and 30 h of alamethicin treatment of Ws wild type and *TPS02* RNA_i plants. Normalized peak areas are shown for each compound as analyzed by GC-MS (see "Materials and Methods"). MeSA is the predominant compound induced by alamethicin. Small amounts of (*E*)-

β-ocimene were emitted from *TPS02* RNA_i line #1 in agreement with the "knock-down" of *TPS02* expression in this line. Results represent mean values ± SE (n = 3). None of the volatiles was detected in mock controls.

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3. Chapter II. The major volatile compound emitted from *Arabidopsis thaliana* flowers, (*E*)-β-caryophyllene, is a defense against bacterial pathogens

(To be submitted)

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Chapter overview and authors' contributions

This manuscript demonstrated the novel anti-microbial function of (*E*)-β-caryophyllene, an abundant sesquiterpene volatile from *Arabidopsis thaliana* flowers. Under the supervision of Dr. Dorothea Tholl and Prof. Jonathan Gershenzon, I characterized transgenic lines with overexpressed (*E*)-β-caryophyllene synthase and performed all experiments and analyses. Dr. Adela M. Sanchez-Moreiras quantified foliage pool size of (*E*)-β-caryophyllene from transgenic plants. With help from Jonathan Gershenzon, Dorothea Tholl and I planned and designed all experiments. With help from Jonathan Gershenzon, I wrote the manuscript.

3.1 Abstract

The sesquiterpene (*E*)-β-caryophyllene is one of the most common volatile compounds emitted from flowers and the major volatile produced by *Arabidopsis thaliana* flowers where it is released from the stigma. We investigated the role of (*E*)-β-caryophyllene in defense against pathogens since flowers have a high risk of pathogen attack due to the presence of nutritive tissues, elevated moisture content, and a high frequency of insect visitors. Plant lines lacking a functional (*E*)-β-caryophyllene synthase and constitutively over-expressing this gene were challenged with the native *A. thaliana* bacterial pathogen, *Pseudomonas syringae* pv. *tomato* DC3000. Lines lacking (*E*)-β-caryophyllene emission suffered more bacterial infection on their stigmas and the seeds produced were lighter and often misshapen. On the other hand, lines with ectopic (*E*)-β-caryophyllene emission from vegetative parts had increased resistance to pathogen infection in leaves, reduced cell damage and higher seed production when infected. Based on *in vitro* experiments, (*E*)-β-caryophyllene seems to act by direct inhibition of bacterial growth, rather than by triggering the salicylate or jasmonate signaling pathways. (*E*)-β-Caryophyllene appears to serve as a defense against floral pathogens, but like other floral volatiles may have multiple roles in defense and pollinator attraction.

3.2 Introduction

Plants synthesize and release a large variety of volatile organic compounds (VOC) into their environment (Pichersky and Gershenzon, 2002). Volatiles are emitted from all organs, but floral volatiles have long been a chief focus of research because of their pleasant smells and important role in plant reproduction (Dudareva et al., 2006). The primary function of floral volatiles is usually assumed to be the attraction and orientation of pollinators (Knudsen et al., 2006). However, these substances are being increasingly implicated in floral defense as well. Floral rewards, both pollen and nectar, are attractive to many organisms besides pollinators, and non-pollinators may feed on other floral tissue as well as disturbing legitimate pollinators (McCall and Irwin, 2006; Junker and Bluethgen, 2010). Floral volatiles have been shown to defend flowers against feeding by ants, beetles and other insects (Junker and Bluethgen, 2008; Willmer et al., 2009).

The largest group of floral volatiles is the terpenes, which include both monoterpenes (C_{10}) and sesquiterpenes (C_{15}) (Knudsen et al., 2006). One of the most widespread sesquiterpene floral volatiles is (*E*)-β-caryophyllene which occurs in floral blends in more than 50% of angiosperm families, and is one of twelve most common volatile compounds in floral scents (Knudsen et al., 2006). For instance, the volatiles of mature carnation (*Dianthus caryophyllus*) flowers contain 23% (*E*)-β-caryophyllene (Lavy et al., 2002), while *Nicotiana sylvestris* volatiles include 35% caryophyllene (Loughrin et al., 1990). (*E*)-β-Caryophyllene accounts for over 40% of the total volatiles emitted from *Arabidopsis thaliana* flowers (Chen et al., 2003), where this compound is almost released exclusively from the stigma (Chen et al., 2003; Tholl et al., 2005).

Curiously, (*E*)-β-caryophyllene has not yet been directly demonstrated to serve in pollinator attraction, but has been reported to act in defense against herbivores when it occurs in non-floral tissues. For example, this compound decreases the growth and survival of insects feeding on cotton and *Hymenaea* (Langenheim, 1994), and also serves as an indirect defense in maize, attracting parasitic wasps to oviposit on lepidopteran larvae feeding on leaves (Koellner et al., 2008), and attracting nematodes to attack coleopteran larvae feeding on maize roots (Rasmann et al., 2005). Given the antimicrobial activity of (*E*)-β-caryophyllene, it may also act in defense against pathogens (Cowan, 1999). A previous study

showed that caryophyllene-rich rhizome oil from *Zingiber nimmonii* had significant inhibitory activity against the bacteria *Bacillus subtilis* and *Pseudomonas aeruginosa* (Sabulal et al., 2006). However, no study has yet been conducted to investigate whether (*E*)-β-caryophyllene benefits plant reproductive organs by its antimicrobial role.

Plant reproductive organs, as well as vegetative organs are continuously exposed to various microorganisms. Daily arrivals of insect-carried bacterial pathogens are common, and airborne pathogens are carried by raindrops or by the wind as aerosols (Hirano and Upper, 2000). Compared to leaves, flowers are richer in nutrients and moisture and therefore carry higher densities of microorganisms. Bacterial populations can reach up to 10^{10} CFU g^{-1} on stigmas, and about 4 log units more than those on leaf surfaces (Johnson & Stockwell, 1998; Stockwell, 2005). Thus, volatiles with antimicrobial activity such as (*E*)-β-caryophyllene that are emitted from floral tissues might be expected to have a function in pathogen resistance.

Here we studied the antimicrobial role of (*E*)-β-caryophyllene in *Arabidopsis thaliana* where it is a major floral volatile (Chen et al., 2003; Tholl et al., 2005). *A. thaliana* like other Brassicaceae plants has dry-type stigmas with approximately 150 finger-like papillar cells (Heslop-Harrison and Shivanna, 1977; Tung et al., 2005). During pollen adhesion, hydration and germination on stigmas, the superficial pellicle consisting of exudates and a proteinaceous matrix provides an excellent humid and nutritive environment for microbial pathogens (Ngugi and Scherm, 2006). As a pathogen, we chose *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000), a natural bacterial pathogen of *Arabidopsis thaliana* (Whalen et al., 1991; Alfano and Collmer, 1996; Jakob et al., 2002). This species causes disease symptoms like necrotic lesions with chlorotic halos on leaves, but seldom on flowers. In this contribution, we demonstrate the role of volatile (*E*)-β-caryophyllene in bacterial pathogen resistance in *A. thaliana* flowers using lines knocked-out in emission or over-producing this sesquiterpene. Our work suggests that, among the mixture of floral volatiles, (*E*)-β-caryophyllene volatiles serve as an antimicrobial substance to protect flowers directly. The study provides support for the idea that floral volatiles, such as sesquiterpenes, help defends flowers against microorganisms in addition to or instead of attracting pollinators.

3.3 Results

3.3.1 *A. thaliana* mutant lines that lack (*E*)-β-caryophyllene emission suffer more bacterial growth on their stigmas

In *A. thaliana*, (*E*)-β-caryophyllene is produced by terpene synthase 21 (TPS21), which cyclizes the C_{15} isoprenoid pathway intermediate, farnesyl diphosphate, to the sesquiterpene (*E*)-β-caryophyllene with a small amount of α-humulene (Chen et al., 2003). TPS21 is almost exclusively expressed in the stigma, especially in mature, open flowers (Tholl et al., 2005). To determine the possible antimicrobial activity of (*E*)-β-caryophyllene, loss-of-function plants without any (*E*)-β-caryophyllene emission from stigmas were used. We inoculated two independent *TPS21* loss-of-function lines (*tps21*) with GFP-labeled *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) under high humidity. After inoculation, petals, sepals, and anthers displayed wilting symptoms and stigmas/styles became slightly yellowish. Bacterial growth on stigmas was observed by confocal laser scanning microscopy (Fig. 3.1). Bacterial abundance correlated with the intensity of GFP fluoresecence. Forty eight hours post inoculation, stigmas of both *tps21* lines carried more GFP-labeled cells than those of wild type plants (Fig. 3.1) Serial scanning of stigmas at different depths (using the z-stack technique) confirmed this impression. Less bacterial fluorescence appeared at sites located deeper in the tissue indicating that bacteria were mostly present on the surface of the stigma. However, some areas of strong bacterial accumulation were also observed in the center of the stigmatic tissue of the *tps21-1* line (Fig. 3.1, B, B1-4).

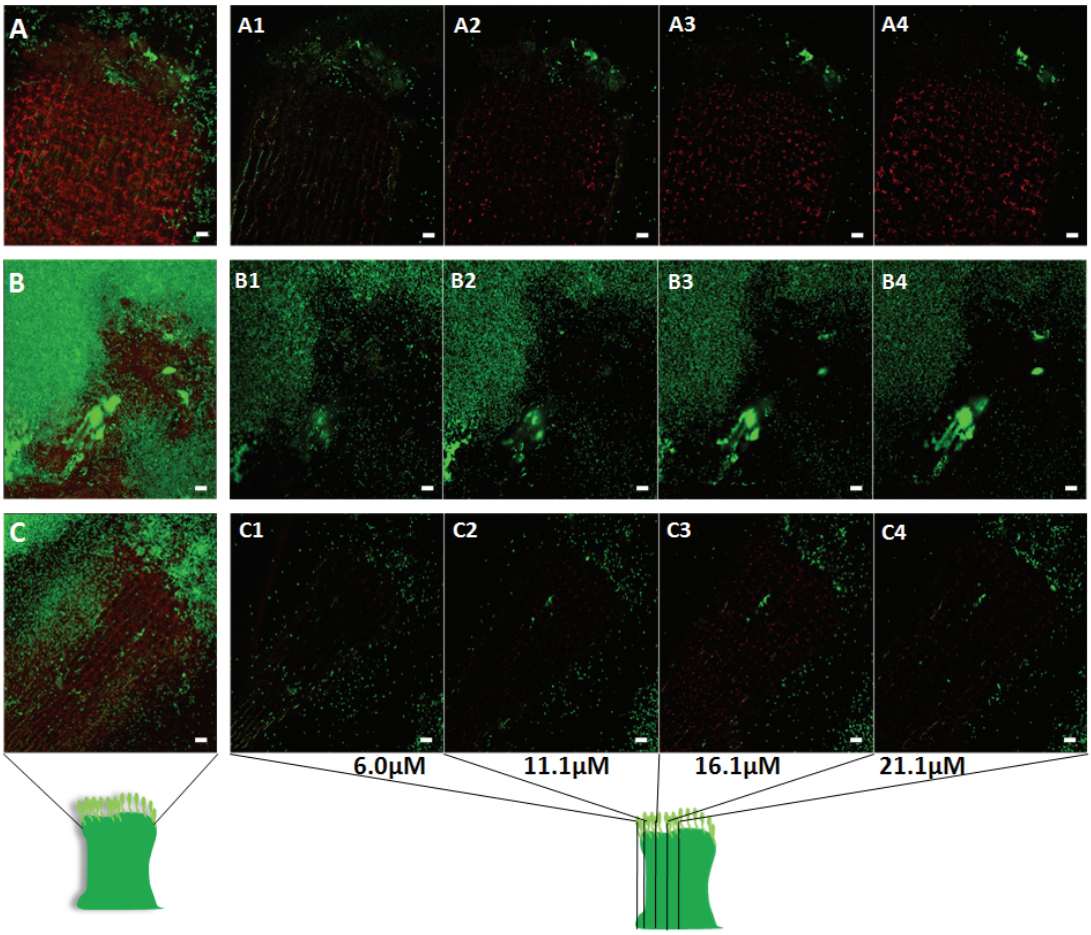

Figure 3.1. Comparison of *P. syringae* pv. *tomato* DC3000 (*Pst* DC3000) growth on stigmas of (*E*)-β-caryophyllene emitting *Arabidopsis* wild-type plants (A) and non emitting *tps21*-1 and *tps21*-2 mutants (B and C) using confocal laser scanning microscopy. Images were taken 48 h post inoculation. Chlorophyll autofluorescence appears in red; GFP fluorescence from GFP-labeled *Pst* DC3000 appears in green. Numbered images are from serial scanning at various depths within the sample: 6.0 µM (1); 11.1 µM (2); 16.1 µM (3); 21.1 µM (4). A, B and C are merged z-stack images. The sketch below indicates the approximate position of each image on a longitudinal section of the stigma. The images shown are representative for at least three independent replicates. Scale bars: 20 µM.

3.3.2 Non-(*E*)-β-caryophyllene-emitting lines have lower seed weight after stigmatic bacterial infection

To determine if loss of (*E*)-β-caryophyllene emission from the stigma would affect future seed development, wild type and loss-of-function mutants were infected with *Pst* DC3000 and their seed weight and appearance monitored. Infection of inflorescences dramatically reduced seed production of both wild type and loss-of-function lines (data not shown). Comparing the weight of 100 randomly-chosen seeds of each plant revealed a similar average seed weight of uninoculated wild type and mutant plants. However, after

infection of inflorescences, *tps21* mutant lines produced significantly lighter seeds compared to wild type plants (Fig. 3.2A). Loss-of-function seeds also showed a high frequency of abnormal shape compared to wild-type seeds (Fig. 3.2B).

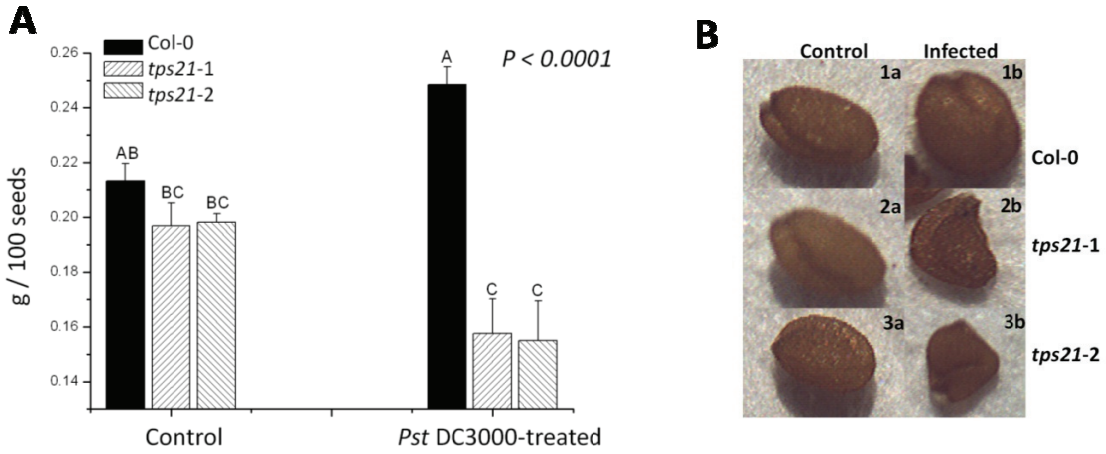

Figure 3.2. Effect of floral (*E*)-β-caryophyllene emissions on seed production of wild type Col-0 and *tps21* mutants (*tps21*-1, *tps21*-2) after inoculation of inflorescences with *Pst* DC3000. A) Average weight of 100 seeds of each line. Results represent mean values ± SE (n = 5, P < 0.0001 according to ANOVA with a Tukey post-hoc test). B) Appearance of seeds of wild type Col-0 (1a and1b), *tps21*-1 (2a and 2b) and *tps21*-2 (3a and 3b). Infection of Col-0 flowers had no effect on seed weight and shape, but infection of non-(*E*)-β-caryophyllene-emitting lines led to significantly lighter seeds with abnormal shape.

3.3.3 Transgenic lines ectopically expressing (*E*)-β-caryophyllene synthase are less damaged by bacterial infection

The effect of (*E*)-β-caryophyllene on *Pst* DC3000 infection was also investigated using (*E*)-β-caryophyllene over-producing *Arabidopsis* lines generated by transformation with the *TPS21* gene under the control of the constitutive CaMV 35S promoter. While expression of *TPS21* in wild-type plants is normally restricted to the floral stigma (Chen et al., 2003; Tholl et al., 2005), transgenic lines showed ectopic expression of *TPS21* in rosette leaves (Fig. 3.3A). Two transgenic lines were chosen with different *TPS21* transcript levels. Southern blot analyses indicated that both lines contained more than one insertion of the transgene (data not shown). The emission rates of (*E*)-β-caryophyllene from these lines at the rosette stage were 4.7 ng gFW^{-1} h^{-1} and 2.4 ng gFW^{-1} h^{-1}, respectively (Fig. 3.3B), somewhat less than the rate of emission observed from flowers (28.2 ng gFW^{-1} h^{-1}). In addition, the pool size of (*E*)-β-caryophyllene in the foliage was barely detectable (< 4 pg

gFW^{-1}) (Fig. 3.3C). Both *TPS21*-expression lines showed no gross morphological differences from wild-type plants.

