# Chemical and Molecular Analysis of Induced and Constitutive Volatile Formation in *Arabidopsis thaliana* and *A. lyrata*

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# Contents

# 1 Introduction

The aim of this thesis was to investigate the biochemistry of volatile compounds and its molecular regulation in the two closely related species *A. thaliana* and *A. lyrata*. The differences and similarities of volatile terpene biochemistry in flowers and foliage of *A. thaliana* and A. *lyrata* were determined. The natural variation of floral volatiles among populations of *A. lyrata* in Central Europe was investigated. Different *A. thaliana* ecotypes were employed to study molecular mechanisms controling the variation of insect-induced terpene volatiles.

## 1.1 Scent, Flavour & Fragrances

Unraveling the roles of plant volatiles in natural ecosystems is a continuing and fascinating challenge requiring a wide range of different approaches. Volatile organic compounds are common in the living world and are often used as signaling molecules. For a compound to be volatile under ambient temperatures, a high vapour pressure is required. Most lipophilic molecules with a molecular mass of up to ca. 270 atomic mass units (amu) are volatile at standard atmospheric pressure and temperature.

Mobile organisms such as animals use sensory systems to detect volatiles as guidance to food resources, mating partners, or help them to survive by provoquing avoidance reactions. Plants are sessile organisms for most of their life cycle. Hence, the use of volatiles is of crucial importance for plants to gain information about their physiological status and potential stress factors in the environment as well as to communicate with other organisms, enemies and mutualists. A well studied case in which emission of volatiles is directed at mutualists, is the attraction of pollinators to flowers with visual and olfactory cues [43]. Flowering plants usually emit mixtures of volatiles with up to 100 different compounds per blend [89] of which the most widely occuring compounds are shown in Figure 1. It is believed that by providing species-specific signals, plants enable insect pollinators to learn about convenient food sources which increases their foraging efficiency [131]. However, to identify these species-specific signals is a challenge. It is unclear whether pollinating insects use only one main compound in a floral scent mixture or if they use the combined information of a set of compounds. For the honeybee *Apis mellifera*, investigations revealed that this hymenopteran is able to use the complete range of floral volatiles to differentiate between four snapdragon cultivars which all emitted the same compounds but in different quantities [171].

Volatiles can also function in direct as well as in indirect defense mechanisms [63]. Direct growth inhibiting effects are obvious against other plants in the vicinity or as deterrent effects to feeding insects [15]. Indirect effects are apparent in attraction of herbivore enemies (also called tritrophic interactions). A well documented case

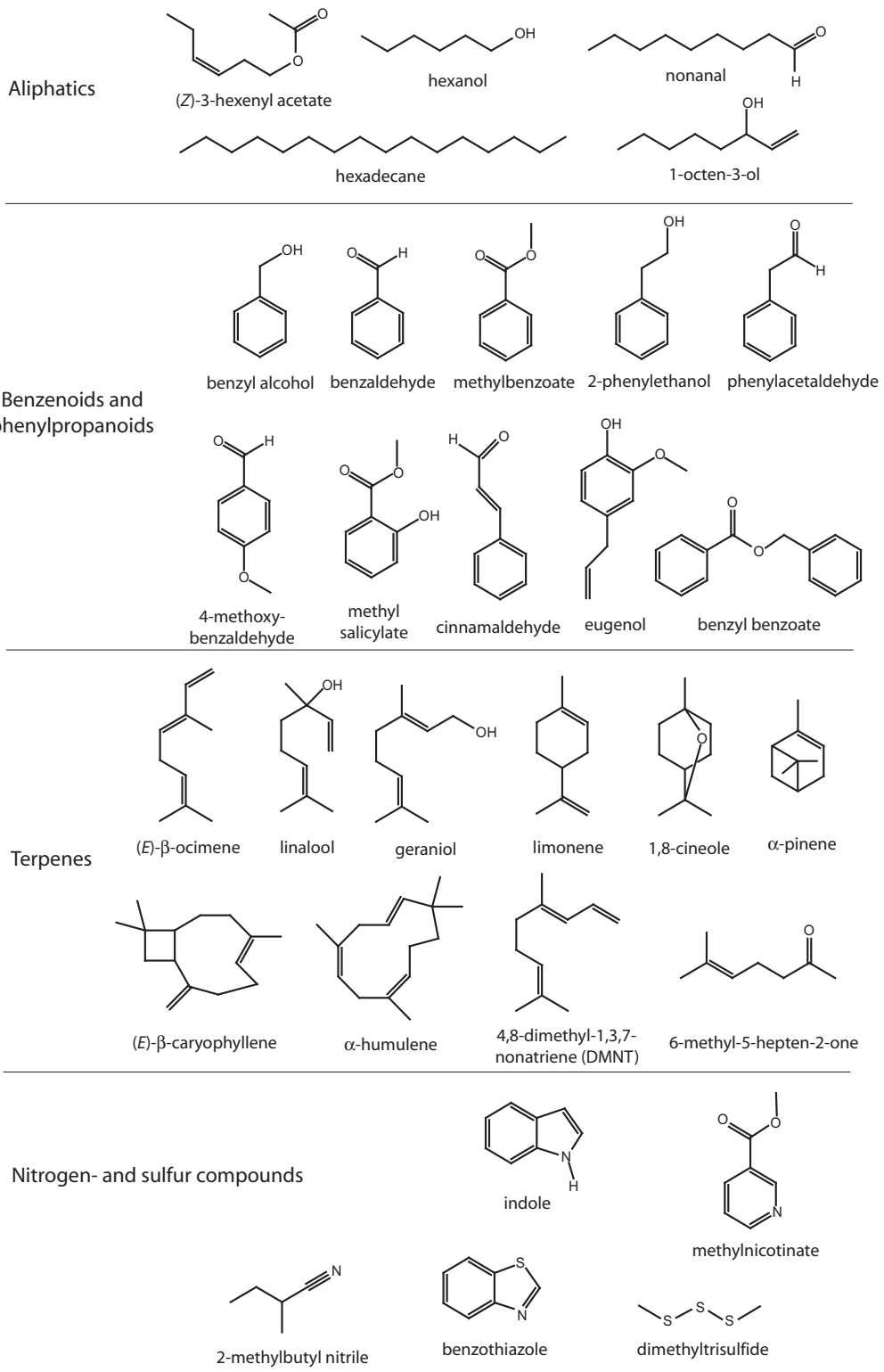


Figure 1: Major compounds of floral scents. Shown are compounds representative of the major chemical classes of natural products which are most frequently encountered according to a recent summary of the literature [88].

for a tritrophic system involves *Zea mays* under attack by caterpillars of *Spodoptera littoralis*. Upon feeding damage, maize produces a blend of volatile terpenes [163]. These attract females of the parasitic wasp *Cotesia marginiventris* which deposits its eggs into the feeding larvae [146]. Parasitoid development reduces caterpillar feeding and survival and so is believed to enhance the reproductive success of the volatile emitting maize plants.

## 1.2 The Terpene World

Terpenes, also known as terpenoids or isoprenoids, are a very large group of plant natural products formed by the condensation of branched five-carbon units. Intramolecular ring closures, hydride shifts, methyl shifts, and other rearrangements during the terminal steps of terpene biosynthesis as well as secondary transformations by the addition of functional groups lead to highly variable chemical structures. This explains why terpenes belong to the structurally richest class of natural compounds with more than 25,000 reported structures [27]. Terpenes are classified according to the number of pairs of five carbon units they contain. An overview of the different terpene classes is shown in Figure 2. The basic units for a biosynthetic terpene formation are the so-called isoprene units, isopentenyl diphosphate (IPP) and its double-bond isomer dimethylallyl diphosphate (DMAPP). They are synthesized in plants by two different pathways – the mevalonate (MVA) pathway in the cytosol and the methylerythritol phosphate (MEP) pathway in plastids [105].

The cytosolic MVA pathway is also present in archaebacteria, fungi and humans and was originally thought to be the only IPP producing pathway in plants [29]. The other more recently discovered MEP pathway is known also from eubacteria [140], green algae [150] and *Plasmodium* sp. [80]. The relationship of the two pathways in a plant cell and their supply of five-carbon units to various terpene classes according to current knowledge [138] is shown in Figure 3.

The MVA pathway has been known in plants for many years [110]. This pathway produces isoprenoid units used for the biosynthesis of sterols, brassinosteroids, polyprenols, dolichols, sesquiterpenes and farnesyl or geranylgeranyl moieties used for protein modifications [34, 79]. IPP and DMAPP units synthesized via the MVA pathway are derived from acetyl coenzyme-A (Ac-CoA) in 6 enzymatic steps (Figure 3). The primary regulatory step is the formation of MVA catalyzed by hydroxymethylglutaryl coenzyme-A (HMG-CoA) reductase (HMGR) [104]. Potent inhibitors of HMGR, including statins like mevinolin or lovastatin, are important in medical applications. The statins have become pharmaceutically valuable due to their ability to reduce the formation of cholesterol, one of the end products derived from MVA pathway precursors in animals [153].