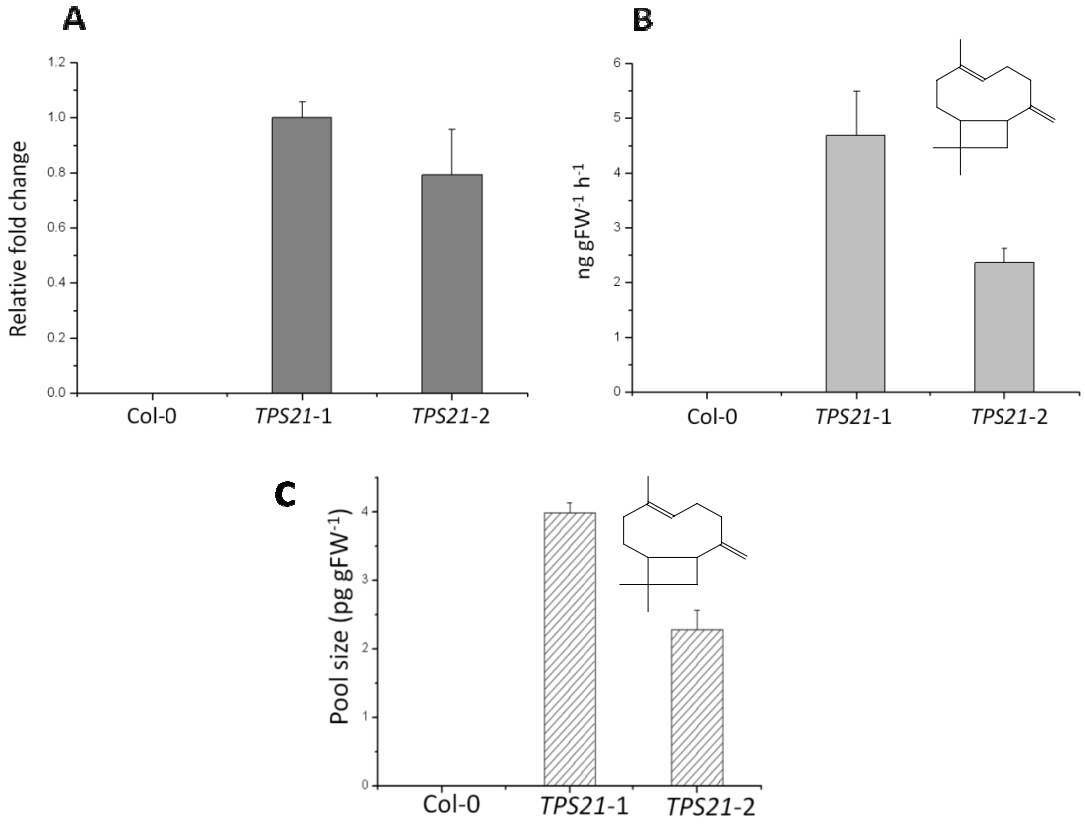

Figure 3.3. Comparison of (*E*)-β-caryophyllene synthase over-expression lines (*TPS21*-1, *TPS21*-2) based on (A) transcript levels of *TPS21* using quantitative RT-PCR, (B) volatile (*E*)-β-caryophyllene emission and (C) foliar pool size of (*E*)-β-caryophyllene. Bars are means ± SE (n = 3).

To analyze resistance to bacterial infection in the (*E*)-β-caryophyllene synthase expressing plants, *Pst* DC3000 was sprayed on leaf surfaces of both wild type and transgenic lines. Inoculation resulted in yellowish, water-soaked lesions on all plants. The size of bacterial populations in wild-type and transgenic plants was quantified by titering living cells in leaves over a time course of three days post-inoculation.

Bacterial growth continuously increased after inoculation in both transgenic and wild-type plants (Fig. 3.4). However, on day 3 post-inoculation, bacterial growth was significantly less in both transgenic lines than in wild type plants (Fig. 3.4) indicating that (*E*)-β-caryophyllene emission retarded or at least delayed growth of the pathogen.

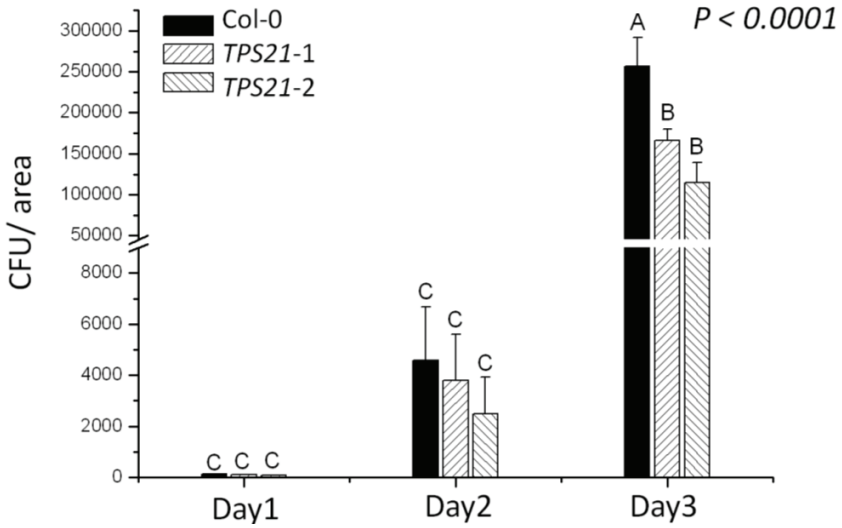

Figure 3.4. Effect of (*E*)-β-caryophyllene emission on growth of *Pst* DC3000. Bacterial population size was measured in leaves of transgenic plants with constitutive expression of (*E*)-β-caryophyllene synthase (*TPS21*-1, *TPS21*-2) in comparison to wild-type controls at day1, 2 and 3 post-inoculation. Results represent mean values ± SE (n = 3). Different letters represent significant differences (P < 0.0001 according to ANOVA with a Tukey post-hoc test).

The extent of bacterial damage to the plant was estimated by cell death staining performed on day 3 after inoculation. In this semi-quantitative approach, leaves of (*E*)-β-caryophyllene emitting lines showed fewer damaged cells than wild type (Fig. 3.5). These results are consistent with the differences in bacterial population size and support the premise that (*E*)-β-caryophyllene may increase resistance to invasion of a phyllosphere pathogen.

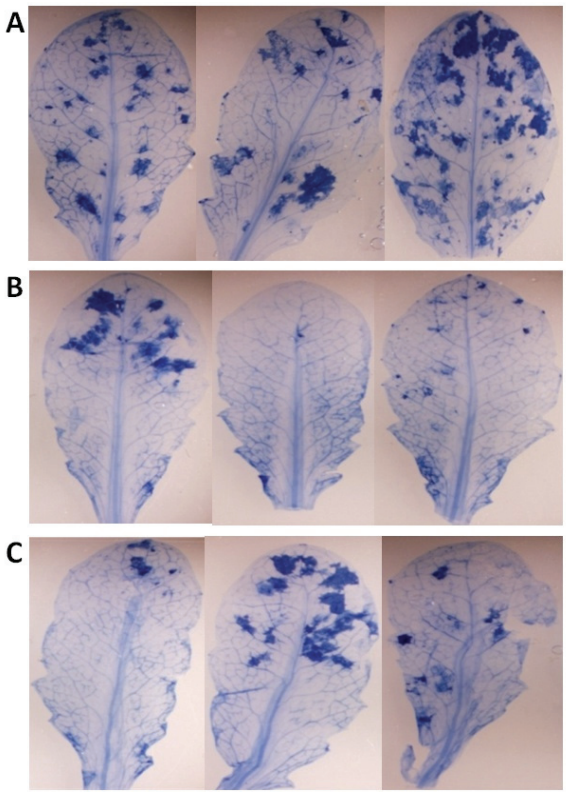

Figure 3.5. Effect of (*E*)-β-caryophyllene emission on *Arabidopsis* resistance to infection by *Pst* DC3000. Cell death was measured using lactophenol-trypan blue staining. Comparisons were made among wild type Col-0 (A) and transgenic plants over-expressing (*E*)-β-caryophyllene synthase (*TPS21*-1, *TPS21*-2) (B, C) at day 3 after inoculation. Three representative images are shown from at least three independent replicates of each plant line. Dark stained areas indicate dead cells.

The benefit of (*E*)-β-caryophyllene emission to plant defense may be canceled out if terpene emission represents a significant loss of energy and fixed carbon (Vickers et al., 2009). To determine the net value of (*E*)-β-caryophyllene production to the plant, we compared the reproductive fitness of over-expressing and wild-type lines with and without *Pst* DC3000 infection using seed production as a measure. In the absence of the pathogen, transgenic plants produced a similar amount of seeds compared to wild types (Fig. 3.6). However, when leaves were inoculated with *Pst* DC3000, transgenic plants produced significantly more seeds than wild type. The seed output of wild-type plants was not affected by *Pst* DC3000 infection. By contrast, *TPS21*-expression lines showed greater seed production after leaf infection in comparison to wild type.

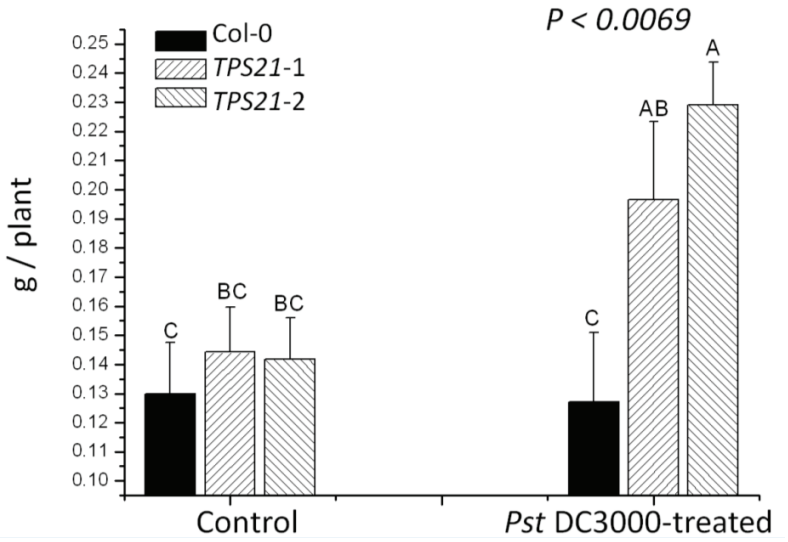

Figure 3.6. Effects of (*E*)-β-caryophyllene emission on seed production of *Arabidopsis* wild type plants and *TPS21*-expression lines 5 weeks post inoculation with *Pst* DC3000. Results are average values ± SE (n = 4). Different letters represent significant differences (P < 0.0069 according to ANOVA with an LSD test).

3.3.4 (*E*)-β-Caryophyllene inhibits bacterial growth *in vitro*

To gain a better understanding of how (*E*)-β-caryophyllene might act to increase plant resistance to *Pst* DC3000, a direct *in vitro* assay was performed to test the activity of the sesquiterpene against this pathogen. In a previous study, (*E*)-β-caryophyllene dissolved in DMF (N,N-dimethylformamide) showed moderate antimicrobial activity to *Propionibacterium acnes* and weak activity to *Streptococcus mutans* (Kubo et al., 1992). Thus we explored the use of organic solvents such as DMF and DMSO (dimethyl sulfoxide) to deliver the highly lipophilic (*E*)-β-caryophyllene into aqueous solution, but found DMSO to show unexpected enhancing effects on *Pst* DC3000 *in vitro* growth (data not shown). As another approach, we calculated the solubility of authentic (*E*)-β-caryophyllene in water and in King's B liquid medium, since isoprene and other terpene hydrocarbons dissolve slightly in water (Milne et al., 1995; Fichan et al., 1999). We estimated that up to 7×10^{-4} mmol and 2×10^{-4} mmol of (*E*)-β-caryophyllene could dissolve in 1L water and King's B liquid medium, respectively. We tested (*E*)-β-caryophyllene at a concentration of 1×10^{-5} mmol L^{-1} and 2×10^{-5} mmol L^{-1} in King's B liquid medium containing *Pst* DC3000. At these concentrations, the sesquiterpene significantly inhibited *Pst* DC3000 growth in a dose-dependent manner (Fig. 3.7). A concentration of 1×10^{-5} mmol L^{-1} (*E*)-β-caryophyllene led to 28% reduction of

bacterial growth, and 2×10^{-5} mmol L^{-1} inhibited *Pst* DC3000 growth by 74%. Thus (*E*)-β-caryophyllene had antibacterial activity *in vitro*.

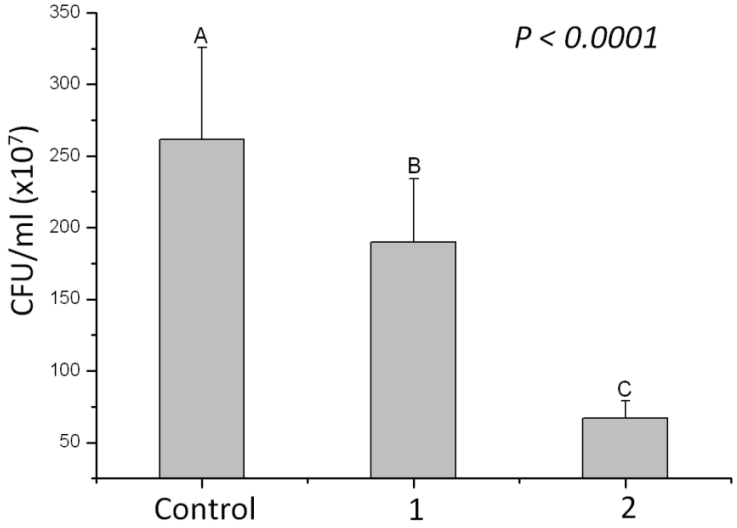

Figure 3.7. Inhibitory effect of (*E*)-β-caryophyllene on *Pst* DC3000 growth in liquid medium. (*E*)-β-Caryophyllene was applied at two different concentrations (1, $1x10^{-5}$ mmol L^{-1}; 2, $2x10^{-5}$ mmol L^{-1}). Results are average values ± SE (n = 3). Different letters indicate significant differences among treatments ($P < 0.0001$ according to ANOVA with a Tukey test).

3.3.5 The defensive function of (*E*)-β-caryophyllene against *Pst* DC3000 is independent of JA or SA signaling

An alternative to the direct action of (*E*)-β-caryophyllene on *Pst* DC3000 is that this sesquiterpene instead triggers a signaling pathway leading to increased pathogen defense. Both salicylic acid (SA) and jasmonic acid (JA) signaling pathways play important roles in plant resistance against pathogens (Truman et al., 2007). To assess whether these signaling cascades are activated by (*E*)-β-caryophyllene, the expression levels of two genes, *PR1* (*pathogenesis-related gene 1*) associated with SA signaling and *LOX2* (*lipoxygenase 2*) associated with JA signaling (Beckers and Spoel, 2006) were determined by quantitative RT-PCR on wild-type *A. thaliana* and transgenic plants with (*E*)-β-caryophyllene emission.

Pst DC3000 inoculation induced *PR1* expression in all plant lines, but the expression levels in both two transgenic lines showed no consistent difference from the wild type (Fig. 3.8). One of the transgenic lines, *TPS21*-2, showed a slower induction of the *PR1* gene, while the other line, *TPS21*-1, exhibited no significant difference from wild-type expression during the entire period.

The induction of *LOX2* transcript also increased after *Pst* DC3000 inoculation but on a much smaller scale than *PR1* (Fig. 3.9). However, no statistically significant difference between transgenic plants and wild-type expression was observed. Taken together, these transcript results indicate the activation of SA and JA signaling pathways after pathogen challenge in both transgenic and wild-type plants, but no consistent differences between (*E*)-β-caryophyllene-emitting and wild-type lines were noted. If (*E*)-β-caryophyllene expression triggered resistance against *Pst* DC3000 by signaling via the SA or JA pathways, induction of *PR1* and *LOX2* would be expected to be higher and/or sooner in the transgenic lines compared to the wild type. This was not the case. In fact, there was even a trend (not statistically significant) to lower expression in transgenic plants (Figs. 3.8, 3.9). We conclude that activation of JA or SA pathway are not the mechanism by which (*E*)-β-caryophyllene emission causes less pathogen growth, less leaf damage and more seed production.

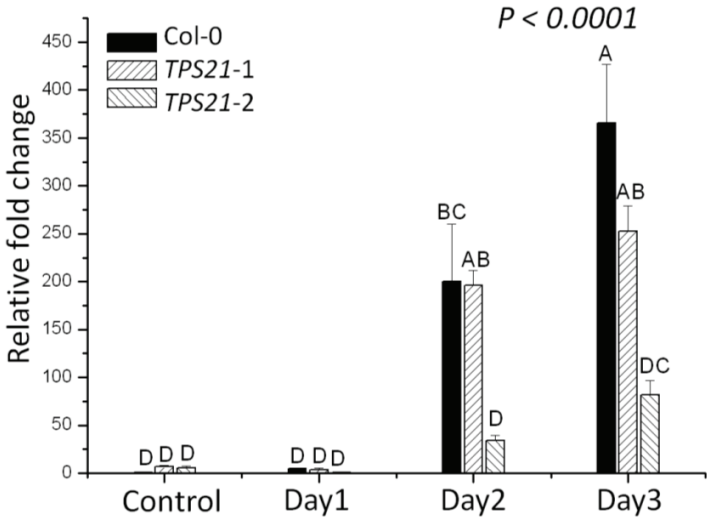

Figure 3.8. Comparison of *PR1* gene expression in Col-0 wild-type and (*E*)-β-caryophyllene synthase over-expression lines (*TPS21*-1, *TPS21*-2) after *Pst* DC3000 infection. Transcript levels of *PR1* in leaves before and 1, 2 and 3 days after inoculation were quantified by qRT-PCR. Bars represent the means ± SE (n = 3). Different letters indicate significant differences (P < 0.0001 according to ANOVA with Tukey post hoc tests).

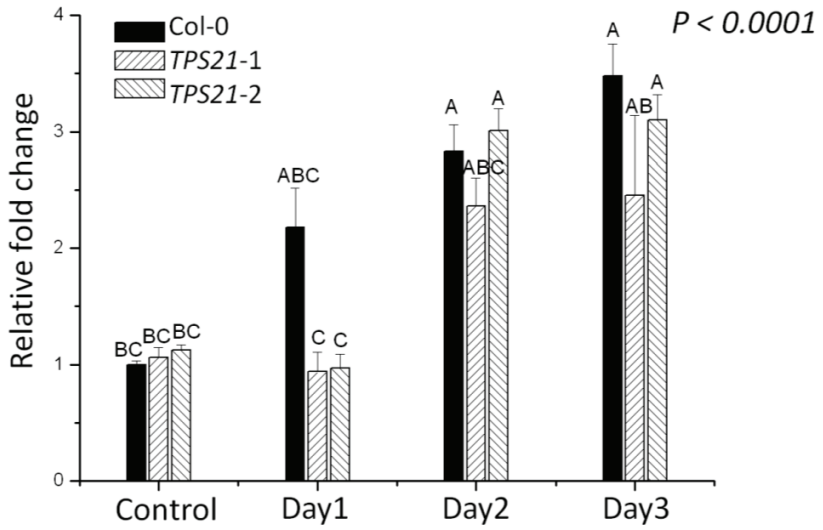

Figure 3.9. Comparison of *LOX2* gene expression in Col-0 wild-type and (*E*)-β-caryophyllene synthase over-expression lines (*TPS21*-1*, TPS21*-2) after *Pst* DC3000 infection. Transcript levels of *LOX2* in leaves before and 1, 2 and 3 days after inoculation were quantified by RT-PCR. Bars represent the means ± SE (n = 3). Different letters indicate significant differences (P < 0.0001 according to ANOVA with Tukey post hoc tests).

3.4 Discussion

3.4.1 (*E*)-β-Caryophyllene increases the resistance of *A. thaliana* flowers to bacterial pathogens

The sesquiterpene (*E*)-β-caryophyllene is the major volatile compound emitted by *Arabidopsis thaliana* flowers. In this investigation, we demonstrated that mutants lacking (*E*)-β-caryophyllene emission suffered greater infection on their stigmas by the natural bacterial pathogen, *Pseudomonas syringae* pv. *tomato* DC3000 (Fig. 3.1) and had reduced seed output (Fig. 3.2). On the other hand, constitutive expression of (*E*)-β-caryophyllene emission throughout the plant reduced pathogen infection (Fig. 3.4), decreased cell death due to the pathogen (Fig. 3.5) and led to increased seed production (Fig. 3.6). Thus (*E*)-β-caryophyllene released from flowers helps defend against bacterial pathogens.

Flowers may have a higher risk of pathogen attack than other plant tissues because of their high nutrient content (due to the presence of pollen and nectar) and elevated moisture level (Morris et al., 2008). Stigmas may be especially vulnerable due to their humid, nutritive environment (Ngugi and Scherm, 2006) which is designed to facilitate the germination and growth of pollen grains, but may be equally conducive to the growth of pathogens. Bacterial

pathogens are capable of causing severe disease to reproductive parts. For instance, *Erwinia amylovora* is the causative agent of severe fire blight disease on apple and pear flowers (van der Zwet and Keil, 1979).

The population of floral pathogens depends not only on the local environment in the flower, but also on the availability of agents, such as water drops, aerosols or insects, to transfer pathogens. Bacterial pathogens including *P. syringae* are frequently vectored between plant tissues by insects in a process independent of either insect species or bacteria species (Hirano and Upper, 2000). For example, *Erwinia amylovora* can be transported by various insects (Emmett and Baker, 1971). Since flowers are a beacon for insect visitors, they may have especially high pathogen loads.

The optimal defense theory hypothesizes that allocation to plant defense among different plant parts is based on the value of those parts to plant fitness as well as the risk of attack. Since flowers obviously make a high contribution to plant fitness, it is not surprising that they contain high amounts of defense compounds (McKey, 1974; Zangerl and Rutledge, 1996). As we have seen, flowers also face high risks of pathogen attack. Hence it is reasonable that they possess effective anti-pathogen defenses that are present constitutively without the time delay needed for the production of inducible defenses. (*E*)-β-Caryophyllene emission in *A. thaliana* is not only constitutive, but is also confined to the flowers; this sesquiterpene is not released at all from the vegetative organs (Chen et al., 2003; Tholl et al., 2005).

3.4.2 (*E*)-β-Caryophyllene acts directly to inhibit bacterial growth

To gain insight into the mode of action of (*E*)-β-caryophyllene against *Pst* DC3000, we tested whether this compound had any direct action against the pathogen or instead triggered one of the known anti-pathogen signaling cascades. (*E*)-β-Caryophyllene did not appear to induce the SA or JA signaling pathways (Figs. 3.8, 3.9), but did in fact act directly against *Pst* DC3000 when added to the culture medium (Fig. 3.7). It is difficult to compare the concentration used in our tests with the amount of volatiles a pathogen would experience on the plant surface. The concentration of (*E*)-β-caryophyllene tested in culture (1- 2 × 10^{-5} mmol L^{-1}, that is 2000- 4000 ng L^{-1} or 2- 4 ng gFW^{-1} of culture) is very similar to the amount of (*E*)-β-caryophyllene produced in the (*E*)-β-caryophyllene synthase over-expression lines

(2.4- 4.7 ng gFW^{-1} h^{-1}) on an hourly basis. However, the amount released by flowers (28.2 ng gFW^{-1} h^{-1}) on an hourly basis is higher, suggesting that (*E*)-β-caryophyllene is very likely to have a direct defensive role in nature.

(*E*)-β-Caryophyllene has been previously shown to have activity against a wide range of bacteria, including species of *Bacillus*, *Pseudomonas* and *Streptococcus* (Kubo et al., 1992; Sabulal et al., 2006; Delamare et al., 2007; Amor et al., 2008; Kim et al., 2008). Terpenes are generally recognized as antimicrobial metabolites (Cowan, 1999), and the mechanism of action of (*E*)-β-caryophyllene on a bacterium like *Pst* DC3000 might be similar to that of other terpenes on microorganisms. It has long been suggested that lipophilic compounds like terpenes act to disrupt membranes leading to ion leakage, membrane potential reduction, proton pump dysfunction and ATP pool depletion (Bakkali et al., 2008). But, recently it has been reported that terpenes like citral, a mixture of the monoterpenes geranial and neral, can disrupt cell microtubules (Chaimovitsh et al., 2010). Moreover, the monoterpenes carveol and carvone effectively disperse bacterial cells from aggregation in liquids by decreasing the percentage of fatty acids of bacteria cell membranes (de Carvalho and de Fonseca, 2007).

The mode of action of (*E*)-β-caryophyllene might also be influenced by its rapid chemical reaction with certain oxygen species. The atmospheric lifetime of (*E*)-β-caryophyllene with hydroxyl radicals (42min), O_3 (2min) and NO_3 (3min) is very short (Atkinson and Arey, 2003). The oxidation products of (*E*)-β-caryophyllene are found to reside almost 100% into the aerosol phase (Hoffmann et al., 1997) and are much stabler than non-oxidized (*E*)-β-caryophyllene (Howard et al., 1989). The rapid reactivity of (*E*)-β-caryophyllene in the atmosphere should not affect pathogen growth on the plant surface. But, it is possible that this reactivity creates new products that are themselves active antimicrobial agents. A recent study with photooxidized (*E*)-β-caryophyllene shows that oxidation products have strongly enhanced activity against *Vibrio parahaemolyticus* and *Streptococcus aureus* compared to the parent compound (Sabulal et al., 2006; Delamare et al., 2007; Amor et al., 2008; Kim et al., 2008). This could mean that oxidized (*E*)-β-caryophyllene exerts its effect after deposition back on *A. thaliana* stigmas, as hypothesized for reactive sesquiterpenes emitted from leaves (Himanen et al., 2010). Whether active as parent compound or oxidized product, the activity of (*E*)-β-caryophyllene may be enhanced by synergistic interactions

(Kubo et al., 1992) with other components of the complex blend of volatiles emitted from *A. thaliana* flowers.

3.4.3 (*E*)-β-Caryophyllene may serve in floral attraction as well as defense

Serving as an inhibitor of pathogen growth in *A. thaliana* flowers does not exclude (*E*)-β-caryophyllene from also acting as a pollinator attractant. *A. thaliana* flowers are thought to be mostly self-pollinated under natural conditions, but cross-pollination occurs with a low frequency as a result of insect visitors, such as flies, beetles or solitary bees (Chen et al., 2003) (Hoffmann et al., 2003). These insects may be attracted to floral volatiles, such as sesquiterpenes. In previous work, higher emission of (*E*)-β-caryophyllene from flowers of *Cucurbita pepo* ssp. *texana* resulted in more visitation from beetles (Ferrari et al., 2006). (*E*)-β-Caryophyllene has also been shown to serve as an attractant for other types of insects, such as herbivore enemies (De Moraes et al., 1998; Rasmann et al., 2005; Koellner et al., 2008). Some insects may have an inherent attraction for this compound since it serves as an aggregation pheromone for the multi-colored Asian lady beetle (*Harmonia axyridis*) (Brown et al., 2006; Verheggen et al., 2007) and a sex pheromone for the European grape berry moth (*Eupoecilia ambiguella*) (Schmidt-Busser et al., 2009).

(*E*)-β-Caryophyllene is also a deterrent as we have demonstrated for a plant bacterial pathogen in this study. In fact, this compound is even used by microorganisms themselves in their own defense. For example, *Serratia* spp. use (*E*)-β-caryophyllene to inhibit *Rhizoctonia solani*, a plant pathogen (Kai et al., 2007). Volatile (*E*)-β-caryophyllene is also reported to be a long distance antagonistic agent of one strain of *Fusarium oxysporum* against another (Minerdi et al., 2009). This deterrence extends to insects as well. When volatilized from the foliage of certain tree species, (*E*)-β-caryophyllene repels leaf-cutting ants and termites (Hubbell et al., 1983; Messer et al., 1990). This sesquiterpene also has the potential to deter unwanted floral visitors, as it repelled the ant *Lasius fuliginosus* in olfactometer tests (Junker and Bluethgen, 2008).

Given the reported activities of (*E*)-β-caryophyllene, it could well serve in *A. thaliana* flowers as both an attractant and a deterrent depending on the context. Other floral volatiles also seem to have dual attractive and repellent roles. For example, cones of the cycad *Macrozamia lucida* attract thrips (*Cycadothrips*) in one phase of flowering when the

monoterpenes (*E*)-β-ocimene, (*Z*)-β-ocimene and low concentrations of myrcene are present. But, in a later phase of flowering, high concentrations of myrcene emitted by the thermophilic cones repel the thrips and cause them to transfer pollen to another cycad (Terry et al., 2007).

A dual role of floral volatiles may reflect their evolutionary origin. It has been theorized that volatiles were first emitted from flowers as herbivore feeding deterrents and only later came to be attractive to pollinators who began to associate the scent with a reward (Pellmyr and Thien, 1986; Terry et al., 2007). To this scenario, we can now add that volatiles may have also functioned originally as pathogen defenses and still do today. Since the advent of pathogenic microorganisms in evolutionary history long predates the rise of flowering plants, flowers have likely had to adapt to these invaders from a very early point in their history.

3.5 Materials and Methods

3.5.1 Plant materials, growth conditions

Seeds of *Arabidopsis thaliana* ecotype Col-0 (CS 6000) were obtained from ABRC (Arabidopsis Biological Resource Center, OH, USA). Seeds of At5g23960 (*TPS21*) T-DNA insertion mutant lines (*tps21*) *SALK_133613* (N636913) and *SALK_138212* (N638212) (Alonso et al., 2003) were from the NASC stock center (Nottingham Arabidopsis Stock Centre). For experiments with vegetative stage plants, wild type and transgenic lines were grown on soil (Sunshine Growing Mix No.1 : sand, 8:1) for 5 to 6 weeks under controlled growth conditions (10 h-light/14 h-dark photoperiod with 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, 23 °C, 55% relative humidity). At the end of this period, plants were still in the rosette stage and had not yet bolted. For experiments with flowering plants, wild type and mutant lines were cultivated on soil for up to 7 weeks under a 16 h-light/8 h-dark photoperiod. Kanamycin-resistant transgenic plants were selected on 1 × Murashige and Skoog (Duchefa, Haarlem, The Netherlands) plates with 1% sucrose and 100 $\mu g\ mL^{-1}$ kanamycin prior to being transferred to soil.

3.5.2 Reagents

All reagents or solvents were obtained from Fisher Scientific, Sigma-Aldrich, Invitrogen or Fluka, unless otherwise stated.

3.5.3 Bacterial cultures and plant treatments

Pseudomonas syringae pv. *tomato* DC3000 (*Pst* DC3000) and a GFP-labeled *Pseudomonas syringae* pv. *tomato* DC3000 strain were obtained from Boris A. Vinatzer, Virginia Tech, Blacksburg, VA. Bacteria strains were cultivated on King's B solid medium with 15 µg mL^{-1} tetracycline or 50 µg mL^{-1} kanamycin at 28 °C for 24 h (Katagiri et al., 2002). One day prior to inoculations, plants were sprayed with distilled water and placed in separated containers under controlled growth conditions with 100% relative humidity. Bacterial suspensions at OD_{600} 0.01 (leaves, 1×10^6 CFU mL^{-1}) or 0.1 (flowers, 5×10^7 CFU mL^{-1})in 10 mM $MgCl_2$ with 0.025% Silwet L-77 (Vac-In-Stuff, Lehle Seeds, Round Rock, TX, USA) were applied on surfaces of leaves or flowers as a fine mist until the suspension ran off. Control plants were sprayed with 10 mM $MgCl_2$ solution containing 0.025% Silwet L-77. After spraying, plants were returned to their containers, placed under controlled growth conditions, and leaf infection symptoms were quantified at 1 day to 3 days post-inoculation. The effects of infection on flowers were quantified by measuring seed production. To visualize the population of bacteria on stigmas, inflorescences of wild type and *tps21* lines were cut off and placed in 5 ml glass beakers filled with tap water. The flowers were inoculated by applying drops of GFP-labeled *Pst* DC3000 at OD_{600} 0.1 with 0.025% silwet L-77 on top of stigmas of newly opened flowers. The beakers with flowers were then placed separately into covered 400 ml beakers to maintain high humidity, and returned back to controlled growth conditions.

3.5.4 Confocal microscopy of GFP-labeld *Pst* DC3000

Bacterial growth on stigmas of *Arabidopsis* wild type Col-0 and *tps21* mutants at 2 days post-inoculation was analyzed by confocal laser scanning microscopy using a LSM 510 microscope (Carl Zeiss, Jena, Germany). Cut stigmas were mounted in water on a micro slide (Corning Glass Works) with a microscope cover glass (#1.5, Fisher Scientific). Scans were performed by a Zeiss LSM 510 water-immersion C-Apochromat 40× / 1.2 W corr objective

lens. The GFP signal was obtained using the 488-nm argon laser line set at 11% and a BP505-550 emission filter. Autofluorescence of chlorophyll was visualized using an LP560 emission filter. Confocal images with a resolution of 512 × 512 pixels were obtained from at least three independent plants of each line. Scanned images of z-stack sections of each sample were made at 6.0 µM, 11.1 µM, 16.1 µM and 21.1 µM. Images of all scanned z-stack images of a stigma were converted into a merged single image by the Zeiss LSM Image Browser 3.2.0.

3.5.5 cDNA cloning of *TPS21*, vector construction and plant transformation

The coding sequence of *TPS21* (At5g23960, Col-0) was amplified by PCR using the forward primer P1 and the reverse primer P2 introducing the restriction sites *Bam*HI (5') and *Sac*I (3'), respectively (Table 3.1). The PCR fragment was cloned into *Bam*HI/*Sac*I restriction sites of the binary vector pBIN420 under control of a CaMV 35S promoter. The resulting vector construct was transformed into *Agrobacterium tumefaciens* GV1301 and used to transform *A. thaliana* ecotype Col-0 by vacuum infiltration (Bechtold et al., 1993). Transformed seeds carrying the pBIN420 derived fragment were selected on agar plates for kanamycin resistance and confirmed by PCR. Selfing and selection was carried out for three more generations to obtain nonsegregating homozygous plants.

3.5.6 Seed production measurements

The rosette leaves of *A. thaliana* wild type Col-0 and *TPS21* expressing transgenic plants were inoculated with *Pst* DC3000 as described and placed in separate containers with 100% relative humidity for 7 days, prior to being returned to 55% relative humidity. Two weeks before the final stage of seed ripening, shoots of each plant were placed into a paper bag and plants was kept under controlled growth conditions without any further watering. To determine seed production from flowers of wild-type and *tps21* mutants, flowers were inoculated with *Pst* DC3000 and plants were placed in separate containers with 100% relative humidity for 14 days before seeds were harvested into paper bags and dried under room temperature. In the experiment with *tps21* mutants, it was difficult to clearly separate the seeds from other plant debris. Therefore, one hundred seeds of each plant were randomly

chosen and weighed. The total amount of seeds from each individual plant was collected from the bag and weighed to an accuracy of 0.01 g.