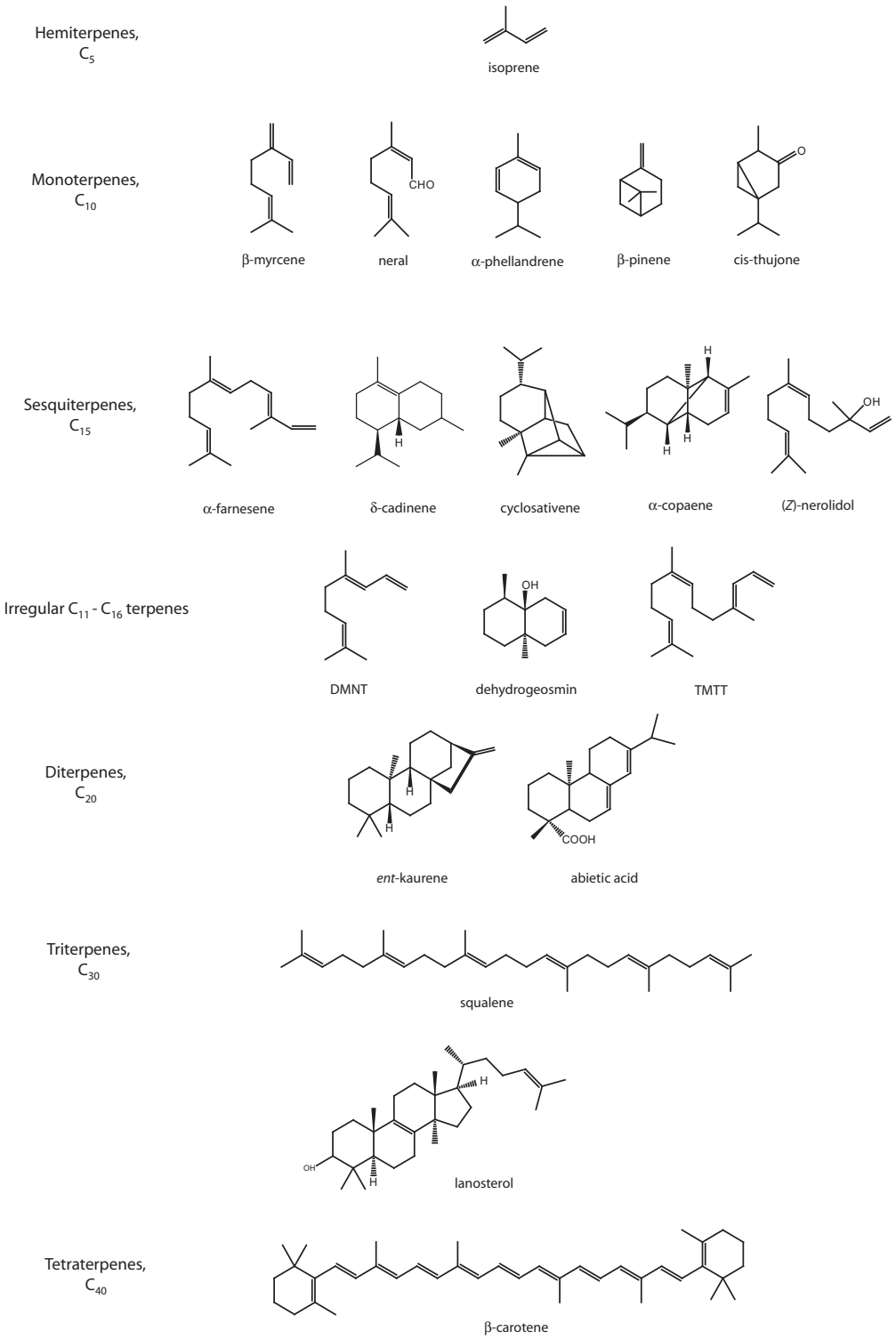


Figure 2: Representative structures of the different classes of terpenes synthesized in plants.

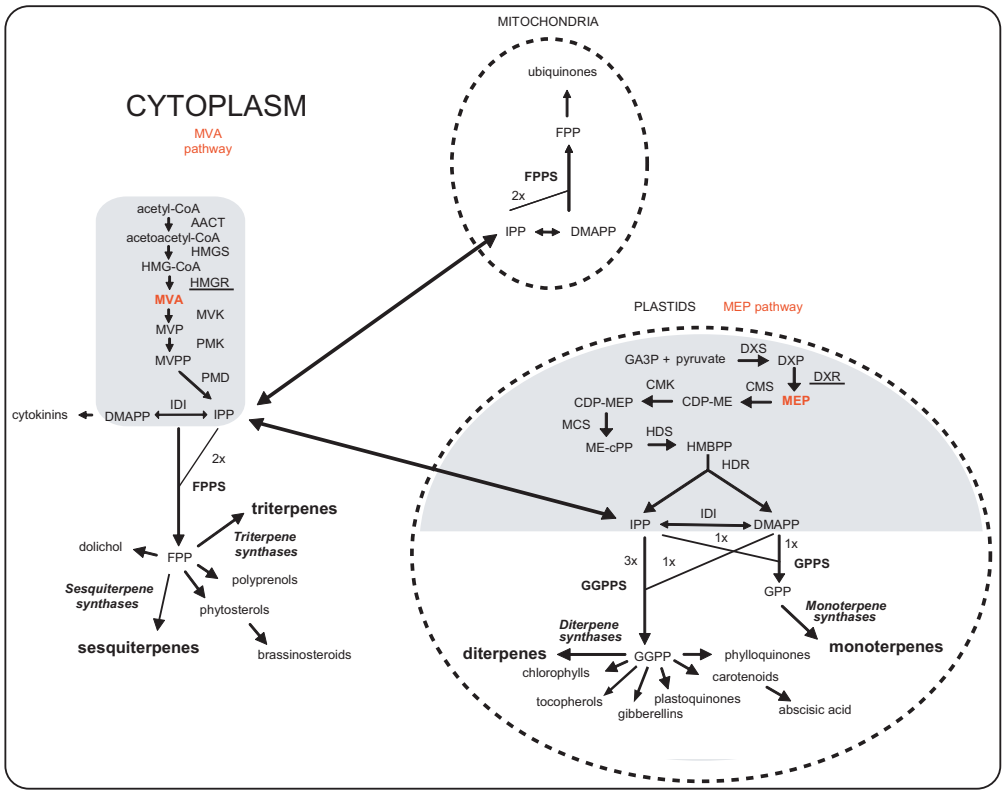


Figure 3: Schematic drawing of terpene synthesis in a plant cell with emphasis on the different compartments.

Compounds: HMG-CoA: hydroxymethylglutaryl coenzyme A; MVA: mevalonic acid; MVP: 5-phosphomevalonate; MVPP: 5-diphosphomevalonate; IPP: isopentenyl diphosphate; DMAPP: dimethylallyl diphosphate; FPP: farnesyl diphosphate; GA3P: glyceraldehyde 3-phosphate; DXP: deoxyxylulose 5-phosphate; MEP: methylerythritol 4-phosphate; CDP-ME: 4-diphosphocytidyl-methylerythritol; CDP-MEP: CDP-ME 2-phosphate; ME-cPP: methylerythritol 2,4-cyclodiphosphate; HMBPP: hydroxymethylbutenyl 4-diphosphate; GPP: geranyl diphosphate; GGPP: geranylgeranyl diphosphate

Enzymes: AACT: acetoacetyl CoA thiolase; HMGS: HMG-CoA synthase; HMGR: HMG-CoA reductase; MVK: MVA kinase; PMK: MVP kinase; PMD: MVPP decarboxylase; IDI: IPP isomerase; DXS: DXP synthase; DXR: DXP reductoisomerase; CMS: CDP-ME synthase; CMK: CDP-ME kinase; MCS: ME-cPP synthase; HDS: HMBPP synthase; HDR: HMBPP reductase; GPPS: geranyl diphosphate synthase; FPPS: farnesyl diphosphate synthase; GGPPS: geranylgeranyl diphosphate synthase

The MEP pathway leads to the building units which are used for the biosynthesis of isoprene, monoterpenes, carotenoids, plastoquinone, gibberellins, abscisic acid, phytol, tocopherols, phylloquinones, and some sesquiterpenes. The first enzyme in the pathway is 1-deoxyxylulose 5-phosphate (DXP) synthase (DXS), a thiamin pyrophosphate dependent transketolase that condenses pyruvate and glyceraldehyde 3-phosphate to form DXP (Figure 3, [100]). The next step yields methylerythritol 4-phosphate (MEP) by the enzyme DXP reductoisomerase (DXR) [155]. Many studies aimed at identifying the main regulatory control points of the MEP pathway have demonstrated that both DXS and DXR affect flux through the pathway. However, a single rate-limiting step has not been identified and rather seems to depend on the plant species organ and developmental stage in question. DXR can be specifically inhibited with the transition state analog fosmidomycin (Fos) [99], which results *in planta* in reduced production of α-tocopherol, chlorophylls, and carotenoids. MEP, once incorrectly proposed to be the first dedicated isoprenoid intermediate [156], is further converted in 5 enzymatic steps into a mixture of IPP and DMAPP in a fixed ratio of 6:1 [49, 139].

Once IPP and DMAPP are formed, the prenyltransferases condense them to elongated allylic diphosphates. Specific prenyltransferases produce each one of these products, geranyl diphosphate (GPP, $C_{10}$), farnesyl diphosphate (FPP, $C_{15}$), and geranylgeranyl diphosphate (GGPP, $C_{20}$). The prenyl diphosphate intermediates are further converted into $C_{10}$-monoterpenes, $C_{15}$-sesquiterpenes, and $C_{20}$-diterpenes by enzymes called terpene synthases (TPS) (Figure 3). The GPP synthase (GPS) is believed to be primarily located in plastids and to some extent in the cytosol [24], whereas FPP synthase (FPS) is found predominantly in the cytosol and mitochondria [35], but may also be located in plastids [144]. Different isoforms of the GGPP synthase (GGPS) were found in plastids, mitochondria and the endoplasmic reticulum [119]. Interestingly, the chain length specificity of GPS and FPS can be interconverted by mutation of a single amino acid. Sequence comparison and functional studies suggest that FPS is the phylogenetic progenitor of GPS [128].

TPS occur in form of large protein families with high sequence similarities as identification of many TPS from multiple plant families and species has shown. A remarkable feature of terpene synthases is the multi-product specificity. TPS are reported which convert one substrate into 14 different volatiles [160]. Similar to prenyltransferases, few amino acid changes in key active site positions can have drastic effects on the product spectrum of terpene synthases [94]. *Tps* genes from a wide range of different plant families have been cloned and characterized. Monoterpene synthases use GPP to form single or multiple monoterpenes, while sesquiterpene synthases act on FPP, and diterpene synthases use GGPP. Although each class of terpene synthases

displays a $K_m$ toward its native substrate in the low micromolar range, sesquiterpene and diterpene synthases can occasionally convert the shorter prenyl diphosphate substrates into hydrocarbon products albeit at low efficiencies. However, in the plant cell compartmentation of terpene synthases generally restricts access to specific substrates. The latter has not been proven yet without doubt.

Many essential plant compounds are derived from terpene biosynthesis. For example the diterpenoid-derived gibberellins are a class of important phytohormones with highly similar structures. Carotenoids and chlorophyll are also derived from terpene biosynthesis. However, our knowledge of specialized or ecological roles of many mono- and sesquiterpenes is limited. The reason for this may be the long held belief, that terpenes and other natural products are metabolic wastes and lack any further function for the producing organism. Starting from the 1970s, this opinion began to change because of results that suggested plant terpenes are toxic, growth inhibiting or repellent to other organisms. That let assume that they might serve prominent ecological roles [101]. A review covering the current knowledge on functions of selected terpenes was published recently [60].

## 1.3 Brassicaceae – a plant family to study variability and biology of plant volatiles

Members of the mustard family, the Brassicaceae (also called Crucifers or Cruciferae), have a long history as important crops. Representatives like *Brassica oleracea*, *B. rapa*, *B. nigra*, *Sinapis alba*, *Camelina sativa*, *Eruca sativa*, or *Lepidium sativum* are widely cultivated, and breeding has resulted in the development of a variety of important cultivars. Other genera like *Alyssum*, *Aubrieta* or *Matthiola* have become cultivated as ornamental plants and are widespread in garden culture. The species included in the Brassicaceae family are sometimes difficult to distinguish using only morphological features. Characteristics of the siliques are essential for determination of species [38]. Another important characteristic for the distinction of certain taxa is the presence and morphology of trichomes [129]. This monophyletic family, which is assumed to contain from 3350 [149] to 3709 species [7] is currently divided into 338 genera which sometimes have only one or very few species per genus [7]. However, traditional morphology-oriented taxonomy may not necessarily reflect a natural phylogeny. Due to the availability of large scale sequencing methods some years ago, the brassicaceaous *Arabidopsis thaliana* became the first flowering plant with a completely sequenced genome [76]. Traditional systematics gave way to comparative sequence analysis with other Brassicaceae species. Efforts are being undertaken to build a more natural taxonomy based on nuclear and plastidic gene sequences [19, 92, 93]. A broad taxonomic overview of the current tribal assignments within the Brassicaceae is given

by Al-Shehbaz *et al.* [7]. From a selected number of 48 taxa from 24 genera, the sequences of the genes *mat*K and *Chs* were compared and timepoints of divergence were inferred (Figure 4, [93]). Based on these results, hypotheses to reconstruct the phylogenetic history of the Brassicaceae were formulated (Figure 4). According to

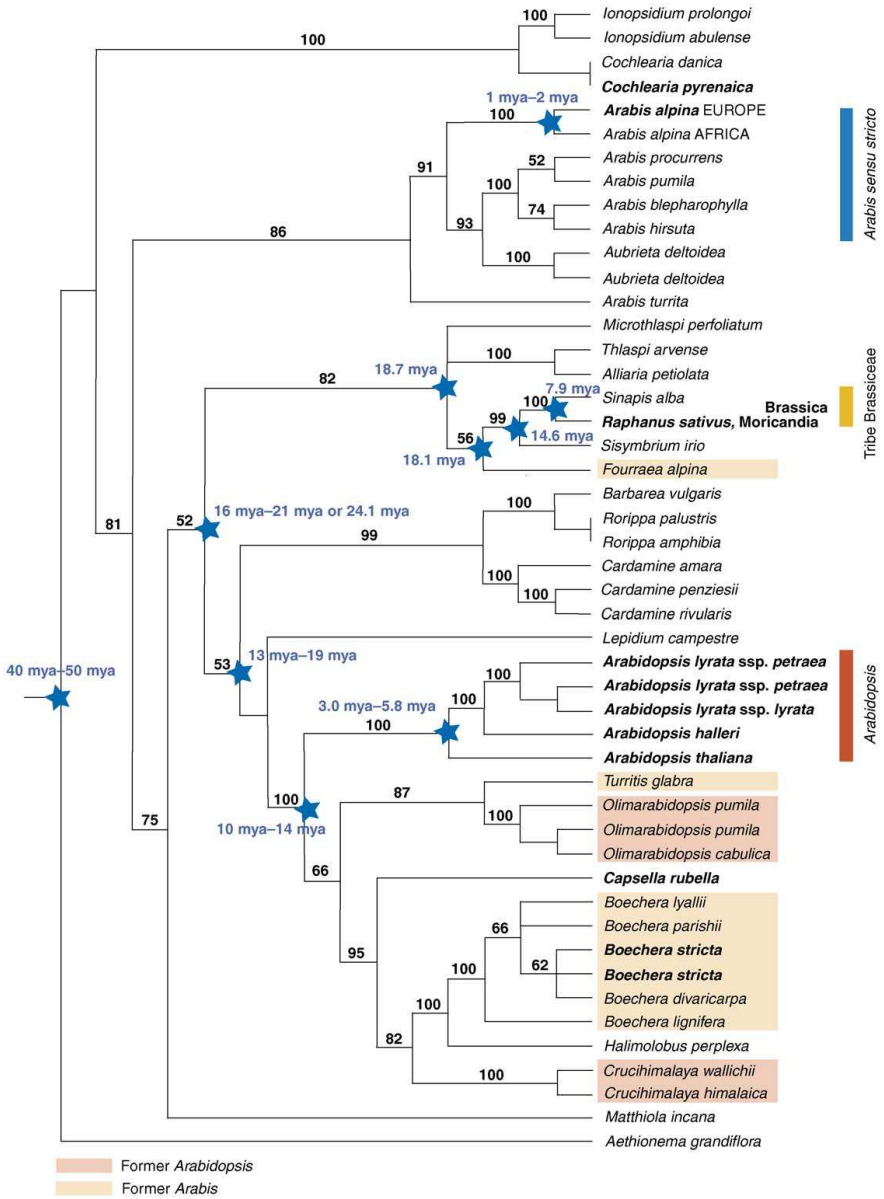


Figure 4: Phylogenetic context of the genus *Arabidopsis* within the Brassicaceae. A strict consensus tree from nine most-parsimonious trees based on *matK* and *ChsA* sequences is presented. Bootstrap values are indicated as a percentage of 1000 replications above the branches, asterisks indicate the presumed timepoints of divergence in million years before the present (mya). Figure is based on results from [93] and is reprinted from [32]. Permission to reprint the figure was obtained from [93] and [32].

this model, after the appearance of the Brassicaceae approximately 50 million years ago (mya), the lineage of the family split unequally at around 40 mya giving rise to modern day *Aethionema grandiflora* [90]. *Aethionema* is the most "basal", which does not mean most primitive, taxon in the Brassicaceae, as supported by all sequencing analyses up to now [19, 58, 64, 92]. All other members investigated diverged more recently, the basal ones being *Cochlearia* first and the european *Arabis* species (*Arabis* s. str.) with *Aubrieta* being next. Before the next estimated timepoint of divergence (16 – 21 mya), a group with modern *Matthiola*, *Braya*, *Hesperis*, *Bunias* and other genera split from the others [19]. At 16 – 21 mya, the Tribe Brassiceae with genera like *Thlaspi*, *Alliaria*, *Sinapis*, *Brassica*, *Raphanus*, *Sisymbrium*, and *Fourraea* split from the remaining branch. From that branch, another group is made up of the genera *Barbarea*, *Rorippa*, and *Cardamine* which split presumably around 13 – 19 mya ago. Around 10 – 14 mya ago, the remaining branch split up into the present *Arabidopsis* clade on the one side and on the other side the North American *Boechera* (named after the botanist Böcher, the genus was formerly included in *Arabis* [6]), *Turritis*, *Olimarabidopsis*, *Capsella*, and *Crucihimalaya*. The model plant *A. thaliana* is included in the *Arabidopsis* clade.

Traditionally, *Arabidopsis* was treated as monophyletic, but now it is regarded as paraphyletic [130] with the closest sister group being *Cardaminopsis* [122]. The newly

Figure 5: *Arabidopsis lyrata* ssp. *petraea*. Plants growing in a typical habitat in northern Thuringia on gypsum rock. Photo taken by the author.

described *Arabidopsis* clade comprising the genera *Cardaminopsis* and *Arabidopsis* represents the natural phylogeny according to current knowledge [121]. That resulted in the renaming of former *Cardaminopsis* species to *Arabidopsis* species, *A. lyrata*, *A. halleri* and *A. arenosa*. The current knowledge about these three species is comprehensively reviewed in [32]. Currently three subspecies are recognized of *A. lyrata*: *A. lyrata* ssp. *petraea* (Figure 5) distributed in central and boreal Eurasia, *A. lyrata* ssp. *lyrata* in North America and *A. lyrata* ssp. *kamchatica* in eastern Asia and northwestern North America [5]. In recent years, beside *A. thaliana,* these other *Arabidopsis* species have attracted more scientific attention and opened up new fields of research which could not be studied using one model plant alone. One obvious reason for this direction of research was certainly the high sequence similarity to the model plant *A. thaliana*, but other reasons were the different breeding systems, ecological preferences and life forms. *A. thaliana,* for example, has a generation time of 6 – 8 weeks and is inbreeding, with flowers having a weak scent (to human noses) composed mainly of sesquiterpenes [30, 160]. With knowledge about close relatives like the outcrossing *A. lyrata*, it is possible to infer ecological functions of particular traits. The research described here follows this approach.

## 1.4 Primary objectives of this study

The present work comprises three chapters which describe investigations of the chemistry and biosynthesis of volatile compounds emitted from *A. thaliana,* an in-breeding annual and *A. lyrata*, an outcrossing perennial. The results of the presented studies provide a basis for further investigations of the evolution and functional ecology of volatiles of the two Brassicaceae species. The primary objectives of the presented study were

(1) to determine differences and similarities of volatile terpene biochemistry in flowers and foliage of *A. thaliana* and *A. lyrata*,

(2) to examine the natural variation of floral volatiles among populations of *A. lyrata* in Central Europe, and

(3) to investigate the molecular mechanisms controling the variation of insect-induced terpene volatiles in different *A. thaliana* ecotypes.

# 2 Chapter 1: Comparative Chemical and Molecular Analysis of Induced and Constitutive Volatile Formation in *Arabidopsis lyrata* and *A. thaliana*

**Abstract**

Little is known about differences in constitutive and stress induced plant volatile emissions and the mechanisms controlling these differences among related plant species with significantly different life histories. The model plant *Arabidopsis thaliana* and its close relative *A. lyrata* ssp. *petraea*, provide a promising system for such studies since, compared to the self-pollinating annual *A. thaliana*, *A. lyrata* ssp. *petraea* is a perennial that is strictly outcrossing.