3.5.7 *In vivo* quantification of bacterial growth

Bacterial populations in leaves were analyzed by harvesting four leaf discs per plant at 1, 2 and 3 days post-inoculation, homogenizing the discs in 200 µl of 10 mM $MgCl_2$ solution and plating appropriate serial dilutions on King's B medium plates with antibiotics for 2 days at 28 °C (Katagiri *et al.*, 2002). Bacterial growth was calculated on the third day of tissue harvest by determining number of colony forming units (CFU) (Katagiri et al., 2002). Experiments were repeated at least three times with similar results. Each data point represents the average CFU from three biological replicates ± standard error of the mean. Data were analyzed by the statistical methods described below.

3.5.8 Volatile Collection and GC-MS Analysis

Volatile collection from intact rosette plants with their root balls wrapped in aluminum foil was performed in 3 L bell jars by using the closed-loop stripping method (Donath and Boland, 1995) under controlled growth conditions as described previously (Chen et al., 2003). Volatiles were collected in the light for 8 h on 1.5 mg activated charcoal and eluted with 40 µL of CH_2Cl_2 containing 80 ng of 1-bromodecane as an internal standard.

The eluted samples (1 µL) were injected in a splitless mode into a GC-2010 gas chromatograph (Shimadzu, Japan) coupled with a QP2010S mass spectrometer (Shimadzu). Sample compounds were separated on an Rxi-XLB column (Restek, Bellefonte, PA, USA) of 30 m × 0.25 mm i.d. × 0.25 µm film thickness. Helium was used as the carrier gas (1.4 mL min^{-1} flow rate), and a temperature gradient of 5 °C/min from 40 °C (hold for 2 min) to 220 ° C was applied.

The identities of volatile compounds were confirmed by comparison of their retention times and mass spectra with those of authentic standards and with mass spectra in the National Institute of Standards and Technology and Wiley libraries. For absolute quantification of (*E*)-β-caryophyllene, the primary ion peaks of (*E*)-β-caryophyllene were integrated (total ion method) and the amounts were calculated in relation to the response of 1-bromodecane. Response curves for the quantified (*E*)-β-caryophyllene relative to the

internal standard were generated by injecting a mixture of equal amounts of authentic standards and internal standard.

3.5.9 Quantitative RT-PCR analysis of gene expression

The expression levels of (*E*)-β-caryophyllene synthase (*TPS21*, At5g23960), *PR1* (At2g14610) and *LOX2* (At3g45140) in wild type Col-0 and transgenic plant leaves were measured by quantitative RT-PCR with Brilliant SYBR Green PCR Master Mix (Stratagene, USA). Total RNAs from treated and untreated leaves of wild type Col-0 and transgenic plants were extracted by the RNeasy Plant Mini Kit (Qiagen, USA), and DNA contamination was eliminated by DNase treatment for 15 min at room temperature (Qiagen, USA). The DNA-free total RNA was reverse transcribed into cDNA as previously described (Chen et al., 2003). A 1 mL aliquot of 50 times diluted cDNA was analyzed with 25 mL SYBR Green Master Mix (Stratagene, USA) in triplicate using primers P3 and P4, P5 and P6, and P7 and P8 for genes *TPS21*, *PR1* and *LOX2*, respectively (Table 3.1). Fold change differences of *TPS21*, *PR1* and *LOX2* expression were calculated according to the efficiency corrected by Pfaffl's method with *APT1* (adenine phosphoribosyltransferase1, At1g27450) as the reference gene (Pfaffl, 2001). Each data point represents the mean of three independent biological replicates and three technical replicates.

Table 3.1 Primer sequences

Primer	Sequence (5' to 3')
P1	ATAGGATCCATGGGGAGTGAAGTCAACCG
P2	ATAGAGCTCTCAAATGGGTATAGTTTCAATGT
P3	AGTAACATACACAAGGCATAGGATAACAG
P4	TCTCATCAGGAACAACGGGAAGC
P5	TTCACAACCAGGCACGAGGAG
P6	CCAGACAAGTCACCGCTACCC
P7	GTTTCTGGAGGGCATAACTTGGTC
P8	TGGTATTGGTTCTGAATCTTGATGGC

3.5.10 Leaf cell death staining

Leaf cell death symptoms after infection were observed by applying the lactophenol-trypan blue staining method on day 3 post-inoculation as previously described. (Koch and Slusarenko, 1990).

3.5.11 *In vitro* antibacterial assay

Antibacterial activity of (*E*)-β-caryophyllene against *Pst* DC3000 was tested by a modified broth dilution method according to Kubo et al. (1992) and Muroi and Kubo (1993). *Pst* DC3000 (pLARR3) (kindly provided by Christiane Gatz, University of Goettingen, Germany) was cultivated in King's B liquid medium with 25 µg mL^{-1} rifampicin and 5 µg mL^{-1} tetracycline at 28 °C until an OD_{600} of 0.8. Cultures were supplemented with (*E*)-β-caryophyllene at concentrations of $1x10^{-5}$ mmol L^{-1} and $2x10^{-5}$ mmol L^{-1} and incubated at 220 rpm for another 24 h. Then, 100 µL aliquots were sampled and diluted by a 10-fold dilution series. One hundred microliter of each dilution were incubated on King's B solid medium at 28 °C for 48 h and the colony forming units of each plate counted. (*E*)-β-Caryophyllene concentrations were tested in three replicates with three aliquots sampled per replicate. Serial dilutions were performed with three technical replicates..

3.5.12 Statistical analysis

Statistical significance was determined according to ANOVA with Tukey or LSD post-hoc tests using SAS9.1 (SAS Institute Inc., Cary, NC, USA).

3.5.13 Acknowledgements

We are thankful to Katrin Luck for technical assistance. We are grateful to Christiane Gatz, University of Goettingen, Germany and Boris A. Vinatzer (Virginia Tech, Blacksburg, VA) for providing *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst* DC3000) and the GFP-labeled *Pseudomonas syringae* pv. *tomato* DC3000 strain.

4. Chapter III. (*E*)-β-caryophyllene-induced molecular and physiological responses in *Arabidopsis thaliana* suggest a role in resistance to oxidative stress

(In preparation)

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Chapter overview and authors' contributions

This manuscript described the effects of (*E*)-β-caryophyllene on transcriptome and redox systems in *Arabidopsis thaliana* leaves. Under the supervision of Dr. Dorothea Tholl and Prof. Jonathan Gershenzon, I performed all experiments and analyses. With help from Jonathan Gershenzon, Dorothea Tholl and I planned and designed all experiments. With help from Jonathan Gershenzon, I wrote the manuscript.

4.1 Abstract

Certain volatile hemiterpenes (C5) and monoterpenes (C10) have been shown to protect plants against high light, high temperature and oxidative stress. The potential of a common sesquiterpene (C15), (*E*)-β-caryophyllene, to protect plants in the same fashion was tested by using transgenic *Arabidopsis thaliana* over-expressing (*E*)-β-caryophyllene synthase. Physiological analyses showed reduced hydrogen peroxide contents in (*E*)-β-caryophyllene-producing plants suggesting protection against oxidative stress. However the content of the important antioxidant ascorbate and total chlorophyll were not changed. Moreover, expression of genes encoding the ROS-scavenging enzymes, ascorbate peroxidase and catalase, were not significantly altered. To explore what other mechanisms might be responsible for (*E*)-β-caryophyllene-mediated oxidative stress protection, we examined transcriptome changes in plants with constitutive (*E*)-β-caryophyllene production. Microarray gene expression profiling suggested that over 154 transcrpits were significantly down-regulated upon induction of (*E*)-β-caryophyllene emission, among which was a high representation of transcription factors and other regulatory proteins. One significantly down-regulated gene is an F-box family transcription factor.

4.2 Introduction

Photosynthetic organisms may all experience photooxidative stress under certain environmental conditions. For example, under high light regimes, photooxidative stress results in the generation of reactive oxygen species (ROS) (Foyer et al., 1994). ROS production is caused from perturbed photosynthesis processes in which light harvesting and light energy consumption are out of balance. Excess light energy induces the generation of reactive oxygen species (ROS), such as singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\cdot}$) (Niyogi, 1999). ROS are lethal at high concentration by damaging cellular proteins, nucleic acids, membrane lipids and other molecules, whereas sublethal levels of ROS may serve as signaling molecules in plant development and defense responses (Apel and Hirt, 2004; Foyer and Noctor, 2005).

Excess ROS are detoxified by a variety of antioxidant mechanisms, including both enzymatic and non-enzymatic antioxidation processes (Apel and Hirt, 2004). Water soluble antioxidants, including ascorbate (Vitamin C) and gluthathione (GSH) serve as direct ROS reductants or cofactors for peroxidases (Niyogi, 1999; Apel and Hirt, 2004). Lipid soluble antioxidants such as carotenoids and tocopherols (Vitamin E) (Fryer, 1992; Munne-Bosch and Alegre, 2002) are crucial for cellular membrane protection because of their direct quenching or scavenging of ROS on membranes (Niyogi, 1999). Interestingly, most lipid antioxidants such as zeaxanthin (a xanthophyll-type carotenoid), tocopherols (Vitamin E), and carnosic acid (diterpene) (Munne-Bosch and Alegre, 2001) belong to the terpene family, but are non-volatile (DellaPenna and Pogson, 2006; Vickers et al., 2009).

In addition to nonvolatile terpenes, some volatile terpenes have also been shown to protect photosynthesis under various abiotic stresses including oxidative stress (Loreto et al., 2001; Behnke et al., 2010a). Fumigation with the C5 terpenoid, isoprene, leads to less cellular and photosynthetic damage and less ROS accumulation in ozone (an oxidative air pollutant, O_3)-treated plants (Loreto et al., 2001). Fosmidomycin, an inhibitor of the DXR enzyme in the MEP pathway, inhibits isoprene emissions and makes plants more sensitive to ozone (Loreto and Velikova, 2001). Similarly, the antioxidant activities of monoterpenes (C10) have been demonstrated by inhibition and fumigation experiments (Loreto et al., 2004; Loreto and Fares, 2007). Taking advantage of transgenic plants emitting volatile terpenes, transgenic tobacco plants (*Nicotiana tabacum*) emitting isoprene constitutively by over-

expressing an isoprene synthase gene were shown to be more resistant to ozone treatment than wild-type tobacco (Vickers et al., 2009).

Despite many studies of the antioxidant properties of isoprene (C5) and monoterpenes (C10), the properties of sesquiterpenes (C15) have not yet been investigated in this regard. Sesquiterpenes have reactive conjugated or terminal double bonds which may mediate ROS scavenging (Vickers et al., 2009). However, whether plants benefit from this antioxidant activity, and how sesquiterpenes influence metabolism in general are still open questions. Here, we report an investigation of transgenic *Arabidopsis thaliana* that was engineered to over-express the widespread sesquiterpene, (*E*)-β-caryophyllene. Large-scale transcriptome analyses demonstrated that 154 genes were significantly down-regulated in leaves of the transgenic plant compared to the wild-type control. One gene (At3g26000) that is most significantly down-regulated belongs to the F-box protein family. We also showed that hydrogen peroxide (H_2O_2) content is significantly lower in transgenic lines under normal growth condition, while ascorbate and chlorophyll contents are similar in both transgenic and wild-type plants. The transcript level of the ROS scavenging enzymes, ascorbate peroxidase (APX1) and catalase (CAT2) was unchanged in transgenic lines under normal and oxidative stress. Taken together, our work suggests that the sesquiterpene (*E*)-β-caryophyllene may be directly involved in the ROS scavenging network in plants and provide resistance to photooxidative stress owing to its high chemical reactivity.

4.3 Results

4.3.1 Over-expression of (*E*)-β-caryophyllene synthase TPS21 leads to down-regulation of many genes

(*E*)-β-Caryophyllene over-expressing lines were generated by transforming Col-0 flowers via *Agrobacterium tumefaciens* GV3101with a construct of *TPS21* full-length cDNA in pBin420 vector under the control of the CAMV35S promoter. Compared to wild type Col-0 leaves, transgenic plants showed constitutive emissions of (*E*)-β-caryophyllene. The (*E*)-β-caryophyllene emissions of two over-expression lines (*TPS21*-1 and 2) were 4.7 ng gFW^{-1} h^{-1} and 2.4 ng gFW^{-1} h^{-1}, respectively and the accumulation of (*E*)-β-caryophyllene in the plant tissue was negligible (see Chapter II for more details). Southern blot analysis indicated that the transgenic lines *TPS21*-1 and 2 contained more than one transgene insersion (data not shown). All transgenic lines analyzed in this work were nonsegregating T4 homozygous plants. The transgenic and wild-type plants have identical growth phenotypes under the growth conditions used.

Microarray analyses were done with (*E*)-β-caryophyllene over-expression plants and the transcriptome profiles were compared to wild type Col-0 plants. The analysis was performed with 4-week old rosette leaves and repeated with three biological replicates, each of which was a sample pool of four independent plants of each line. Transcript signals from Affymetrix GeneChips were reproducibly detected and were analyzed statistically by a set of gene-specific *t* tests (Tusher et al., 2001). With a significance threshold of 0.7, to give a low false discovery rate (Tusher et al., 2001), there were 154 significantly down-regulated genes in (*E*)-β-caryophyllene over-expressing lines (green spots in Fig. 4.1A) compared to wild-type plants, but no significant up-regulated genes. The 15 most significantly down-regulated genes (Table 4.1) are listed with putative function, expression time and location in plants in Table 4.2. After the threshold was changed to 0.18 to include more up and down-regulated genes, 1773 differentially up-regulated genes and 3106 differentially down-regulated genes were detected (Fig. 4.1B) although the false discovery rate (FDR) could be as high as 61%. This set included the over-expressed (*E*)-β-caryophyllene synthase gene, with a score of 3.8.

Figure 4.1. Transcriptome profiles of *A. thaliana* transgenic lines which over-express (*E*)-β-caryophyllene synthase (TPS21) analyzed by the methods of (Tusher et al., 2001). (**A**) Significantly down-regulated genes (154 genes) are shown in the graph as green dots in using a threshold Delta=0.7. There were no significantly up-regulated genes according to this threshold. (**B**) With the threshold Delta=0.18, 1773 differentially up-regulated genes and 3106 differentially down-regulated genes were identified.

Table 4.1. Significantly down-regulated genes in (*E*)-β-caryophyllene synthase over-expressing transgenic lines according to the methods of the significant analysis of microarrays (Tusher et al., 2001). Down-regulated genes are shown with negative number; the lowest score represent the most significant down-regulation. False discovery rate are shown as q values (q-val).

	Gene Accession	Expressed Protein	Score(d)	q-val(%)
Down-regulated (significant, according to SAM)				
1	At3g26000	F-box family protein	**-10.9**	0
2	At1g76110	DNA-binding domain-containing protein	**-7.66**	7.78
3	At3g47460	Arabidopsis thaliana structural maintenance of chromosome 2	**-7.13**	7.78
4	At1g30200	F-box family protein	**-6.68**	7.78
5	At3g57040	Transcription regulator (Response reactor 4)	**-6.48**	7.78
6	At3g59940	F-box family protein	**-6.30**	7.78
7	At1g47270	Transcription factor; Phosphoric diester hydrolase	**-6.25**	7.78
8	At2g43820	UDP-glucosyltransferase	**-6.24**	7.78
9	At5g55970	Zinc finger family protein	**-6.06**	7.78
10	At5g18600	Glutaredoxin family protein	**-6.06**	7.78
11	At3g27350	Unknown protein	**-6.01**	7.78
12	At2g29670	Binding protein	**-5.85**	13.2
13	At5g48490	Protease inhibitor family protein	**-5.81**	13.2
14	At5g49730(At5g49740)	Ferric reduction oxidase	**-5.59**	13.2
15	At5g59030	Copper ion transporter	**-5.57**	13.2

Table 4.2. The function and expression pattern of significantly down-regulated genes in (*E*)-β-caryophyllene synthase over-expressing transgenic lines

Gene	Protein	Functions (known)	Expression time, involved in	Location
Down-regulated				
At3g26000	F-box family protein		15 growth stages	24 plant structures
At1g76110	DNA-binding domain-containing protein	Transcription factor activity	14 growth stages	Intracellular, nucleus, 20 plant structures
At3g47460	Arabidopsis thaliana structural maintenance of chromosome 2	Transporter activity, chromosom organization	10 growth stages	Chromosome; 19 plant structures
At1g30200	F-box family protein		14 growth stages	23 plant structures
At3g57040	Transcription regulator (Response reactor 4)	Transcription regulator activity, circadian rhythm, two-component signal transduction system (phosphorelay), response to cytokinin stimulus, cytokinin mediated signaling pathway	13 growth stages	Nucleus; 24 plant structures
At3g59940	F-box family protein		13 growth stages	23 plant structures
At1g47270	Transcription factor; Phosphoric diester hydrolase	Phosphoric diester hydrolase activity, transcription factor activity, regulation of transcription	15 growth stages	24 plant structures
At2g43820	UDP-glucosyltransferase	UDP-glycosyltransferase activity, transferase activity, transferring hexosyl groups, para-aminobenzoic acid metabolic process	7 growth stages	9 plant structures
At5g55970	Zinc finger family protein	Protein binding, zinc ion binding	5 growth stages	Chloroplast, 15 plant structures
At5g18600	Glutaredoxin family protein	Electron carrier activity, protein disulfide oxidoreductase activity, arsenate reductase (glutaredoxin) activity, cell redox homeostasis	10 growth stages	18 plant structures
At3g27350	Unknown protein		15 growth stages	Plasma membrane, 24 plant structures
At2g29670	Binding protein	Binding	15 growth stages	Chloroplast, 23 plant structures
At5g48490	Protease inhibitor family protein	Lipid binding and transport	13 growth stages	Endomembrane system, 20 plant structures
At5g49730 At5g49740	Ferric reduction oxidase	Oxidoreductase activity, ferric-chelate reductase activity, response to light stimulus and cell differentiation		Plasma membrane, 3 plant structures: green aerial tissues (shoot, flower and cotyledon)
At5g59030	Copper ion transporter	Copper ion transport, pollen sperm cell differentiation, root development	15 growth stages	Membrane, 23 plant structures

To learn more about the transcriptome affected by over-expressing (*E*)-β-caryophyllene synthase, we sorted the significantly down-regulated 154 genes according to the TAIR Gene Ontology (GO) categorization system (www.arabidopsis.org). Genes were sorted by three criteria: cellular location, biological process and molecular function (Fig. 4.2). In the category of cellular location, transcripts within chloroplasts, nucleus, membranes and intracellular components were highly represented in transgenic plants (Fig. 4.2A). In the "biological processes" category, transcripts associated with transcription, protein metabolism and abiotic/biotic stress were more frequently present than transcripts associated with other biological processes (Fig. 4.2B). In the category of "molecular functions", the transcription factors had the highest representation, and the relative abundances of protein binding and DNA/RNA binding factors were also high (Fig. 4.2C).