A comparative analysis of floral and herbivore induced volatile emission from plants of German *A. lyrata* ssp. *petraea* populations and *A. thaliana* indicates that the different life histories of the two closely related species are also reflected in their volatile profiles: whereas *A. thaliana* flowers emit only low amounts of sesquiterpenes, the floral scent of *A. lyrata* is characterized by benzenoids like benzaldehyde and phenylacetaldehyde. These compounds are released by a diurnal rhythm and known to be attractants of insect pollinators. Another difference is apparent after induction by insect feeding: *A. lyrata* emits (*E*)-β-caryophyllene from vegetative tissue, whereas wound-induced emissions from *A. thaliana* are dominated by TMTT and (*E*)-β-ocimene. Using a homologous PCR based approach with primers based on sequence information from *A. thaliana*, we found two terpene synthase (*TPS*) genes in *A. lyrata* vegetative tissue that are upregulated after herbivory. The transcripts showed a sequence identity of 85% compared to known sequences from *A. thaliana*. An (*E*)-β-ocimene synthase and a (*E*)-β-caryophyllene synthase could be functionally identified by *in vitro* enzyme assays. In floral tissues of *A. lyrata* populations in Germany, no *tps*-like genes were found to be expressed.

## 2.1 Introduction

*Arabidopsis thaliana* has become a valuable plant model system to investigate basic plant physiology as well as ecological aspects of plant biology [161]. However, the specialized niche of *A. thaliana* as a self-compatible annual of disturbed habitats, limits the range of ecological studies one can untertake with this species. To go further, some of the resources developed for *A. thaliana* can be applied to other species of the Brassicaceae. Within the *Arabidopsis* clade, *Arabidopsis lyrata* is a close relative to *A. thaliana* whose ancestors diverged ca. 5 million years ago [92]. During that time the species separated enough to develop different breeding systems and different life

histories. Meanwhile the genome was reduced from presumably 8 ancestral chromosomes as in modern *A. lyrata* to 5 chromosomes in *A. thaliana*. Still, on an individual gene nucleotide sequence level there is an overlap of ca. 85% between *A. thaliana* and *A. lyrata*. This close similarity makes this species pair an ideal system for comparative biological studies. Chief characteristics of the nearly worldwide occurring annual *A. thaliana* are its small weedy habit and self pollination. Thus this species is not appropriate for studying the features of perennials and floral biology of outcrossing systems. In comparison to *A. thaliana*, *A. lyrata* is different in several aspects. Three subspecies from different continents can be distinguished [121]. The subspecies *A. lyrata* ssp. *petraea* can be found in isolated habitats of central Europe, but it is mainly distributed in Eurasian boreal regions [143, 147]. The other two subspecies *lyrata* and *kamchatica* occur in North America and in East Asia, respectively.

Habitats of *A. lyrata* ssp. *petraea* are characterised by the absence of grassy vegetation. Plantlets can often be found rooting in small fissures directly on rocks. Types of rocks supporting growth are gypsum stone or basalt gravel, or dolomitic calcareous soil. The density of individuals rarely reaches more than 1 individual per $m^2$. Due to its ability to reproduce vegetatively by shoots that protrude from roots, clustering of rosettes is usually observed in the field. Each rosette produces one to several flowering shoots, with the terminal flower on the shoots available for pollination. If not pollinated, the flower senesces and new terminal flowers appear, a process that leads in a greenhouse to long unbranched shoots. If pollination leads to seed production, slender capsules with a length of 2 – 3 cm are produced.

The flowers are similar in shape and colour to the flowers of *A. thaliana*, but are two to three times larger with a size of 8 – 12 mm. They can be observed in the field from April until August, and extended flowering until October is described [32]. The species is strictly outcrossing, being pollinated in the summer mainly by bees and small flies.

Given the fundamental differences in life histories and breeding systems of *A. thaliana* and *A. lyrata*, we were interested in whether there would also be differences in their volatile profiles. A principal function of volatiles is in attraction of floral visitors. While the outcrossing *A. lyrata* depends on floral visitors to produce seeds, the self-compatible flowers of *A. thaliana* are also sometimes visited by insects [72]. Both species have white-coloured flowers. However, *A. lyrata* flowers usually produce a much stronger scent than *A. thaliana* flowers which is not unexpected given the self-incompatibility of the species and the need for pollinator attraction. The major floral volatiles of the North American subspecies *Arabidopsis lyrata* ssp. *lyrata* were shown to be benzenoids, benzaldehyde and phenylacetaldehyde [126], but nothing is known for *A. lyrata* ssp. *petraea*. Based on the different life forms of the two closely related

crucifers, any differences in floral volatiles may allow us to hypothesize a function for these chemical cues. Since floral scent may not only attract visitors for pollination, it may repel foraging insects searching for food or breeding opportunities. Therefore we also investigated whether scent emissions change during night and day. Diurnal emission patterns may also help shed light on the activity patterns of insects that visit flowering *A. lyrata*.

Another major function of volatiles is to serve as signals from herbivore-damaged foliage to attract herbivore enemies. Feeding by caterpillars and other insects is well known to induce the emission of volatiles in cultivated plants like maize [40], beans [13], or cotton [141] which attracts wasps that are parasitoids of the feeding larvae [146]. Unfortunately most of the studies on herbivore-induced volatiles have employed crop plants that have been uncoupled from natural evolutionary forces by breeding efforts. We investigated the herbivore-induced volatiles of *A. lyrata* to compare with the previously described volatiles from *A. thaliana* (Chapter 3 and [160]) to determine if life history, breeding system or habitat influence this trait using a species pair that has not been subjected to selection by humans.

## 2.2 Materials and Methods

### Plants

We sampled *A. lyrata* ssp. *petraea* (L.) Hiitonen from Germany, one of three subspecies of *A. lyrata*. The subspecies *A. lyrata* ssp. *lyrata* occurs only in North America and *A. lyrata* ssp. *kamchatica* (Fisch. ex DC.) O'Kane & Al-Shehbaz can be found in eastern Asia. *A. lyrata* ssp. *petraea* is also known as *Cardaminopsis hispida* (Myg.) Hayek, *C. petraea* (L.) Hiitonen or *Arabis petraea* (L.) Lam. In this study we apply the classification of O'Kane & Al-Shehbaz [121] and Koch et al. [91], who refer to it as *Arabidopsis lyrata* ssp. *petraea*. Plants and seeds were derived from populations in southern Germany (Germany, northern Bavaria, Fränkische Schweiz). One population was close to Neutras (NT), and another one was located nearby in Plech. For the designation of the Plech plants see Table 1. Plants were cultivated in plant climate rooms with a constant 22°C, 55% relative humidity (rel. H.) and 10-h light/14-h dark cycles with a light intensity of 160 $\mu$mol $m^{-2}$ $s^{-1}$ PAR.

Seeds from a controlled crossing experiment performed with the Plech population [33] were treated for germination as follows: seeds were placed on wet filter paper in petri dishes and vernalized in the dark at 4°C for 2 – 3 weeks. The substrate for growing plants was an autoclaved mixture of vermiculite, fertilizer, and Arabidopsis soil. Germinated seeds from which lines could be established were placed in plant climate chambers on wet substrate and covered with a translucent plastic dome to preserve high humidity conditions.

As *A. lyrata* is self-incompatible and leads by cross-pollination to highly variable successors, clonal plant lines had to be established. Plants were propagated vegetatively to obtain clonal lines by separating shoots that emerged from the same rootstock. The shoots were further cultivated on soil. Mature plants were available two months later. Designations for seeds and lines used are given in Table 1.

Table 1: Designations for seeds and lines of *A. lyrata* from a controlled crossing experiment which were used in this study. The designated male and female plants were derived from field collections described elsewhere [33]. Crossing of the male and female plants led to seeds from which several individual plant lines could be established.

| Male | Female | From 5 seeds germinated | More than 3 clones could be established from lines |
|---|---|---|---|
| 59-2 | 59-5 | 4 | Gr4A1, Gr4A2, Gr4A4 |
| 61-5 | 50-1 | 5 | 6A4 |
| 56-3 | 61-4 | 4 | 8A4 |
| 80-1 | S-4 | 5 | 11A2, 11A4 |
| S-5 | D-3 | 2 | 14A1 |
| 73-2 | 83-2 | 3 | 22A1, 22A2 |
| R-9 | 50-3 | 2 | 24A1, 24A2 |
| FR-3 | I-4 | 4 | 2B1 |
| R-3 | C-4 | 5 | 16B3 |
| M-4 | R-7 | 5 | 23B1, 23B3 |

## Insects

Insect-feeding experiments were performed with larvae of a *Plutella xylostella* G88 colony. Larvae were reared on artificial diet with a wheat germ base at 27°C with 18-h light/6-h dark cycle [134] and were used at their third or fourth instar, when they had a size of ca. 8 – 10 mm.

A dynamic headspace volatile collection system (see Volatile Collection) was used for the experiments. Potted plants were placed at day 0 in the collection system. In the morning of day 1, 20 – 30 larvae of *Plutella xylostella* were taken from the artificial diet and placed on the leaf surface of plants. Volatiles were collected for 8 h. Twenty four hours later, larvae were removed from the plants and volatiles collected for another interval of 8 h. On day 3 a final volatile collection of 8 h was performed.

## Volatile Collection

A dynamic headspace volatile collection system was employed for herbivory experiments and other longer-term collections. The system was installed in a plant climate chamber operating at 22°C, 70% r. H. and 150 $\mu$mol $m^{-2}$ $s^{-1}$ PAR with a 12-h light/12-h dark cycle. The design of airflow allowed collection from 6 plants to be sampled in parallel with defined flow rates for each sample. The soil and root balls of plants were wrapped in aluminium foil and the complete plant was placed in a dessicator of 3 L volume. Continuous dry air was passed over charcoal filters and purified by a Whatman Balston Zero Air Generator (Whatman, Tewksbury, MA, USA) with a maximum flow rate of 18 L/min. The flowrate of air into the plant container was 1.2 L/min, while air was actively pumped out with a rate of 1.0 L/min. Air moisture was regulated by passing half of the incoming air through a flask containing distilled water. Volatiles were collected by passing the exiting air over a trap with 5 mg of activated charcoal placed directly at the outlet of the glass container.