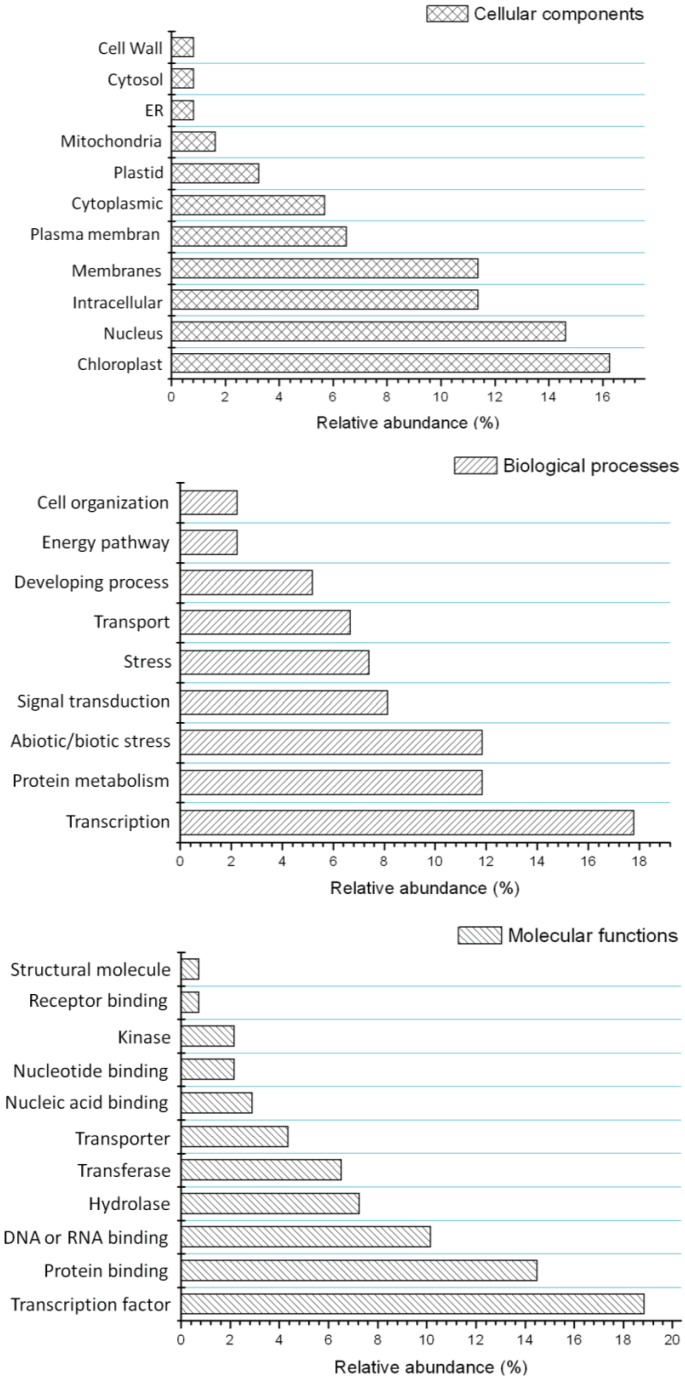

Figure 4.2. Distribution of transcripts significantly down-regulated in (*E*)-β-caryophyllene synthase over-expression lines according to the criteria of cellular location, biological processes, and molecular functions. Genes are sorted by the TAIR Gene Ontology (GO) categorization system.

In (*E*)-β-caryophyllene synthase over-expressing transgenic plants, 26 different transcription factors were significantly down-regulated (Table 4.3). Most of these are members of the zinc finger family (23.1% of the 26 significantly down-regulated transcription factors). Transcription factors in the auxin response and the MYB families both account for 19.2% of the down-regulated transcription factors (Fig. 4.3).

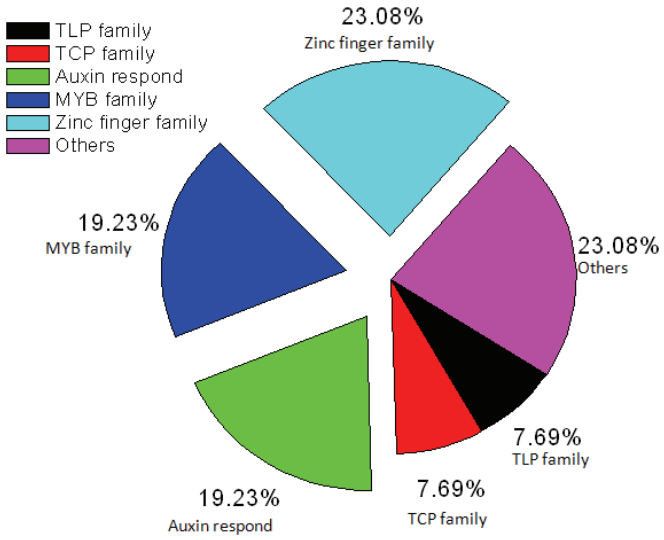

Figure 4.3. Distributions of significantly down-regulated transcription factors in (*E*)-β-caryophyllene-emitting plants among major families of transcription factors.

Table 4.3. Significantly down-regulated transcription factors in (E)-β-caryophyllene synthase over-expressing transgenic lines

	Gene Accession	Name	Transcription factor family and known functions
1	AT1G47270	ATTLP6 (Tubby like protein 11)	TLP family
2	AT5G18680	ATTLP11 (Tubby like protein 11)	TLP family
3	AT3G47620	ATTCP14	TCP family (regulates seed germination)
4	AT5G60970	TCP5	TCP family (heterochronic control of leaf differentiation)
5	AT1G04250	Auxin resistant 3	Aux/IAA protein family (response to auxin stimulus)
6	AT1G52830	IAA6	Aux/IAA protein family (response to auxin stimulus)
7	AT4G14550	IAA14	Aux/IAA protein family
8	AT4G14560	IAA1 (Auxin resistant 5)	Aux/IAA protein family (response to auxin stimulus)
9	AT4G32280	IAA29	Aux/IAA protein family (response to auxin stimulus, red/far red light)
10	AT1G22640	MYB domain protein 3	MYB family (represses phenylpropanoid biosynthesis gene expression)
11	AT1G68670	F24J5.9	MYB family
12	AT1G71030	ATMYBL2	MYB family (putative)
13	AT5G08520	F8L15.2	MYB family
14	AT5G52260	MYB domain protein 19	MYB family
15	AT1G68190	T22E19.18	Zinc finger family (B-box type)
16	AT2G25900	ATTZF1	Zinc finger family (putative Cys3His protein)
17	AT5G44260	K9L2.1	Zinc finger family (CCCH type)
18	AT5G54630	MRB17.13	Zinc finger family (related)
19	AT5G57660	ATCOL5	Zinc finger family (B-box type)
20	AT5G60850	MAE1.2	Zinc finger family
21	AT5G02840	LHY/CCA1-LIKE 1	CCA1 and LHY function (synergistically in regulating circadian rhythms)
22	AT5G28770	ATBZIP63 (basic leucine zipper 63)	bZIP protein (BZO2H3, regulation of transcription, DNA-dependent)
23	AT5G61590	K11J9.4	ERF/AP2 transcription factor family (response to water deprivation)
24	AT1G76110	T23E18.4	High mobility group (HMG1/2) family protein
25	AT2G01930	Basic pentacysteine1 (BPC1)	Regulator of homeotic SEEDSTICK (STK), controls ovule identity
26	AT2G18300	bHLH	Basic helix-loop-helix family protein (response to cytokinin stimulus)

4.3.2 (*E*)-β-Caryophyllene production reduces hydrogen peroxide but does not affect ascorbate or chlorophyll content

Since (*E*)-β-caryophyllene was suspected of being able to react readily with ROS *in vivo*, we compared the oxidative status of (*E*)-β-caryophyllene synthase-over-expressing lines with wild-type controls by measuring their hydrogen peroxide levels. H_2O_2 is a less reactive ROS compared to singlet oxygen ($^1O_2$), superoxide radicals ($O_2^{\cdot-}$) and hydroxyl radicals ($OH^{\cdot}$). Because it is a non-charged molecule, it can diffuse throughout the cell and thus damage cellular components, signal stresses or even recruit defense responses in a wide variety of cellular locations (Ledford and Niyogi, 2005). The quantification of hydrogen peroxide (H_2O_2) in rosette leaves was performed by using the potassium titanium oxalate method. A significant decrease of H_2O_2 was observed in the two over-expression lines (*TPS21*-1, 2) with constitutive (*E*)-β-caryophyllene emission compared to the wild-type control (Fig. 4.4). The H_2O_2 level in *TPS21*-1 and *TPS21*-2 was 2.9 and 1.8-fold lower than those of wild type, respectively. Thus, the cellular redox environments in transgenic lines appear to be less oxidized than in plants without (*E*)-β-caryophyllene.

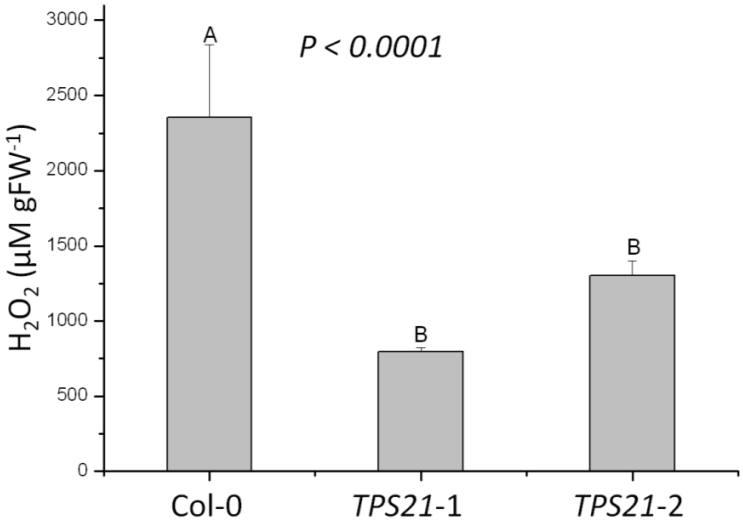

Figure 4.4. Hydrogen peroxide (H_2O_2) contents in leaves of *A. thaliana* Col-0 and transgenic lines over-expressing (*E*)-β-caryophyllene synthase (*TPS21*-1, -2). Results represent mean values ± SE (n = 4). Letters represent significant differences (P < 0.0001 according to Tukey, ANOVA).

In addition to H_2O_2, we compared the levels of ascorbate between the (*E*)-β-caryophyllene-emitting lines and wild-type plants. Ascorbate (vitamin C) is an important photoprotective antioxidant in quenching $^1O_2$, $O_2^{\cdot -}$, $OH^{\cdot}$ directly, serving as a cofactor of ascorbate peroxidase for scavenging H_2O_2, and participating in the regeneration of tocopherols (Asada, 1999; Niyogi, 1999). Plant tissues can accumulate ascorbate in up to millimolar concentrations and the total amount of ascorbate represents one of the most important pools of reduced antioxidants in cells (Noctor and Foyer, 1998). In our measurements, both transgenic and wild-type plants had micromolar concentrations of ascorbate in their leaves without any significant difference between them (Fig. 4.5A). Thus the pool of this antioxidant is not affected by over-expressed (*E*)-β-caryophyllene.

Since chlorophyll is protected from oxidative stress by carotenoids, a type of non-volatile terpenes (Anderson and Robertson, 1960), we investigated if the volatile terpene (*E*)-β-caryophyllene could also act in this manner. Total chlorophyll contents in over-expression lines and wild-type controls were measured according to the DMF method, but the total chlorophyll contents of transgenic lines were not significantly different from those of wild type under the growth conditions used (Fig. 4.5B).

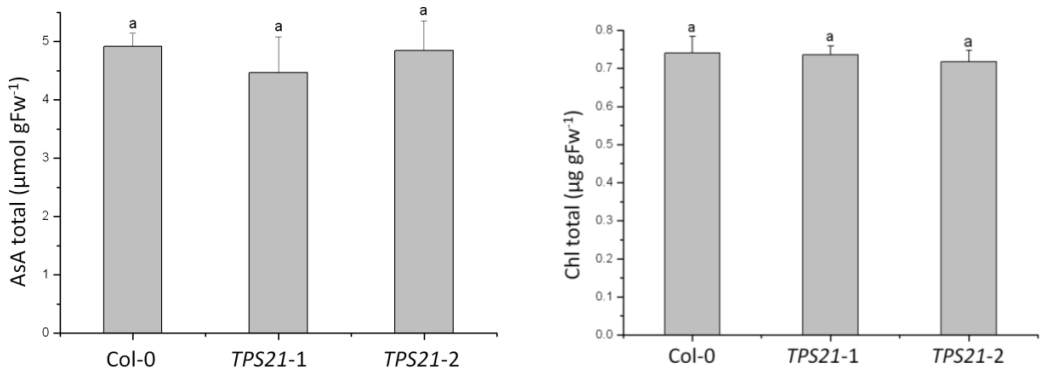

Figure 4.5. A) Ascorbate (AsA) contents of leaves from wild-type *A. thaliana* and transgenic plants with (*E*)-β-caryophyllene emission. Results represent mean values ± SE (n = 4). Letters represent significant differences ($P<0.0001$ according to Tukey, ANOVA). **B**) Chlorophyll (Chl) content. Results represent mean values ± SE (n = 12). Letters represent insignificant differences according to ANOVA.

4.3.3 (*E*)-β-Caryophyllene production has no effect on antioxidative scavenging enzymes

ROS scavenging relies on both enzymatic and non-enzymatic process. Two of the important enzymes that scavenge H_2O_2 in plants are ascorbate peroxidase (APX) and catalase (CAT). APX and CAT also play key roles in signaling in adaptation to oxidative stress. Detoxification of H_2O_2 to H_2O by APX occurs by oxidation of ascorbate (ASA) to monodehydroascorbate, while CAT converts H_2O_2 into H_2O and O_2 (Apel and Hirt, 2004). In *A. thaliana*, the cytosolic ascorbate peroxidases are APX1 (At1g07890) and APX2 (At3g09640), with APX1 playing a key role in protecting the chloroplastic H_2O_2-scavenging system (Davletova et al., 2005). The catalase (CAT) multigene family is composed of CAT1 (At1g20630), CAT2 (At4g35090) and CAT3 (At1g20620). CAT2 is the most predominant highly expressed catalase in peroxisome of leaves (Vandenabeele et al., 2004). In order to evaluate the influence of (*E*)-β-caryophyllene on the ROS scavenging system, the transcript levels of ascorbate peroxidase (*APX1*, At1g07890) and catalase (*CAT2*, At4g35090) were determined by quantitative RT-PCR. The relative transcript abundance of both genes was decreased in both transgenic lines (*TPS21*-1 and 2), although this was not statistically significant (Fig. 4.6).

The expression of *APX1* and *CAT2* were also compared using the microarray data. For *CAT2*, the results of microarray analysis and quantitative RT-PCR were consistent, both showing a weak, statistically insignificant decline in (*E*)-β-caryophyllene over-expressing lines (Table 4.4). *APX1* showed weak down-regulation in the quantitative RT-PCR analysis, but up-regulation from microarray results (Table 4.4). However, neither of these differences is statistically significant and so the expression of these genes can be concluded not to differ between transgenic and wild-type plants.

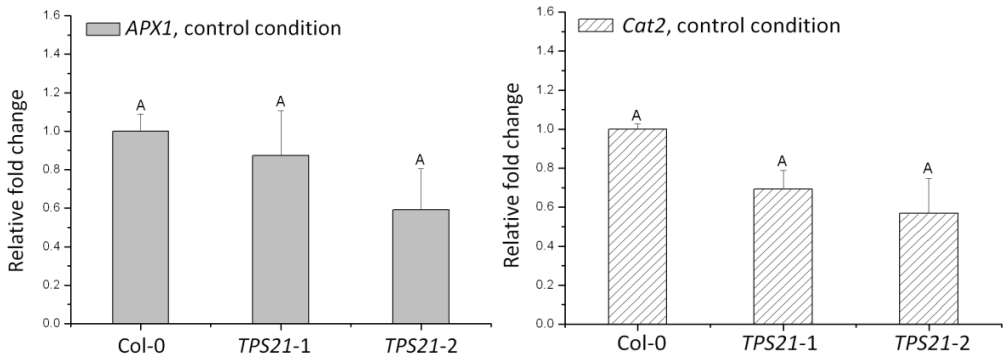

Figure 4.6. Transcript levels of H_2O_2-scavenging-enzymes ascorbate peroxidase (APX1) and catalase (CAT2) from wild-type *A. thaliana* and transgenic lines with (*E*)-β-caryophyllene production as determined by qRT-PCR. Results represent mean values ± SE (n = 3). Letters represent insignificant differences according to ANOVA.

Table 4.4. Microarray expression scores of *APX1* and *CAT2* in (*E*)-β-caryophyllene over-expressing transgenic lines

Gene Accession	Expressed Protein	Score (d)	q-val (%)
At1g07890	APX1 (ascorbate peroxidase)	**2.18**	50.4
At4g3509	CAT2 (catalase)	**-1.71**	52.2

4.3.4 Treatment with Paraquat to induce oxidative stress has no effect on expression of genes encoding antioxidative scavenging enzymes

To determine if the expression of *APX1* and *CAT2* genes would be altered by oxidative stress, we treated plants with Paraquat (methyl viologen, 1,1'-dimethyl-4,4'-bipyridinium chloride). This non-selective toxic herbicide is photoreduced rapidly by photosystem I under light, and then transfers electrons to oxygen to form the superoxide anion ($O_2^{\cdot -}$). The production of other toxic ROS ($OH^{\cdot}$ and H_2O_2) leads to lethal oxidative damage (Bowler et al., 1992). We compared Paraquat-induced oxidative stress on wild type and (*E*)-β-caryophyllene over-producing plants, treating both types of plants under high light intensity. However, quantitative RT-PCR revealed again that there was no significant difference in gene expression between transgenic and wild-type plants, although most transcript levels of antioxidant enzymes remained lower than wild type 3 and 24 h after Paraquat treatment (Fig. 4.7). Taken together, our results indicate that scavenging enzyme systems are not regulated significantly by (*E*)-β-caryophyllene with or without oxidative stress.

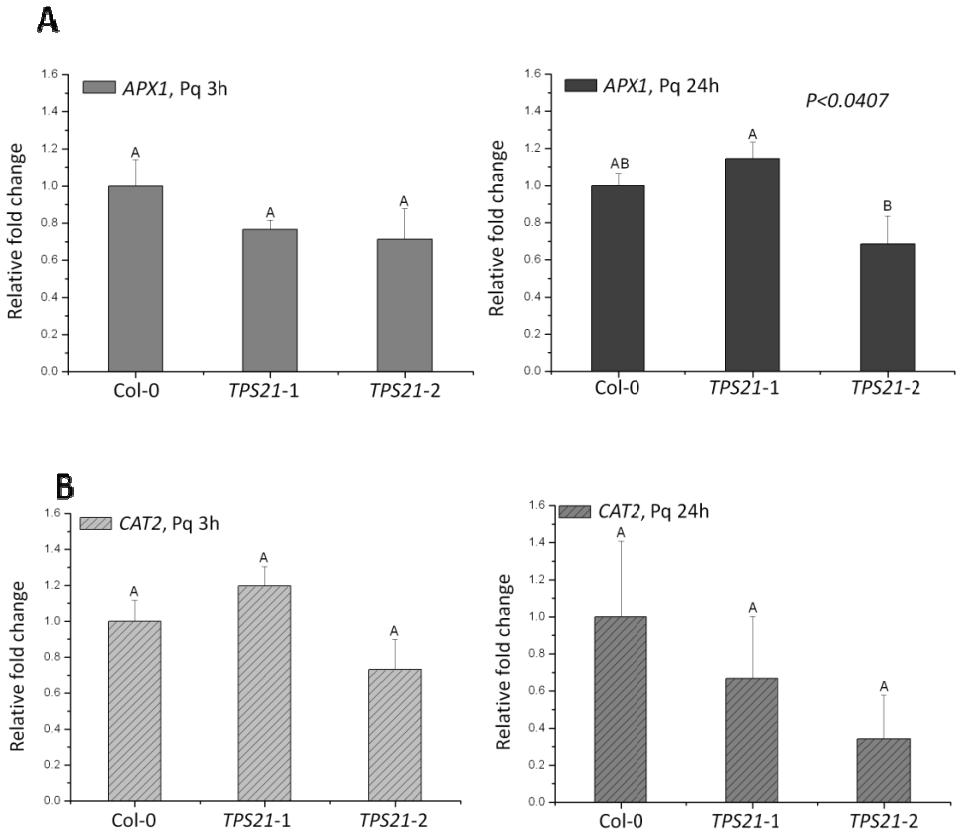

Figure 4.7. Comparisons of the expression levels of APX1 (A) and CAT2 (B) from wild-type *A. thaliana* and transgenic plants with (*E*)-β-caryophyllene emission. Left panels are for leaves harvested 3 h after treatment and right panels for leaves harvested 24 h after treatment. Results represent mean values ± SE (n = 3). Letters represent significant differences (P < 0.0407 according to Tukey, ANOVA).

4.4 Discussion

4.4.1 (*E*)-β-Caryophyllene decreases expression of many regulatory genes, one of which encodes an F-box protein

(*E*)-β-Caryophyllene is a widespread plant sesquiterpene that readily reacts with reactive oxygen species (ROS). To determine if this substrate could protect plants against oxidative stress, we over-expressed (*E*)-β-caryophyllene synthase in *A. thaliana* leading to constitutive production (and emission) from leaves. In this species, the compound is normally synthesized only in flowers. Transcriptional changes occurring in over-expressing lines can give valuable information on the effects of (*E*)-β-caryophyllene. This sesquiterpene caused down-regulation of 154 genes, among which are many involved in responses to abiotic or

biotic stress, protein metabolism and transcription (Fig. 4.2). It is interesting to compare this list with one of *A. thaliana* genes found to be up- or down-regulated after fumigation with monoterpenes (Godard et al., 2008). Monoterpenes applied to *A. thaliana* leaves were found to induce more up-regulated transcripts than down-regulated ones (Godard et al., 2008) in contrast to our work which showed that (*E*)-β-caryophyllene only triggered significant down-regulation (Fig. 4.1B). Sesquiterpenes might be expected to cause different responses than monoterpenes, but another important difference is that our study involved ectopic expression of (*E*)-β-caryophyllene within the plant while (Godard et al., 2008) fumigated plants externally with monoterpenes.

According to our microarray analysis, the most significantly down-regulated transcript (At3g26000) in the transgenic plant encodes a protein that belongs to the F-box family (Table 4.1). F-box proteins are major components of the E3 ubiquitin ligase complex termed SCF (Skp1/Cdc53/F-box protein) and serve as special biding factors to recruit protein substrates for ubiquitination and subsequent degradation (Elledge and Harper, 1998). F-box proteins are named after the first identified member of the family, "human cyclin F" (Xiao and Jang, 2000). In plants, ubiquitin-mediated proteolysis is of great importance in turnover of proteins, including various regulatory proteins. F-box proteins are found to be involved in essential signal transduction pathways in plants. For instance, the F-box protein TIR1 (transport inhibitor response 1) is an auxin receptor in *Arabidopsis thaliana* (Dharmasiri et al., 2005; Kepinski and Leyser, 2005). In addition, F-box proteins called SLEEPY1 (Dill et al., 2004) and SNEEZY (Strader et al., 2004) regulate gibberellin induced degradation; F-box proteins named EBF1 and EBF2 (ethylene insensitive 3 (EIN3)- binding F-box protein 1 and 2) repress ethylene action and promote growth by degrading EIN3 (Gagne et al., 2004); and the F-box protein involved in the SCF^{COI1} ubiquitin-ligase complex is essential for jasmonate response to pathogens and insects in *Arabidopsis thaliana* (Xu et al., 2002). Moreover, F-box proteins play important roles in controlling lateral root, leaf and floral development, and self-incompatibility as well as in regulating light signaling, circadian rhythms, and stress responses (Xiao and Jang, 2000; Yu et al., 2007). Our microarray data indicate that the F-box protein (At3g26000) might have a close relation with (*E*)-β-caryophyllene mediated responding process in *Arabidopsis thaliana*, although we may not exclude that the (*E*)-β-caryophyllene-affected ROS may have direct impact on transcriptional changes in transgenic

plants because of the signaling activity of ROS (Mittler et al., 2004) (see discussion below). Here we designate this novel F-box protein as Caryophyllene-Suppressed F-box protein (**CarSF**). Our findings warrant future work to identify whether regulatory responses to volatile sesquiterpenes like (*E*)-β-caryophyllene are mediated through the ubiquitin-mediated proteolytic system in *A. thaliana*, and provide new molecular targets for dissection of the function of (*E*)-β-caryophyllene in plant-abiotic stress interactions.

4.4.2 (*E*)-β-caryophyllene production reduces ROS in transgenic plants without affecting anti-oxidant pools

The formation of ROS in plants in an unavoidable consequence of processes associated with photosynthesis. For example, direct electron transfer at photosystem I towards O_2 generates the superoxide ($O_2^{\cdot-}$) radical at PSI (Mehler reaction). Superoxide is rapidly converted to H_2O_2 by superoxide dismutase (SOD) in the chloroplast and the product H_2O_2 is detoxified by ascorbate peroxidase (APX) in the water-water cycle (Asada, 1999, 2006). ROS are also generated in photorespiration in the chloroplast, where H_2O_2 is produced by glycolate oxidase, and scavenged by catalase and peroxidase reactions in the peroxisome (Apel and Hirt, 2004). If (*E*)-β-caryophyllene can help protect plants against oxidative stress, lines in which (*E*)-β-caryophyllene synthase has been over-expressed should have lower ROS levels. From our results, the (*E*)-β-caryophyllene-producing lines do have significantly lower amounts of H_2O_2 in leaves (Fig. 4.4). (*E*)-β-Caryophyllene is known to react very rapidly with ROS species in the atmosphere, with atmospheric-lifetime with OH (42 min), O_3 (2 min) and NO_3 (3 min) being much shorter than for almost any other plant terpene (Atkinson and Arey, 2003). Thus, reduction in H_2O_2 levels may be accomplished via direct chemical reaction. Moreover, because of its lipophilicity (*E*)-β-caryophyllene may cross membranes and react with different pools of H_2O_2 in the cell. However, the effect of (*E*)-β-caryophyllene may also be realized by reaction with other ROS, which are often in equilibrium with hydrogen peroxide. H_2O_2 may be reduced to hydroxyl radicals ($OH^{\cdot}$) by superoxide ($O_2^{\cdot-}$) radicals via the Haber-Weiss reaction in the presence of transition metal ions (Apel and Hirt, 2004). Hydroxyl radicals ($OH^{\cdot}$) are the most reactive and destructive forms of ROS (Imlay and Linn, 1988; Ledford and Niyogi, 2005). In addition, singlet oxygen ($^1O_2$) is more toxic

than H_2O_2 too. (*E*)-β-Caryophyllene reaction with these species could be of great value to the plant and should impact H_2O_2 levels as well, due to chemical equilibria.

As a major antioxidant in plant cells, ascorbate represents over 10% of the soluble carbohydrate (Noctor and Foyer, 1998), and its abundance is especially high in chloroplasts (~25 mM) (Niyogi, 1999). Our results show that the total ascorbate content in transgenic lines is not affected when (*E*)-β-caryophyllene synthase is over-expressed (Fig. 4.5A). We also investigated chlorophyll content as a way of detecting perturbations to photosynthesis caused by redox changes, but total chlorophyll content was not changed significantly in transgenic lines. In order to know more about the energy status of chlorophyll and changes in the rate of photosynthesis, more studies need to be performed (Foyer et al., 1994; Lichtenthaler et al., 2007).

Recently, transgenic tobacco lines engineered to produce isoprene were shown to have higher amounts of reduced ascorbate than non-producing controls, although the total ascorbate contents showed little difference. Therefore isoprene was suggested to improve a plant's antioxidant capacity (Vickers et al., 2009). However, in (*E*)-β-caryophyllene-producing lines, it seems that the antioxidant capacity in term of total ascorbate content is not reduced. Like isoprene, (*E*)-β-caryophyllene is lipophilic but larger and less volatile, and so may not have the same effect, although its reduction of H_2O_2 levels suggests its ability to influence cellular redox status. It may protect lipid membrane as carotenoids and tocopherols do preventing lipid peroxidations, or stabilize membranes as isoprene is thought to act (Sharkey and Yeh, 2001).

4.4.3 (*E*)-β-Caryophyllene production and regulation of antioxidant genes

Cellular redox status is not only controlled by the levels of ROS and ascorbate, but also by the activity of a variety of enzymes of which four are prominent. Superoxide dismutase (SOD) is the first line of defense against ROS accumulation by dismutating superoxide ($O_2^{\cdot -}$) to H_2O_2. Glutathione peroxidase (GPX), ascorbate peroxidase (APX) and catalase (CAT) then metabolize the resulting H_2O_2 (Inze and Van Montagu, 1995; Apel and Hirt, 2004). In *A. thaliana*, so far six isozymes of APX have been identified. A thylakoid-bound APX, a lumen APX and a stromal APX were found in chloroplast. One peroxisome APX, and two cytosolic APXs have been characterized as well (Davletova et al., 2005). All

APXs require ascorbate and glutathione as cofactors for the detoxification of H_2O_2 (Inze and Van Montagu, 1995), whereas no cofactors are required for three CAT isozymes found in the peroxisome (Apel and Hirt, 2004). During light induced photooxidative stress, cytosolic APX1 is the key scavenging enzyme protecting the chloroplast by reacting with H_2O_2 transported to the cytosol, (Davletova et al., 2005), while CAT2 is the most predominantly expressed catalase (Vandenabeele et al., 2004). According to our results, there were no significant differences in *APX1* and *CAT2* transcript levels in (*E*)-β-caryophyllene-producing *A. thaliana* compared to wild type under normal growth conditions (Fig. 4.6) or under oxidative stress (Fig. 4.7), although the (*E*)-β-caryophyllene lines tended to have lower amounts. The activities of ROS scavenging enzymes are critical to maintain redox status at physiological levels. Mutants of APX or CAT are hypersensitive to stresses, and loss of one scavenging enzyme may up-regulate others (Mittler et al., 1999; Apel and Hirt, 2004). In our (*E*)-β-caryophyllene over-expression lines, the slight reduction of *APX* and *CAT* expression may indicate that there is more anti-oxidative capacity available from other sources, such as (*E*)-β-caryophyllene. Further analysis of gene expression and protein levels in these transgenic lines is necessary to determine the effect of (*E*)-β-caryophyllene on plant oxidative stress.

Transgenic plants with manipulated volatile terpene emission sometimes show complex responses upon oxidative stress. For example, transgenic grey poplar plants (*Populus* × *canescens*) with repressed isoprene emission are not sensitive to oxidative stress (Behnke et al., 2009), suggesting the presence of other plant components that act in protection (Behnke et al., 2010b). In our study, the role of (*E*)-β-caryophyllene as an *in vivo* protectant against oxidative stress is suggested based on the evidence that transgenic plants have significantly lower H_2O_2 content than wild-type controls. These findings are consistent with studies on the antioxidant activities of isoprene and monoterpenes (Loreto and Velikova, 2001; Loreto et al., 2004; Vickers et al., 2009). However, additional work is required to confirm the role of (*E*)-β-caryophyllene and other sesquiterpenes in protecting plants against oxidative stress and defining their mechanism of action.

4.5 Materials and Methods

4.5.1 Plant materials, growth conditions

Seeds of *Arabidopsis thaliana* ecotype Col-0 (CS 6000) were obtained from the ABRC (Arabidopsis Biological Resource Center, OH, USA). Plants were grown on soil (Sunshine Growing Mix No.1 : sand, 8:1) for 4 to 6 weeks until rosette stage under controlled growth conditions (10 h-light/14 h-dark photoperiod with 150 $\mu mol \cdot m^{-2} \cdot s^{-1}$ PAR, 23 °C, 55% relative humidity). Kanamycin resistant transgenic plants were pre-selected on 1 × Murashige and Skoog (Duchefa, Haarlem, The Netherlands) plates with 1% sucrose and 100 $\mu g\ mL^{-1}$ kanamycin prior to being transferred to soil.

4.5.2 Reagents

All reagents or solvents were obtained from Fisher Scientific, Sigma-Aldrich or Fluka, unless otherwise stated.

4.5.3 Plant treatments

For Paraquat treatment, a 50 µM solution was sprayed directly on leaves of *A. thaliana* wild type and *TPS21* transgenic plants. Plants were then placed under controlled growth conditions with a light intensity of 600 $\mu mol\ m^{-2}\ s^{-1}$. Leaf samples were collected on 3 and 24 h after spraying.

4.5.4 Hydrogen peroxide content determination

Hydrogen peroxide (H_2O_2) content in leaves was determined by using the potassium titanium oxalate method (Sellers, 1980). The absorbance was measured at OD 508 nm (Patterson et al., 1984).