Less abundant volatiles from flowers were collected with a more sensitive closed loop stripping method [44]. Four to 15 individual flowers were cut and transferred to 10 mL glass beakers filled with tap water and placed in a 2 L glass dessicator in a plant climate chamber. Air circulated continuously with a flowrate of ca. 1 L/min, passing over a trap with activated charcoal. Floral volatiles were collected over a period of 8 h during the light phase.

Headspace solid-phase micro extraction (SPME) [176] was used for the analysis of flower organ specific volatile emission and for collecting products of enzyme assays. Fibers used were PDMS-100 (100 $\mu$m Polydimethylsiloxane) and CW/DVB-65 (65 $\mu$m Carbowax/Divinylbenzene) fibers (Supelco, Bellefonte PA, USA). Single flowers or parts were enclosed in a 1 mL glass vial sealed with a septum cap. The septum was punctured and the SPME needle inserted without touching any sample material. The complete system was incubated at 25°C and the fiber exposed for 30 min to the headspace of samples. Following exposure, the fiber was inserted into a GC-MS system as described below.

Adsorbed volatiles were eluted from the charcoal trap with 50 $\mu$L $CH_2Cl_2$ with nonylacetate or 1-bromodecane at a concentration of 2 ng/$\mu$L as internal standard (IS). 1 $\mu$L of the eluate was analyzed with a Hewlett-Packard 6890 gas chromatograph cou-

pled to a Hewlett-Packard 5973 quadrupole mass selective detector. Separation was performed on (5%-phenyl)-methylpolysiloxane columns (J&W Scientific, Folstom, CA, USA) of 30 m x 0.25 mm i. d. x 0.25 $\mu$m thickness. Helium was the carrier gas (flow rate of 2 mL/min). A splitless injection was used, and a temperature program starting at 45°C with a ramp of 5°C/min to 180°C followed by a ramp of 60°C/min to 300°C. Mass spectrometry was performed with a transfer line temperature of 230°C, source temperature of 230°C, quadrupole temperature of 150°C, ionization potential of 70 eV, and scan range of 35 to 350 atomic mass units. Compounds were identified using mass spectral libraries NIST98 (Agilent Technologies, Palo Alto, CA, USA) and Wiley275 and confirmed with authentic standards where available. For quantification, selected ion peaks of each compound were integrated and related to the response of the internal standard (SIM, selected ion monitoring).

An unknown benzenoid, detected with charcoal adsorption but not with PDMS-100 and CW/DVB-65 SPME fibers, could not be further identified. The EI mass spectrum of the unknown product had main fragments with m/z 51, 77, and 105, a pattern which is characteristic for a variety of benzenoids. Several compounds suggested by the MS libraries NIST98 and Wiley were analysed by GC-MS, but all of them had retention times different to the unknown benzenoid.

### Proton-Transfer-Reaction Mass Spectrometry (PTR-MS)

Proton-transfer-reaction mass spectrometry (PTR-MS) is a real-time technique for long-term continuous measurements of volatile emission with limited analyte fragmentation due to the low energy input used for protonation. The detection allows a high temporal resolution of less than 1 min. Details of the analytical technique are described elsewhere [39, 106].

A mobile Proton-Transfer-Reaction Mass Spectrometer (PTR-MS from Ionicon Analytik, Innsbruck, Austria) was used to measure continuously the emission pattern of whole flowering *A. lyrata* plants. One flowering plant (A) and one plant in a vegetative state (B) were placed in desiccators of 2 L volume. Desiccators were supplied with scrubbed (free of volatile organic carbons and ozone) and humidified air (~ 50% r. H.) at a flow rate of 1 L/min. Light was obtained from two sodium lamps and the temperature range was between 23°C (night) and 28°C (day). Volatile emission from *A. lyrata* flowers and leaves was measured by alternating the air flow every 20 min between A and B with the help of an automatically controlled valve switch. The emission of floral and leaf volatiles was measured continuously for 46 hours, including two nights and days. The PTR-MS was run at an E/N mode of 120 and compounds of interest were measured in selected ion mode including the protonated masses of benzaldehyde (m107), phenylacetaldehyde (m121), monoterpene olefins (fragments of m81 and m137), methylsalicylate (m153), and sesquiterpene olefins (fragments of m149 and m205). PTR-MS data were corrected for mass discrimination and for the background signal measured from each empty desiccator. Total sesquiterpenes were calculated from the contribution of measured fragments relative to the total fragment masses.

### RNA Extraction and cDNA synthesis

For RNA extraction of leaves, 1 g fresh weight leaf material was ground to a fine powder under liquid nitrogen and incubated with 10 mL TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) for 15 min at 25°C. Two mL chloroform were added and the extract again incubated for 3 min at 25°C. Phases were separated by centrifugation (12.000xg, 15 min at 4°C). The aqueous phase was removed and mixed with 2.5 mL isopropanol and 2.5 mL high salt precipitation buffer (1.2 M sodium chloride, 0.8 M sodium citrate). Precipitated RNA was separated by centrifugation (12.000xg, 10 min at 4°C). After washing the RNA with 80% ethanol, the pellet was air-dried and dissolved in 200 $\mu$L DEPC-treated water. The quality of RNA was determined by separating RNA on a formaldehyde-agarose gel with ethidiumbromide and analyzed under UV-light.

Floral RNA was extracted from 100 mg fresh weight of flowers with the RNA-Extraction Kit RNeasy (Qiagen, Hilden, Germany) according to the manufacturer's protocol. An on-column DNA digestion was performed with DNase (Qiagen, Hilden, Germany). The quality of RNA was analyzed with the microcapillary analyzer Bioanalyzer 2100 (Agilent, Palo Alto, CA, USA).

Synthesis of cDNA was done with 4 $\mu$g of total RNA. cDNA was produced by reverse transcription with an engineered version of M-MLV reverse transcriptase from Invitrogen (SuperScript III RNase $H^-$ Reverse Transcriptase, Invitrogen, Carlsbad, CA, USA). Two hundred units were used in a total volume of 20 $\mu$L at 50°C for 60 min, followed by an inactivation at 70°C for 15 min, according to the manufacturer's protocol.

### PCR and Sequencing

Polymerase chain reaction (PCR) was performed with Platinum*Taq* polymerase (Invitrogen, Carlsbad, CA, USA) for standard procedures or *Pfu*Turbo (Stratagene, La Jolla CA, USA). PCR amplification from cDNA was done in independent triplicates. Each PCR cloned fragment and three clones of each replicate were sequenced with vector- and fragment-specific primers to confirm correct amplification. One $\mu$L of template cDNA was used per reaction; primer concentrations were 5 $\mu$M. Primers used in this study to detect expressed sequence tags are listed in Table 2. Terpene synthase transcripts were detected with primers that were designed based on a homology search from the *A. thaliana* NCBI database (http://www.ncbi.nlm.nih.gov).

A T-Personal and T-Gradient Thermal Cycler (Biometra, Göttingen, Germany) were used with an initial denaturation step of 96°C for 3 min, followed by 30 cycles of 94°C for 20 s, annealing temperature for 30 s, 72°C for 2 min, and a final elongation step of 72°C for 10 min. The annealing temperature was adjusted to the characteristics of each individual primer pair and was in the range of 52–68°C. Initial cloning of PCR amplified fragments was done with the vector pCR4-TOPO (Invitrogen, Carlsbad, CA, USA) and propagated in *E. coli* Top10 (Invitrogen, Carlsbad, CA, USA). Sequencing reactions were performed with the Big-Dye system Version1.1 from ABI (Applied Biosystems, Foster City, CA, USA), purified over Sephadex G50 superfine (Fluka, Buchs, Switzerland) 96 well plates and analyzed on a ABI Prism

Table 2: Primers used in this study to detect terpene synthase transcripts.

| Oligo | Sequence 5'–3': fwd, rev | Specific for |
|---|---|---|
| SL1 | TATATTTGATGTAATCATCG, TTGAACCATAGCAGTGAAGAG | At3g25810 |
| SL2 | TATTTGATGTGATCATCGACC, GGAACACTTAAGATATAAAAGGT | At3g25830 |
| SL3 | CTGGTGGATGGAGACAGGTTT, CGGTGAGGTTACAAGGTCGTT | At2g24210 |
| SL4 | GAGACAAAGGATCAAATGGAG, CCATTGGTCTTTAACATAGAA | At4g16740 |
| SL5 | TAAAGAAGAGGTGAGGAAGAC, CTAGAAATAAGTTTAAGTTCT | At4g16730 |
| SL6 | ATGATCGATGTCATTCAAAGT, TTAAATGTTTGAGACATTTCTC | At1g61680 |
| SL7 | CAGGAAATGGATGATCTTTGGA, GTGGTTAGTGTTTTCAGATCTTG | At1g70080 |
| SL8 | GAAATTGATAGCCTTGGGAGA, TTCACGATAGTTTGAAGCTCT | At5g48110 |
| SL9 | GATATGGATATGCTTACAATAG, CTTTCGACACAATCAAGAAGC | At2g23230 |
| SL10 | GAAATGAATGCCCTTGCCCA, TCAGAAACATTACCGTATCTA | At4g15870 |
| SL11 | GAAATGGATGTTCTTGAAAGA, ATTGATGAGGCTTTCGACCTC | At3g29110 |
| SL12 | GAAATGGATGCGCTTAGGAAA, CTCTCCAGACTATTGGTGAGG | At4g20210 |
| SL13 | ATTTCTTTAGGAGATTCAAAGG, CTCTTCTATTGTGGCACACAC | At3g29190 |
| SL14 | GAAATGGATGCACTTAAAGAA, GTGTATTTCACACTGTAAGAT | At4g20230 |
| SL15 | GAAATGGATGCCCTTAGAAAA, CTCTTCAAAAGTATCCAAAAT | At4g20200 |
| SL16 | ATGTATTCAGAAGATTCAAAGGGAG, TAGAATCGCTAAACAAGTGAAGTAC | At1g66020 |
| SL18 | TGGAGGAAAATATAGTGATAT, CGGTGCTGAGGTATGTGAAGA | At5g44630 |
| SL19 | AAACAAATGTCCAGATGGGAT, TCAAAGAGGTATTGGATGGAG | At3g29410 |
| SL20 | ATGGCAGTAGCAAGAACGGTT, CTCTTGGATGTAATGTAAACG | At3g32030 |
| SL21 | ATGGGTTTTCGAGCTAAAACT, TTGGTGAGAGTTTTTAGCTCT | At1g33750 |
| SL22 | TGAAGCCAAAAGTGAGAGACATG, GCTCTTGGATATAATGTAGCTGGC | At3g14490 |
| SL23 | GAGTTTGATGAGCTAGAAAGAGAGATTG, GTGAGCGTTTTGAGTTCTTGGAC | At1g31950 |
| SL24 | ATGCCTTTGATAGATTCAGAG, CTCGTAGGTTCCTACGTCATT | At3g14520 |
| SL25 | ATGCCTTTGATAGATTCAGAG, CTCGTAGGTTCCTACGTCATT | At3g14540 |
| SL27 | GGAACTGAGACGTTCAAAGAG, CGCTGTGAATAAGATTAGTGC | At5g23960 |
| SL28 | ATGGGAAGGAGAAGAGCTTAA, TTAGTAGAAGCATGGTGCGAAT | At1g61120 |
| SL29 | TCAAAGGGAAGAGAAGCATA, CGATATGAGGTTTTTGTAACTC | At1g79460 |
| SL30 | ATACCAAAAGAGATAATGCA, CTCGCCAGGTAAGTCTTTCAT | At4g02780 |
| SL31 | GTGTATTCAAGAGATTTACAG, CTTTCCACACAATCTACGAGC | At4g13280 |
| SL32 | GCTTGTGAGTCTTGGTCTCGC, AGCGAGGTGCAGTTCCACGAT | At1g48800 |