4.5.5 Ascorbate content measurement

Total ascorbate contents of leaf tissue were measured using the method of analyzing ascorbate and dehydroascorbate (Hewitt and Dickes, 1961) (Foyer et al., 1983). Leaves were ground in liquid nitrogen with 0.75 mL of 6% metaphosphoric acid and centrifuged at maximum speed for 10 min. A 50 µL portion of supernatant was added to 950 µL potassium phosphate buffer (0.1 M, pH 6.9) and the absorbance at OD 265 nm was recorded after

mixing. The absorbance was recorded again after the addition of 1 U of ascorbate oxidase and the stabilization of the reading. The difference between the two values represents the ascorbate content. The amount of dehydroascorbate was measured to account for possible losses of ascorbate due to oxidation. A 50 µL portion of supernatant was added to the 950 µL potassium phosphate buffer (0.1 M, pH 6.9) and the absorbance at OD 265 nm was recorded as previously mentioned. Then after addition of 1 µL of 0.2 M dithiothreitol, the absorbance at OD 265 nm was allowed to rise for approximately 10 min and stabilize, before recording the final absorbance. The difference between these two values represents the amount of dehydroascorbate. The total ascorbate content is then represented by the sum of ascorbate and dehydroascorbate content.

4.5.6 Chlorophyll content analysis

Plant leaves were harvested under normal growth condition, and total chlorophyll contents determined at OD 647 nm and 665 nm according to the DMF (N,N-dimethylformamide) method (Inskeep and Bloom, 1985).

4.5.7 Quantitative RT-PCR analysis of gene expression

The expression levels of ascorbate peroxidase (*APX1*, At1g07890) and catalase (*CAT2*, At4g35090) in wild type Col-0 and transgenic plant leaves were measured by quantitative RT-PCR with a Power SYBR® Green PCR Master Mix (Applied Biosystems, USA). Total RNAs from wild type Col-0 and transgenic plants from both Paraquat-treated and untreated leaves were extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA), and DNA contamination was eliminated with RQ1RNase-free DNase (Promega, Madison, WI, USA). The DNA-free total RNA was reverse transcribed into cDNA as previously described (Chen et al., 2003). A 1 mL aliquot of 50× diluted cDNA was analyzed with 25 mL SYBR Green Master Mix (Applied Biosystems, USA) in triplicate. The primers (P1&P2, P3&P4) used for each gene (*APX1* and *CAT2*) are shown in Table 4.5. The efficiencies of the primers were examined by Pfaffl's standard curve method (Pfaffl, 2001). According to the test of gene candidates (Phillips et al., 2009), Actin8 (At1g49240) (P5&P6, Table 4.5) was selected for normalization of overall expression levels between wild-type and transgenic plant leaves, both treated and untreated. Fold change differences of *APX1* and *CAT2* expression were

calculated according to the efficiency corrected by the Pfaffl method with Actin8 (At1g49240) as the reference gene. Each data point was obtained from three independent biological replicates and three technical replicates.

Table 4.5. Primer sequences

Primer	Sequence (5' to 3')
P1	AGGGGTCGCATTGCATTTGAAC
P2	GTCTAAGCAGCAAAAGCGCAAC
P3	TACAATCTTCATCATGTGGATC
P4	ATCTTTCAAGGAAGAAGGAGC
P5	ATGAAGATTAAGGTCGTGGCAC
P6	GTTTTTATCCGAGTTTGAAGAGGC

4.5.8 Microarray analysis

Leaves of 4 week old *A. thaliana* wild type (Col-0) and *TPS21* transgenic lines (*TPS21*-1 and 2) were collected. Total RNA extracted from 4 plants of each line was pooled together as one biological replicate. Three biological replicates were obtained from each line. RNA was extracted by the Trizol reagent (Invitrogen, Carlsbad, CA, USA), and DNase treatment and RNA purification was performed with the RNeasy Plant Mini Kit (Qiagen, USA). RNA quality was evaluated by agarose gel, spectrophotometry analysis and Agilent 2100 BioAnalyzer assessment in the core laboratory facility (CLF) of Virginia Bioinformatics Institute at Virginia Tech (VBI). Microarray hybridizations were done on Affymetrix GeneChip by VBI CLF (https://www.vbi.vt.edu/core_laboratory_facility/gene_expression_analysis/affymetrix_technology_description). The signal intensity of the probes on the individual arrays was normalized by MAS5.0 for a quick quality check. RMAExpress (Robust Multichip Average) was used for comparing and normalizing different chip signals (Bolstad et al., 2003; Irizarry et al., 2003). Further data processing was performed by applying Significance Analysis of Microarrays (SAM) for identifying significantly changed genes (Tusher et al., 2001).

4.5.9 Statistical analysis

Significance was determined according to ANOVA with a Tukey post-hoc test using SAS9.1 (SAS Institute Inc., Cary, NC, USA).

4.5.10 Acknowledgements

We are thankful to the core laboratory facility (CLF) of Virginia Bioinformatics Institute at (VBI) for the technical assistance. We are grateful to Lei Bao, Virginia Tech, USA, for assistance in RMAExpress and SAM analysis.

5. Discussion

5.1 Various volatile terpenes with diverse ecological functions

Volatile terpenes belong to the category of secondary metabolites. They are historically thought to be waste products and not directly involved in primary processes such as growth, development and reproduction. A growing body of evidence shows that secondary metabolites such as volatile terpenes have direct (De Moraes et al., 2001; Kessler and Baldwin, 2001; Wang et al., 2008) or indirect (Kappers et al., 2005; Rasmann et al., 2005; Schnee et al., 2006) defensive functions against herbivore attack and certain abiotic stresses (Delfine et al., 2000; Loreto et al., 2001; Loreto and Velikova, 2001; Sharkey et al., 2001; Loreto et al., 2004; Copolovici et al., 2005). However, the formation of only a small fraction of plant volatile terpenes has been investigated (Dicke and Loreto, 2010). Advances in molecular biology facilitate functional studies of plant metabolites via transgenic lines with particular biochemical phenotypes. The objectives of this thesis were to understand the molecular mechanisms that are responsible for the intraspecific variation of herbivore-induced terpene emissions from *Arabidopsis thaliana* leaves and to gain insight into the ecological functions of terpene emission from *A. thaliana* flowers.

5.1.1 Mechanisms of various terpene emissions

Volatile terpenes have been discovered from various plants, such as Arabidopsis, rice, maize, cotton, tobacco, cucumber, mint, basil, snapdragon, strawberry, apple, kiwifruits, oak, pine, spruce and other plants. Among theses, *Arabidopsis thaliana* has become a model plant to study the biosynthesis and regulation of terpene emission for its abundant genetic and genomic resources. From the vegetative parts of *A. thaliana*, the ecotype Col-0 was found to release the sesquiterpene (*E,E*)-α-farnesene along with methyl salicylate and the C_{16}-homoterpene 4,8,12-trimethyltridecatetra-1,3,7,11-ene after feeding damage by the crucifer specialists *Pieris rapae* and *Plutella xylostella* or application of the fungal peptide elicitor alamethicin (Van Poecke et al., 2001; Herde et al., 2008). The resulting volatile emissions are suggested to be signals in tritrophic interactions to attract the parasitic wasp *Cotesia rubecula* which increases plant fitness (van Loon et al., 2000; Van Poecke et al., 2001). However, it was unknown whether other *A. thaliana* ecotypes release similar induced volatiles, and if not,

what controls such differences. Intraspecific variation in plant volatiles has been studied in cultivated plants (maize, basil, snapdragon and strawberry) and wild species (wild tobacco, wild maize and horse nettle). *A. thaliana* and its abundant natural ecotypes are suitable tools to discover the molecular mechanisms governing natural diversity of volatile terpenes.

5.1.1.1 Allelic variation contributes to volatile terpene diversities

Induced volatile terpenes among 27 ecotypes of *A. thaliana* were quantified and compared after coronalon induction by using the closed-loop stripping method and gas chromatography- mass spectrometry. Coronalon is a jasmonate-mimic (Schueler et al., 2001) that can induce plant responses similar to those after insect feeding (Herde et al., 2008). It was shown that the monoterpene (*E*)-β-ocimene was emitted as a main volatile by 20 ecotypes, including Ws but not Col-0. Almost all ecotypes release the sesquiterpene (*E,E*)-α-farnesene with lower emission rates than those of (*E*)-β-ocimene. The Ws ecotype was chosen as a representative of high (*E*)-β-ocimene emitters, while the Col-0 ecotype was chosen as a representative of low (*E*)-β-ocimene emitters. From the significant correlation between (*E*)-β-ocimene and (*E,E*)-α-farnesene in most ecotypes, these two volatile terpenes were hypothesized to be produced either simultaneously by a single bi-functional terpene synthase or by two co-expressed enzymes. In order to test these proposals, Ws and Col-0 were analyzed in detail. A previous study had shown that the (*E*)-β-ocimene synthase (TPS03) from *A. thaliana* ecotype C24 has the activity to convert the precursor GPP to (*E*)-β-ocimene (Faeldt et al., 2003), so we started to analyze the *TPS03* gene. Using semiquantitative RT-PCR, we showed *TPS03* induction in Col-0 upon both coronalon and *Plutella xylostella* feeding. In Ws, no induction of *TPS03* transcripts could be found. Since *TPS03* is in close proximity to a similar terpene synthase gene *TPS02* on chromosome 4 (Aubourg et al., 2002), we extended our analysis to include *TPS02* also. *TPS02* and *TPS03* are likely to have emerged by tandem gene duplication. Tandem pairs of *TPS* genes in the *A. thaliana* genome are not rare. The root-specific 1,8-cineole synthases AtTPS-Cin (*TPS23* and *27*) (Chen et al., 2004) and the (*Z*)-γ-bisabolene synthases (*TPS12* and *13*) (Ro et al., 2006) are two examples. After coronalon and insect treatment, transcripts of *TPS02* could be found in both ecotypes along with splicing variation in Col-0. Combining the expression studies with sequencing analyses, the Ws-*TPS03* allele and Col-*TPS02* alleles were found to be inactive because of

frame shift mutations and consequent premature translational terminations; however, both Ws-*TPS02* and Col-*TPS03* have full-length cDNAs which encode terpene synthase proteins. Therefore, *TPS02* is hypothesized to be responsible for (*E*)-β-ocimene and (*E,E*)-α-farnesene formation in Ws, and *TPS03* is responsible for (*E,E*)-α-farnesene formation in Col-0. Analysis of knockout lines and RNAi lines of Ws-*TPS02* and Col-*TPS03* strongly support our hypothesis that the active tandem genes, Ws-*TPS02* and Col-*TPS03,* are responsible for the difference of induced volatile terpenes from Ws and Col-0, respectively. For genes in the *TPS* superfamily and other families of secondary metabolism, it is known that gene diversification occurs rapidly via gene duplication and sequence divergence (Pichersky and Gang, 2000; Kliebenstein et al., 2001; Koellner et al., 2004). Observations from different cultivars of crop plants (tomato, basil and maize) show that inactivation of alleles of *TPS* often contributes to terpene diversity (van der Hoeven et al., 2000; Iijima et al., 2004; Koellner et al., 2004). Our findings provide direct evidence that allelic variation of *TPS* genes in wild gene pools contributes to the natural variation in terpene formation.

5.1.1.2 Subcellular segregation of terpene synthases and their substrates leads to volatile terpene diversity

Whether Ws-TPS02 and Col-TPS03 are bifunctional enzymes depends on their catalytic abilities and access to different substrate pools *in vivo*. We analyzed the *in vitro* catalytic activities of *TPS02* and *TPS03* encoded enzymes by using the heterolgous *E. coli* expression system to clone and express both genes. The mature protein of TPS02 without a predicted plastidial transit peptide converted GPP and FPP into (*E*)-β-ocimene and (*E,E*)-α-farnesene, respectively, as main products, while the full length TPS03 protein converted GPP and FPP into (*E*)-β-ocimene and (*E,E*)-α-farnesene in a similar fashion. Thus both recombinant TPS proteins show bifunctional activity without major kinetic differences *in vitro*. To understand why the bifunctional Col-TPS03 enzyme only produces (*E,E*)-α-farnesene as its main product *in vivo*, while the bifunctional Ws-TPS02 produces (*E*)-β-ocimene as a main product and (*E,E*)-α-farnesene in small amounts, we performed subcellular localization studies by cloning N-terminal signal peptides of Col-*TPS03* and *Ws-TPS02* into GFP constructs. Using confocal laser scaning microscopy, we saw a stable GFP signal for Col-*TPS03* in the cytosol and a signal for Ws-*TPS02* in plastids. Thus differential

subcellular targeting of bifunctional enzymes may lead to compartment-specific formation of monoterpenes and sesquiterpenes in wild *A. thaliana* due to differential availability of substrate.

To investigate substrate availabilities in different compartments, inhibition of terpene biosynthetic pathways was performed together with coronalon induction. Treatments with the MEP pathway inhibitor fosmidomycin (inhibits DXR) and the MVA pathway inhibitor lovastatin (inhibits HMGR) demonstrate that the major substrate supplies of GPP and FPP are located in plastids and the cytosol, respectively although crosstalk between plastid and the cytosol are present as previously described (Hemmerlin et al., 2003; Laule et al., 2003; Schuhr et al., 2003; Dudareva et al., 2005). The presence of plastidial FPP may arise from an import from the cytosol (Schuhr et al., 2003) or from biosynthesis in plastids. The biosynthesis of FPP in plastids is not only supported by our results but also by the description of a (*Z,Z*)-FPP synthase in wild tomato (Sallaud et al., 2009). It has been suggested that terpene biosynthetic intermediates are particularly exported from plastids to the cytosol rather than the other way around (Laule et al., 2003). The trace emission of (*E*)-β-ocimene in Col-0 indicates that the flow of GPP precursor to the cytosol is low, consistent with the results from tobacco and kiwifruits (Wu et al., 2006; Nieuwenhuizen et al., 2009). Together with the investigations of peppermint, snapdragon and strawberry plants (Aharoni et al., 2004; Turner and Croteau, 2004; Nagegowda et al., 2008), we prove that subcellular segregation and differential substrate availabilities to bifunctional terpene synthases are important molecular mechanisms in the natural evolution of the intraspecific volatile terpene diversity.

5.1.1.3 Individual terpene synthase enzymes also contribute to diversity by making multiple products

In studying the biosynthesis of volatile terpenes, terpene synthases play prominent roles. For instance, monoterpene formation and biosynthetic rates in pepperemint are controlled mainly by the relevant monoterpene synthases (McConkey et al., 2000). Interestingly, nearly half of all characterized monoterpene and sesquiterpene synthases produce multiple products from a single substrate (Degenhardt et al., 2009). Starting from the divalent metal ion-dependent ionization, the substrates GPP or FPP undergo a series of cyclizations, hydride shifts and other chemical rearrangements until the reaction is terminated

(Tholl et al., 2005; Degenhardt et al., 2009; Boland and Garms, 2010). The formation of multiple products results from the fact that the carbocationic intermediates of the reaction can have multiple fates depending on the type of terpene synthase and the cellular environment. For example, the biosynthesis of the monoterpenes (*E*)-β-ocimene and myrcene starts with the ionization of the GPP substrate, but removal of a different proton leads to different products (Degenhardt et al., 2009) (Fig. 5.1). Specific amino acid motifs and active site sequences of individual terpene synthases might facilitate multiple product formation.

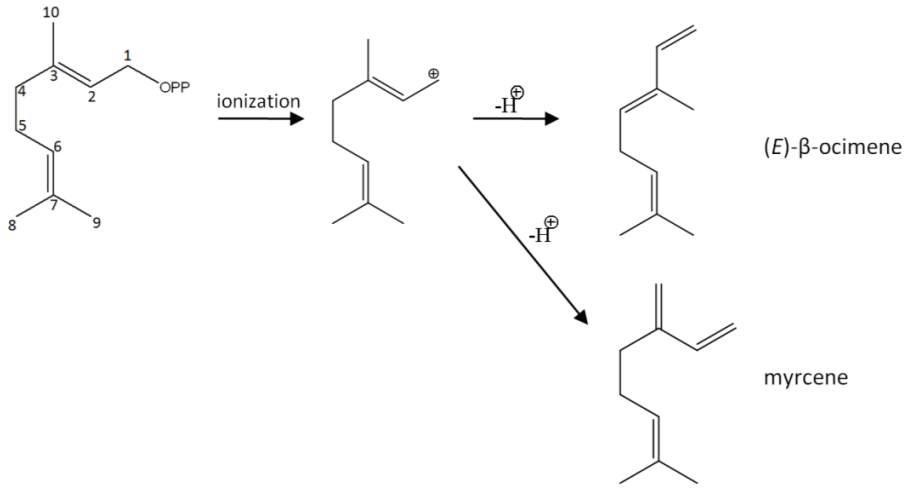

Figure 5.1. The proposed reaction pathway from GPP to (*E*)-β-ocimene and myrcene.

5.1.1.4 Organ, tissue and cellular specialization of terpene biosynthesis is a form of regulation and contributes to patterns of diversity

Terpene synthases are differentially expressed in different plant species, and can be localized in specialized organs or tissues. For instance, the biosynthesis of volatile monoterpenes from peppermint plants is localized to the secretory cells of glandular trichomes on leaf surfaces (Gershenzon et al., 1992; McCaskill et al., 1992). In *A. thaliana*, Ws-*TPS02* and Col-*TPS03* genes are expressed upon herbivore induction in leaves and constitutively expressed in flowers. A similar expression pattern of the *A. thaliana* geranyllinalool synthase is observed in leaves and flowers (Herde et al., 2008). Although terpene synthase expression plays a critical role in the regulation of natural variation in terpene formation, a hierarchy of other factors contributes as well (Fig. 5.2).