Gen-Analysator 3100 sequencer with 16 capillaries (Applied Biosystems, Foster City, CA, USA).

### Protein Expression and *in vitro* Enzyme Assays

Standard protein expression was performed with *E. coli* BL21(DE3) (Invitrogen, Carlsbad, CA, USA) and *E. coli* BL21(DE3) Codon Plus (Stratagene, La Jolla, CA, USA) cells. Plasmid pCRT7/CT-TOPO (Invitrogen, Carlsbad, CA, USA) with the coding sequence of interest was transformed freshly and three liquid cultures were set up from individual clones. Induction was performed at 18°C overnight with 1 mM isopropyl-1-thio-β-D-galactopyranoside in LB medium with ampicillin. Terpene synthase activity was measured by determining the turnover of the substrates geranylpyrophosphate (GPP) or farnesylpyrophosphate (FPP) in an aqueous environment into volatile products. A crude recombinant protein extract was obtained from a 100 mL *E. coli* culture. Cells were harvested at 4°C, washed with 20 mL wash buffer (20 mM Tris-HCl pH 7.5 and 50 mM KCl) and resuspended in 5 mL MOPSO buffer (50 mM MOPSO pH 7.0, 5 mM $MgCl_2$, 10% glycerol, 5 mM DTT, 5 mM sodium ascorbate, 0.5 mM PMSF). Cells were lysed with a sonicator and the cell debris separated from crude extract by centrifugation at 20.000xg for 20 min and 4°C. The supernatant was desalted using a Bio-Rad Econo 25 column. One hundred $\mu$L of the purified eluate were used for crude protein enzyme assays in a total volume of 500 $\mu$L. For partially purified 6xHis tagged protein extract, 10 $\mu$L were used in a total assay volume of 500 $\mu$L. Assays were performed in 1 mL glass vials closed with a septum. A 100 $\mu$m polydimethylsiloxane SPME-fiber was inserted through the septum and exposed to the headspace of the sample. The assay was incubated at 30°C for 1 h and adsorbed volatiles were analyzed with a GC-MS system using conditions as described above.

## 2.3 Results

### Flowers from *A. lyrata* ssp. *petraea* populations in Germany emit benzenoid metabolites but no terpenes

Sampling of floral scent from *A. lyrata* ssp. *petraea* with dynamic headspace collection as well as with closed loop stripping revealed the absence of any floral emitted terpenes in contrast to *A. thaliana*. Instead, we found emission of benzenoid derivatives from whole flowering plants as well as from single flowers using headspace solid-phase micro extraction (SPME) analysis. Figure 6 shows a typical volatile profile of flowering and non-flowering *A. lyrata* ssp. *petraea* determined via dynamic headspace sampling. By conducting SPME analysis of volatiles from separated floral organs, the two main benzenoid compounds, benzaldehyde and phenylacetaldehyde, were found to be emitted exclusively from flower petals, and not from other organs (Figure 7).

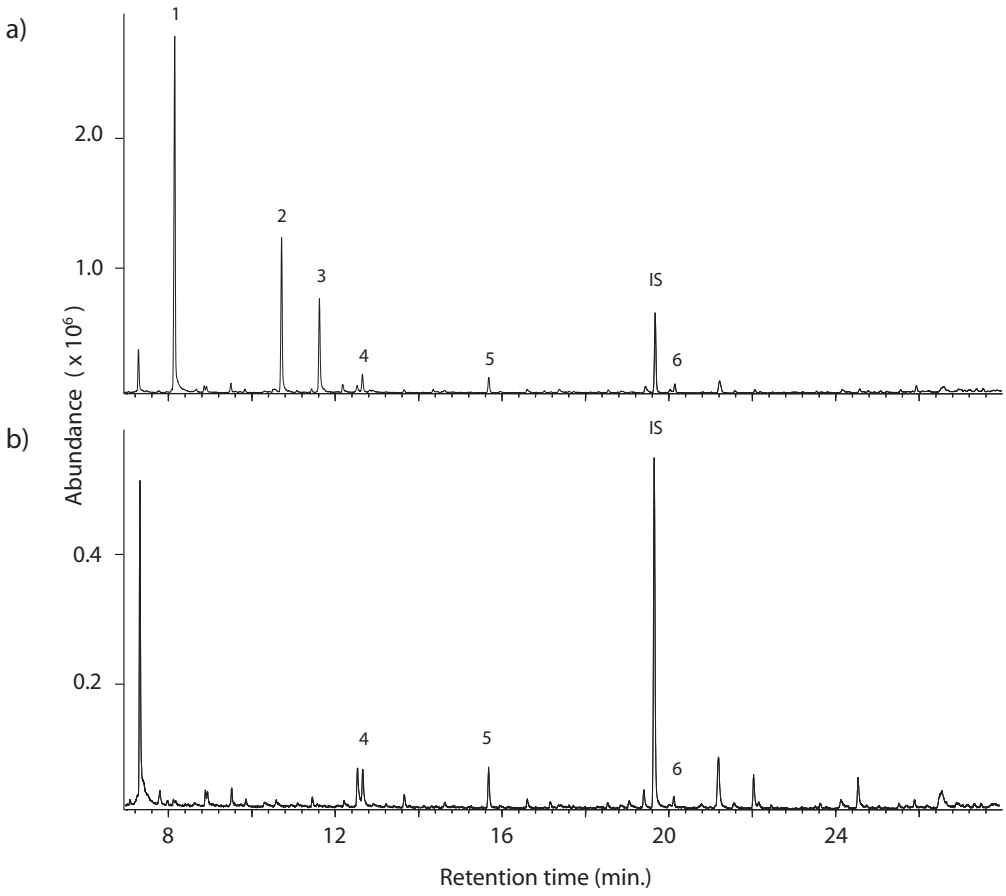


Figure 6: GC-MS chromatogram (total ion current) of volatiles emitted from *A. lyrata* ssp. *petraea* during (a) and prior (b) to flowering. 1: Benzaldehyde, 2: Phenylacetaldehyde, 3: unidentified benzenoid compound, 4: Nonanal 5: Decanal 6: (+)-Cyclosativene

Other compounds, including C-8, C-9, and C-10 aldehydes, were observed in trace amounts from vegetative tissue. In addition, the sesquiterpene (+)-cyclosativene was detected reproducibly from leaf tissue in small amounts. The identification of (+)-cyclosativene was confirmed with an authentic standard (Fluka). To discriminate from the commercially not available isomer (+)-cycloisosativene, which has the same EI mass spectrum, samples and standard were separated on a DB5 and a DB-wax column. The retention times of the standard (+)-cyclosativene and the sample on the DB5 column was 22.03 min and on the wax column 13.59 min. The EI mass spectra of the standard (+)-cyclosativene and the sample were identical (Figure 13).