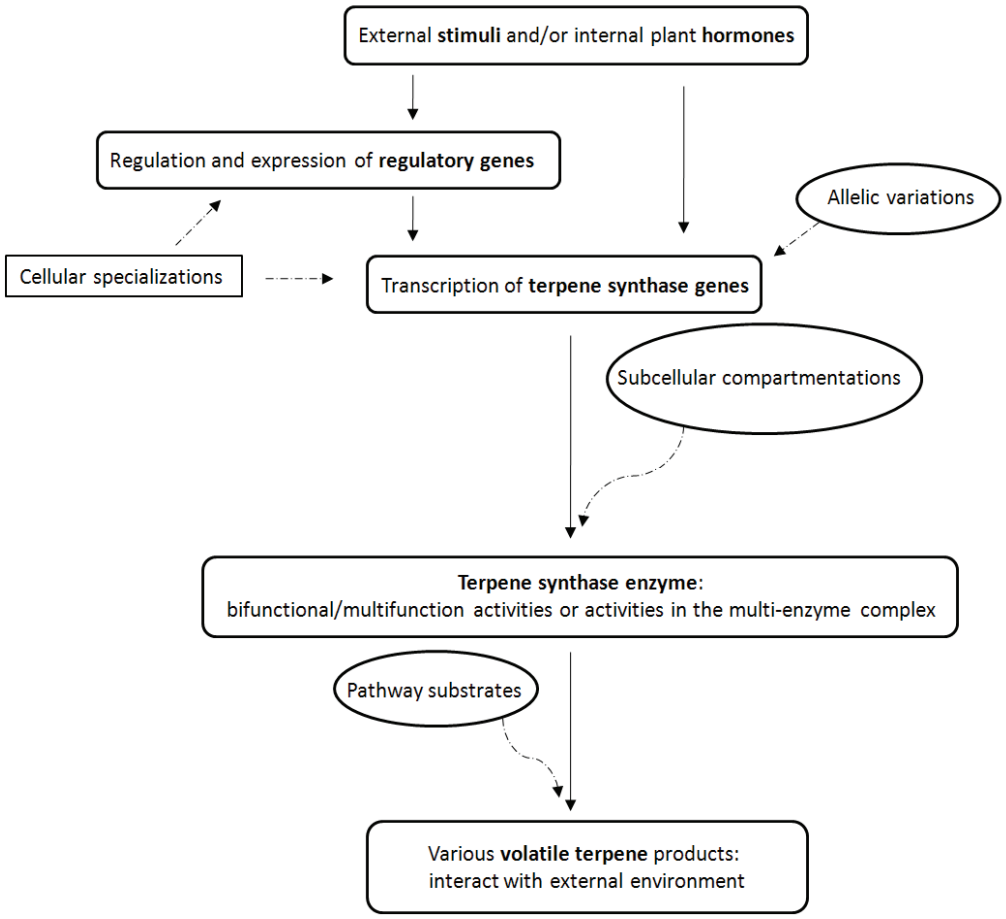

Figure 5.2. The multiple mechanisms regulating volatile terpene biosynthesis

The release of (*E*)-β-ocimene and (*E,E*)-α-farnesene from *A. thaliana* flowers and herbivore-damaged leaves indicates that the functions of these terpenes are probably related to pollinator attraction and tritrophic interactions. Volatile mixtures containing (*E*)-β-ocimene and (*E,E*)-α-farnesene from pathogen-attacked silver birch or aphid-attacked soybean suggest similar ecological defensive functions of these two volatile terpenes (Zhu and Park, 2005; Vuorinen et al., 2007; Holopainen and Gershenzon, 2010). In *A. thaliana*, other secondary metabolites including glucosinolates are known to show widespread intraspecific variation (Kliebenstein et al., 2001). But, in all of these cases, the ecological significance of intraspecific variation is still not understood.

5.1.2 Multiple ecological roles of terpene emissions

It has often been suggested that volatile terpenes play various ecological roles in plants, but rigorous tests of these ideas have not often been carried out. The sesquiterpene (*E*)-β-caryophyllene is one of the most common volatile terpenes and is present in many plant species, such as clove (*Eugenia caryophyllata*) (Zheng et al., 1992), cinnamon (*Cinnamomum* spp.) (Jayaprakasha et al., 2003), hemp (*Cannabis sativa* L) (Gertsch et al., 2008), ginger (*Zingiber* spp.) (Sabulal et al., 2006), and sage (*Salvia* spp.) (Liang et al., 2009). It is a common component of floral scent (Knudsen et al., 1993; Knudsen et al., 2006) as well. In this thesis, we turned our attention to the ecological significance of (*E*)-β-caryophyllene using *A. thaliana* as model plants.

A. thaliana flowers constitutively emit large amounts of volatile terpenes with monoterpenes and sesquiterpenes making up more than 60% of total volatiles (Chen et al., 2003). But, there is extensive variation among ecotypes. In flowers of Col-0, 24 monoterpenes were identified (Rohloff and Bones, 2005) and the major monoterpenes are β-myrcene and linalool; the predominant volatile in Ws flowers is (*E*)-β-ocimene (Chen et al., 2003; Tholl et al., 2005). From Col-0 flowers, 26 sesquiterpenes were recognized (Rohloff and Bones, 2005) which account for more than 95% of the total terpene volatiles from the inflorescences of Col-0 (Chen et al., 2003). In particular, (*E*)-β-caryophyllene and α-humulene together account for 43% of the total terpene volatiles (Chen et al., 2003). Two sesquiterpene synthase genes (At5923960 and At5g44630) were shown to be responsible for the complex mixture of sesquiterpenes emitted from *A. thaliana* flowers and the volatile differences are controlled by differential transcription of terpene synthase (*TPS*) genes and putative posttranslational modifications (Tholl et al., 2005). The characterization of (*E*)-β-caryophyllene synthase (At5923960, *TPS21*) facilitated our functional studies by allowing us to generate transgenic plants that over-express (*E*)-β-caryophyllene synthase and to identify loss-of-function knock-out lines.

5.1.2.1 (*E*)-β-caryophyllene is suggested to be a direct antioxidative compound

Information on the role of volatile terpenes in plants can sometimes be inferred from analysis of gene expression changes when plants are exposed to these substances. Previously, a large-scale transcriptome analysis showed that *A. thaliana* plants fumigated by exogenous volatile terpenes exhibited significant changes in transcript levels (Godard et al., 2008). Here we demonstrated for the first time that substantial transcriptome changes also occur after constitutive endogenous fumigation with (*E*)-β-caryophyllene due to over-expression of (*E*)-β-caryophyllene synthase. In transgenic leaves, 154 genes were significantly down-regulated. The physiological significance of most of these down-regulated genes is unknown and needs further investigation. The gene that is most significantly down-regulated (At3g26000) belongs to the F-box protein family, and we designate it as Caryophyllene Suppressed F-box protein (CarSF) gene. The finding of CarSF and other down-regulated genes provides new molecular targets for dissection of the function of (*E*)-β-caryophyllene in plant-abiotic stress interactions. In plants growing in nature, variability of (*E*)-β-caryophyllene emission is commonly observed (Duhl et al., 2008). Since high temperature and in some cases, high light increase β-caryophyllene emissions, this compound may be postulated to have a natural role in resistance to these abiotic factors. Interestingly, we found out that hydrogen peroxide (H_2O_2) content is significantly lower in (*E*)-β-caryophyllene over-producing lines under normal growth conditions. However, the pool of the antioxidant, ascorbate, is similar in both transgenic and wild-type plants. And, the expression of two enzymes that reduce oxidative stress by scavenging reactive oxygen species, ascorbate peroxidase (APX1) and catalase (CAT2), are insignificantly changed in transgenic lines under normal and oxidative stress conditions. Taken together, our results suggest that (*E*)-β-caryophyllene may be directly involved in a reactive oxygen species (ROS) scavenging network, like that indicted for isoprene and monoterpenes in other plant species (Loreto et al., 2001; Loreto and Velikova, 2001; Loreto et al., 2004; Loreto and Fares, 2007; Vickers et al., 2009). To confirm the antioxidative function of (*E*)-β-caryophyllene, further analyses are required, such as measuring the levels of different reactive oxygen species, determining gene expression and activities of scavenging enzymes in antioxidative networks, and quantifying the amounts of other metabolites that respond to abiotic stress (Behnke et al., 2010b).

5.1.2.2 (*E*)-β-caryophyllene is a defense against bacterial pathogens

Besides abiotic factors, biotic factors also influence (*E*)-β-caryophyllene emission. (*E*)-β-Caryophyllene has been found to serve as induced signal for plant indirect defense. For example, when released from maize leaves, it attracts parasitic wasps to oviposit on larvae of the lepidopteran *Spodoptera littoralis* (Koellner et al., 2008). And, when released from maize roots it attracts nematodes to attack larvae of the beetle *Diabrotica virgifera virgifera* (Rasmann et al., 2005). (*E*)-β-caryophyllene can also directly decrease the growth and survival of insects feeding on cotton and *Hymenaea* (Langenheim, 1994). (*E*)-β-Caryophyllene has been reported to act in defense against herbivores when it occurs in non-floral tissues, but has not yet been directly demonstrated to serve in pollinator attraction in flowers. Knowledge of the role of (*E*)-β-caryophyllene emission in flowers is scarce, especially as a possible defense against pathogen attack. Since floral stigmas provide a humid and nutritive environment for microbial pathogens (Ngugi and Scherm, 2006) and there may be a high frequency of infection due to vectoring by insect visitors, we investigated the ability of (*E*)-β-caryophyllene to serve as an anti-bacterial defense in *A. thaliana* flowers and demonstrated significant antimicrobial activity against the bacterial pathogen *Pseudomonas syringae*. Knockout mutants lacking (*E*)-β-caryophyllene emissions from stigmas were more susceptible to this pathogen, and the seed production and fitness of knock-out mutants were reduced compared to wild-type *A. thaliana*. Moreover, transgenic lines over-expressing (*E*)-β-caryophyllene synthase throughout the plant were more resistant to pathogens applied to leaves. Our results suggest that (*E*)-β-caryophyllene, whose production in flowers is restricted to the stigma, serves as an antimicrobial substance. A previous study of caryophyllene-rich rhizome oil from *Zingiber nimmonii* showed significant inhibitory activity against the bacteria *Bacillus subtilis* and *Pseudomonas aeruginosa* (Sabulal et al., 2006). In our *in vitro* study, (*E*)-β-caryophyllene showed a direct inhibitory effect on the pathogen in a liquid medium. Since the tested concentration of (*E*)-β-caryophyllene in the medium was actually lower than the amount found in *A. thaliana* flowers on an hourly basis, this compound appears to be present in sufficient amounts in nature to have an antimicrobial role. Of course, serving as an inhibitor of pathogen growth in *A. thaliana* flowers does not exclude (*E*)-β-caryophyllene from also acting as a pollinator attractant. Floral (*E*)-β-caryophyllene may be both an attractant and a deterrent because of its attractiveness to

beetles, moths and herbivore enemies (Brown et al., 2006; Verheggen et al., 2007; Koellner et al., 2008; Schmidt-Busser et al., 2009).

5.2 Conclusion

Taken together, our investigations of (*E*)-β-caryophyllene as an anti-oxidant and an anti-bacterial floral volatile suggest multiple roles for this substance in plants. Given the widespread distribution of volatile terpenes in different plant species and organs in nature, a wide range of other roles can be expected. Elucidation of these roles should reveal many new aspects of how plants are adapted to cope with stressful biotic and abiotic factors in their environment.

6. Summary

Plants are a very successful group of organisms over the evolutionary history of our planet developing a range of responses to cope with various stress factors in their environment. Plants are adapted to both biotic stresses (e.g. attacks from herbivores or pathogens) and abiotic stresses (e.g. high light or temperature, poor nutritional conditions, oxidative stress). Volatile terpenes may facilitate resistance to many of these stress factors.

In this thesis, numerous ecotypes of *Arabidopsis thaliana* were investigated to gain a picture of intraspecific variation in volatile formation. We demonstrated that the Col-0 ecotype emits the sesquiterpene (*E*,*E*)-α-farnesene as a major volatile upon herbivore attack, whereas the Ws ecotype releases the monoterpenes (*E*)-β-ocimene in large amounts and (*E*,*E*)-α-farnesene in smaller amounts. Two homologous bifunctional terpene synthase genes (*TPS02* and *TPS03*) were discovered, each of which is active in one ecotype, but present as a non-functional copy in the other. The difference in product formation can be attributed to different subcellular compartmentations of the active enzymes (plastids vs. cytosol) giving each one access to different substrate pools. These results reveal how variation in terpene synthase genes leads to the natural diversity of herbivore-induced volatile terpenes in *A. thaliana*.

One of the well-known ecological functions of volatile terpenes is the attraction of pollinators to flowers. However, pollinator attraction may not be the only function of floral volatiles. This study illustrated that (*E*)-β-caryophyllene – one of the most common sesquiterpene volatile from flowers and found in the flowers of *A. thaliana* Col-0, can inhibit the growth of pathogenic microbes, such as *Pseudomonas syringae*, a natural bacterial pathogen of *A. thaliana*. Transgenic *Arabidopsis* plants lacking (*E*)-β-caryophyllene floral emission are more vulnerable to stigmatic infection than wild type plants, while plants that over-produced (*E*)-β-caryophyllene in leaves are more resistant to pathogen attacks there. The *in vitro* inhibitory effects of (*E*)-β-caryophyllene on bacteria support the idea that floral (*E*)-β-caryophyllene helps defend flowers directly. But (*E*)-β-caryophyllene may have other roles in stress resistance, since lines over-expressing this sesquiterpene had reduced oxidative stress as measured by H_2O_2 content and large scale transcriptome changes. Thus widespread plant volatiles, such as (*E*)-β-caryophyllene, may have multiple roles in defense, pollinator attraction and abiotic stress resistance.

7. Zusammenfassung

Evolutionär gesehen sind Pflanzen sehr erfolgreiche Organismen. Sie haben komplexe, aber effiziente Systeme entwickelt, um unterschiedliche Stressfaktoren zu bewältigen. Pflanzen reagieren sowohl auf biotischen Stress (z.B. Herbivore, Pathogene) als auch auf abiotischen Stress (z.B. Licht, Temperatur, Ernährung, oxidativer Stress). In einer sich verändernden Umwelt haben flüchtige Terpene eine ökologische Bedeutung für die Fitness der Pflanzen.

Anhand der verschiedenen Ökotypen von *Arabidopsis thaliana* sind Untersuchungen zu Änderungen in der Duftstoffzusammensetzung der Pflanzen möglich. Durch die Kenntnis der genomische Sequenz und die Verfügbarkeit zahlreicher Mutanten ist *Arabidopsis thaliana* ein gutes Werkzeug zur Untersuchung molekularer Mechanismen. Durch Blattfraß wird beim Ökotyp Col-0 hauptsächlich das Sesquiterpen (*E, E*)-α-Farnesen induziert, während der Ökotyp WS vor allem (*E, E*)-α-Farnesen und das Monoterpen (*E*)-β-Ocimen freisetzt. Für diese Unterschiede sind 2 homologe bifunktionale Terpensynthasegene verantwortlich. Die Tandem-Allele Col-TPS03 und WS-TPS02 sind aktiv und können beide Terpene bilden. Die Pseudogene Col-TPS02 und WS-TPS03 sind nicht funktionsfähig. Die unterschiedlichen Endprodukte beruhen auf der subzellularen Kompartimentierung der beiden aktiven Proteine und der dort vorhandenen Vorstufen. Es gibt molekulare Beweise, dass die Vielfalt der Pflanzenfraß-induzierten flüchtigen Terpene unter natürlichen Bedingungen auf verschiedenen Ebenen reguliert wird.

Die Blüten der Pflanzen setzen auch ohne äußere Reize kontinuierlich flüchtige Terpene frei. Eine ökologische Funktion dieser Duftstoffe ist die Anlockung von Bestäubern. Dies ist aber nicht die einzige Aufgabe. (*E*)-β-Caryophyllen ist eine Sesquiterpen, das in großen Mengen von *A. thaliana* Col-0 Blüten freigesetzt wird. Es ist in der Lage das Wachstum von *Pseudomonas syringae* zu hemmen und damit die Blütenorgane vor diesem Pathogen zu schützen. *A. thaliana*-Mutanten ohne (*E*)-β-Caryophyllen sind anfälliger als Wildtyp-Pflanzen, während Pflanzen, die (*E*)-β-Caryophyllen überproduzieren, widerstandsfähiger gegen Krankheitserreger sind. (*E*)-β-Caryophyllen hat *in vitro* einen direkten hemmenden Einfluss auf Bakterien. Das zeigt, dass flüchtige Terpene den Blüten bei der direkten Verteidigung helfen können. Darüber hinaus führt die Überproduktion von (*E*)-

β-Caryophyllen zu weitreichenden Transkriptom-Veränderungen sowie zu einer Modifikation des Redoxsystems mit einem niedrigerem Gehalt von H_2O_2 in den Blättern. Die verschiedenen Funktionen von (*E*)-β-Caryophyllen unterstützen die Vorstellung, dass flüchtige Terpene der Verteidigung von Blüten in der Natur helfen.

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9. Curriculum Vitae

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Publications

- **Huang, M.,** Abel, C., Sohrabi, R., Petri, J., Haupt, I., Cosimano, J., Gershenzon, J., and Tholl, D.: Variation of herbivore-induced volatile terpenes among *Arabidopsis* ecotypes depends on allelic differences and subcellular targeting of two terpene synthases, TPS02 and TPS03. Plant Physiology, 2010, DOI:10.1104/pp.110.154864
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11. Eigenständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

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Mengsu Huang