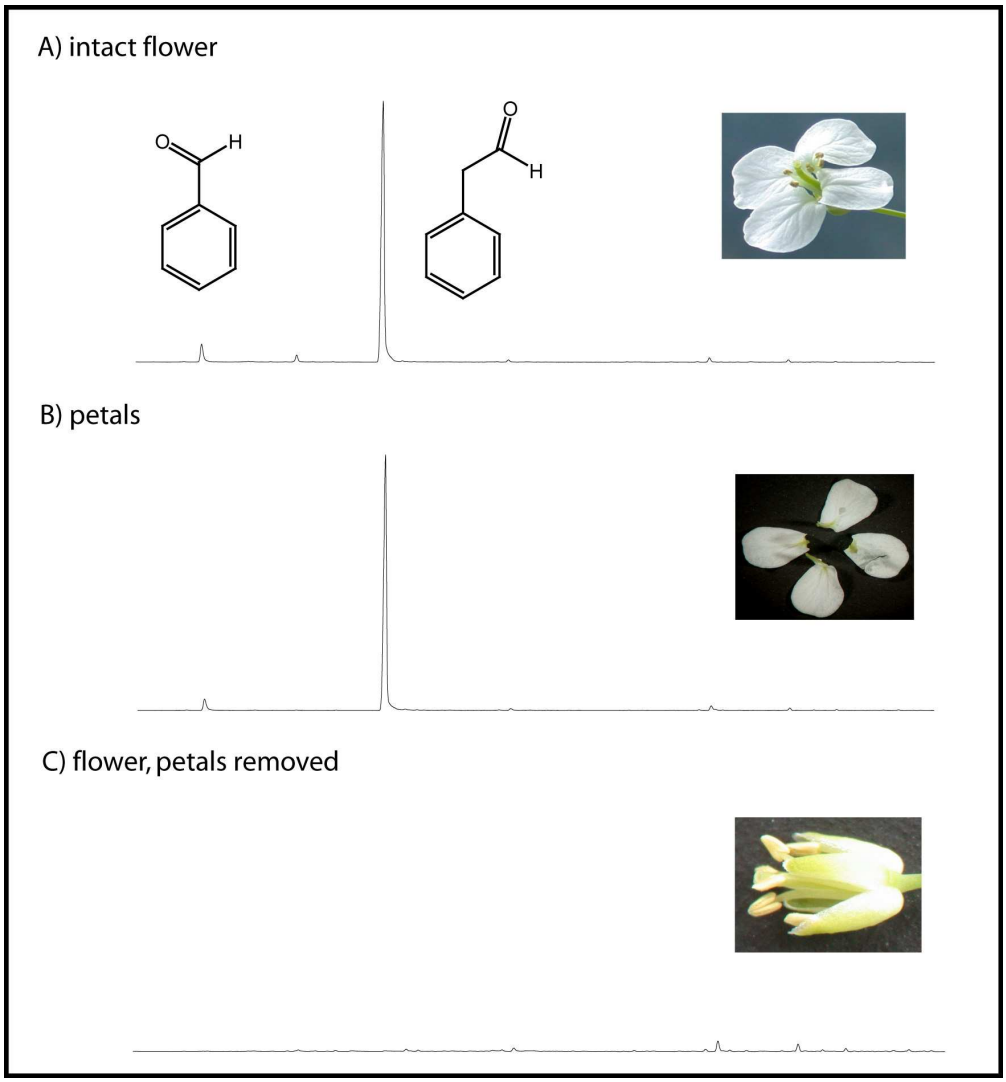


Figure 7: Floral organ volatile analysis. Flowers were manually dissected and scent emission of the different organs was monitored with headspace SPME. The GC-MS analysis revealed that benzaldehyde and phenylacetaldehyde were emitted from petals only.

## Diurnal rhythm of volatile emissions from *A. lyrata*

Floral scent is usually emitted in a temporally variable manner to attract putative pollinators at their time of highest foraging activity. We determined the temporal change of floral emission by continuous volatile measurements over 48 h. A diurnal rhythm of emission for the benzenoids and the sesquiterpene (+)-cyclosativene was evident after continuous monitoring of emission from a plant in full bloom with a dynamic headspace system (Figure 8). Volatiles were collected over 48 h in 2 h intervals and analysed by GC-MS. From flowering plants, the most abundant compound detected during light was benzaldehyde with amounts of roughly six fold higher than during darkness: the emission peaked approximately one hour after light onset and began to decrease around six hours later. At the beginning of darkness, the level was already low and it remained low until 1 – 2 hours before the onset of light. A similar pattern was observed for phenylacetaldehyde, though this compound is app. 10 fold less abundant than benzaldehyde. An unknown benzenoid product had a similar emission pattern and abundance as benzaldehyde. (+)-Cyclosativene was the only sesquiter-

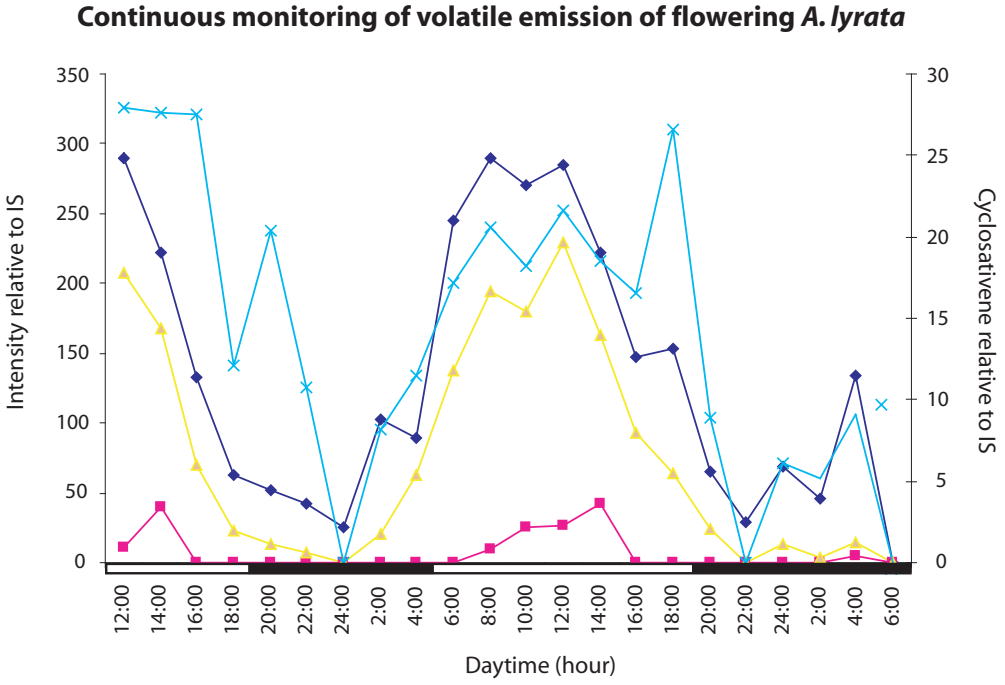


Figure 8: Volatile benzenoid and terpene emission monitored with a dynamic headspace system. Volatiles were collected for 48 h in two hour intervals on active charcoal. Main volatile compounds: benzaldehyde ◊, phenylacetaldehyde □, (+)-cyclosativene ×, an unknown benzenoid compound △. The ratios of the aldehydes was different with PTR-MS and SPME detection, the unknown benzenoid was also not detected with these two methods. The scale for (+)-cyclosativene is on the right.

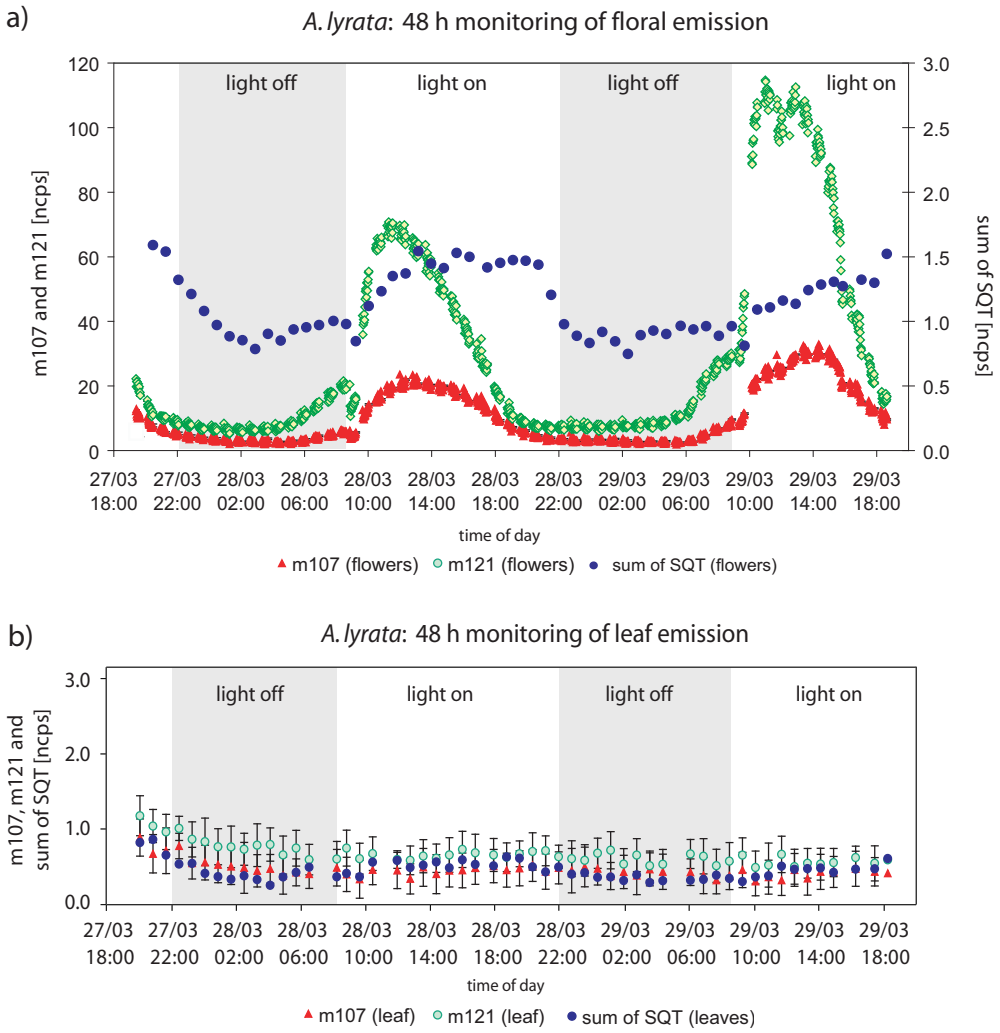


Figure 9: Continuous 48 h high-resolution volatile emission pattern of *A. lyrata* ssp. *petraea* recorded by PTR-MS. A whole flowering plant (a) is compared with a plant without flowering shoots (b). The detected masses of benzaldehyde △ (107), phenylacetaldehyde ♢ (121) and sesquiterpene olefins ◯ (205) are shown. In (a) the scale for the latter is on the right side due to the low amounts of sesquiterpenes. ncps: normalized counts per second

pene detected and its emission was attributed to leaf tissue only. Its emission pattern was similar to benzaldehyde, but the peak emission was shifted to ca. 2 hours later compared to benzaldehyde, whereas the overall abundance was around 100 fold lower.

As the ratio of the aldehydes benzaldehyde and phenylacetaldehyde detected with the dynamic headspace collection was different to that detected with SPME, we used a third volatile detection method to investigate diurnal rhythm. Proton-transfer reaction mass spectrometry (PTR-MS) [159], confirmed the rhythmic emission of volatiles obtained by dynamic headspace sampling. Volatile emissions of a flowering *A. lyrata* (Figure 9 a) in a glass jar were detected on-line over 48 h in comparison to a plant with only vegetative tissue (Figure 9 b). The flowering plant showed a clear light-dependent emission of m/z 107 (benzaldehyde) and m/z 121 (phenylacetaldehyde). The signal intensity of m/z 205 (total of sesquiterpenes) was more than 100 fold lower than m/z 107 or 121 but also follows a light dependent emission pattern. Other m/z signals detected did not show this kind of oscillation and remained lower than background intensities. The analysis of a plant with vegetative tissue only showed the same light-dark pattern for m/z 205 with a similar signal intensity, but neither m/z 107 nor m/z 121 could be detected (Figure 9 b).

## Terpenes are emitted from vegetative tissue of *A. lyrata* upon insect-feeding and show high intraspecific variability

As we concluded that terpenes are not part of the floral scent of German *A. lyrata* populations, we wondered whether they might be induced by insect feeding as it is the case for *A. thaliana*. Feeding experiments on *A. lyrata* were performed with caterpillars of *Plutella xylostella*, a lepidopteran specialist feeding on Brassicaceae species. Volatile analysis after feeding revealed that herbivory also induced volatile emission from *A. lyrata*. Several lines were tested from the Neutras population (Germany, Fränkische Schweiz), and most lines showed inducible emission. Volatiles were detected not earlier than 15 hours after insect feeding began. A qualitative comparison of herbivore-induced plants versus controls (Figure 10) shows intraspecific variability of the volatile blends. The C-11 homoterpene, 4,8-dimethyl-1,3,7-nonatriene (DMNT), was frequently detected during herbivory, although it was not present in every line investigated. When the C-16 homoterpene 4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT) was detected, usually the terpene alcohol nerolidol was detected as well. The monoterpene (*E*)-β-ocimene was emitted from some lines, the only sesquiterpenes detected were (*E*)-β-caryophyllene accompanied by minor amounts of α-humulene. The benzenoid methylsalicylate was present only in few plants .

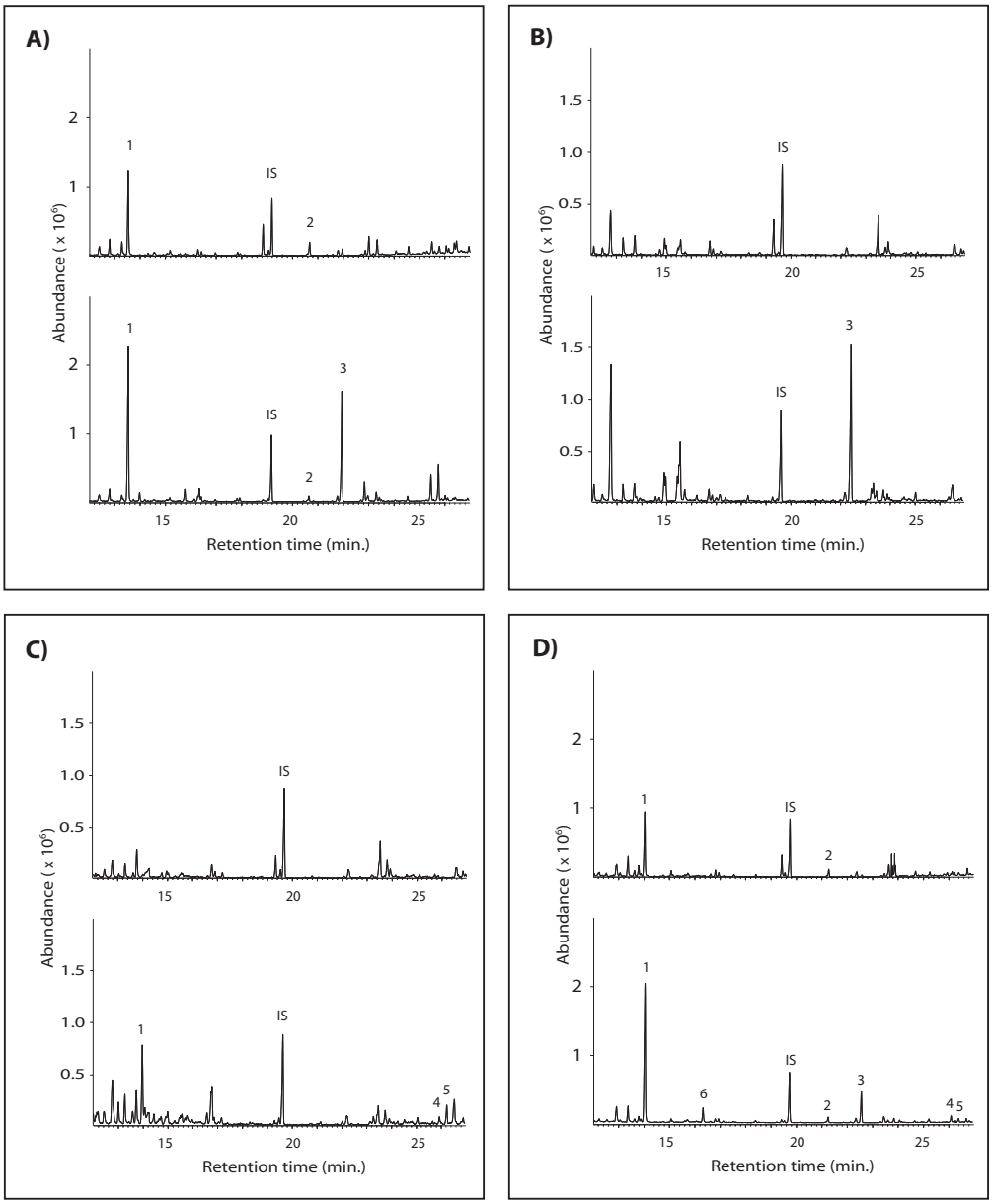


Figure 10: Qualitative comparison of volatiles emitted from *A. lyrata* Neutras (NT) population lines after herbivory by *Plutella xylostella*. Each upper chromatogram is a total ion current chromatogram from control plants, the lower ones show the emission during herbivory. NT-lines presented: A: NT16, B: NT20, C: NT35, D: NT51. Volatiles: 1: DMNT, 2: (+)-Cyclosativene, 3: (*E*)-β-Caryophyllene, 4: Nerolidol, 5: TMTT, 6: Methylsalicylate, IS: Internal Standard

Table 3: Detection of terpene synthase transcripts in *A. lyrata* before and after herbivory by *Plutella xylostella*. Shown are results of experiments with line NT20; in brackets are transcript patterns of other lines: + = transcript present, - = no transcript detected

| Locus | TPS-Nr. | Herbivore damage | Control |
|---|---|---|---|
| At3g25810 | 1 | - | - |
| At3g25830 | 2 | + | + |
| At2g24210 | 3 | - (+) | - (+) |
| At4g16740 | 4 | - (+) | - (+) |
| At4g16730 | 5 | - (+) | - (-) |
| At1g61680 | 6 | - | - |
| At1g70080 | 7 | - | - |
| At5g48110 | 8 | - | - |
| At2g23230 | 9 | - | - |
| At4g15870 | 10 | - | - |
| At3g29110 | 11 | - | - |
| At4g20210 | 12 | - | - |
| At3g29190 | 13 | + | + |
| At4g20230 | 14 | - | - |
| At4g20200 | 15 | - (+) | - (+) |
| At1g66020 | 16 | - | - |
| At4g13300 | 17 | - | - |
| At5g44630 | 18 | - | - |
| At3g29410 | 19 | - | - |
| At3g32030 | 20 | - | - |
| At1g33750 | 21 | - | - |
| At3g14490 | 22 | + | + |
| At1g31950 | 23 | - | - |
| At3g14520 | 24 | + | + |
| At3g14540 | 25 | - (+) | - (+) |
| At1g54660 | 26 | - | - |
| At5g23960 | 27 | + | - |
| At1g61120 | 28 | - (+) | - |
| At1g79460 | 29 | + | + |
| At4g02780 | 30 | + | + |
| At4g13280 | 31 | - | - |
| At1g48800 | 32 | - | - |

### Terpene synthase genes are upregulated in leaves of *A. lyrata* in response to herbivory

The sesquiterpenes detected as herbivore-induced volatiles of *A. lyrata* are direct products of terpene synthases. Therefore we wanted to investigate the possible upregulation of terpene synthase (*TPS*) genes after herbivory. Because of the high sequence similarity between *A. thaliana* and *A. lyrata*, we chose an RT-PCR approach with homologous primers derived from *A. thaliana*. A primerset for all known *TPS* genes of *A. thaliana* was developed based on the available sequence information. The primers were designed to have annealing temperatures of $55\pm5^{\circ}$C and to yield PCR amplified fragments of 300 – 700 bp.

RNA of herbivore-treated and untreated samples was extracted from leaf tissue and cDNA was produced. RT-PCR showed upregulated TPS transcripts after herbivory (Table 3). For the transcripts of TPS2 and TPS3, which were found in herbivore-treated and untreated samples in all lines other than NT20, the signal intensity was clearly stronger in treated samples compared to control samples. All transcripts except the ones detected with primers specific for TPS13, 22, 24 and TPS28 were sequenced for verification. The detection of transcripts for TPS5 and TPS27 correlated with the emission of the terpenes (*E*)-β-ocimene and (*E*)-β-caryophyllene, respectively. Transcripts of the (*E*)-β-caryophyllene synthase homologous gene were only detected after herbivory in lines which emitted (*E*)-β-caryophyllene.

### Terpene synthases from *A. lyrata* show high sequence similarity to terpene synthases from *A. thaliana*

We found *TPS* sequences in *A. lyrata* corresponding to those responsible for the biosynthesis of (*E*)-β-ocimene and (*E*)-β-caryophyllene in *A. thaliana*, and we sequenced the full-length transcripts to determine the degree of sequence similarity. In *A. thaliana* the gene At5g23960 (TPS27) was found to be uniquely responsible for floral emitted (*E*)-β-caryophyllene [160]. At5g23960 has 7 exons which is the same genetic structure as other *TPS* genes in *A. thaliana*. The resulting coding sequence of 1644 nucleotides is translated into 547 amino acids. The homologue from *A. lyrata* also consists of 7 exons with two codons less, *i. e.* 1638 nucleotides and 545 amino acids. DNA sequence identity was 93%, whereas protein sequences were 91% identical and 95% similar. The protein sequence alignment is shown in Figure 11.

A transcript with a high similarity to the pseudogene At4g16730 from *A. thaliana* ecotype Col-0 was found after herbivory in some lines that emitted (*E*)-β-ocimene. The full transcript was amplified from cDNA by PCR and sequenced. The gene consists of 7 exons, and the resulting cDNA has 1647 nucleotides that are translated into 549 amino acids. The *in vitro* analysis of heterologously expressed protein in *E. coli*

showed activity of an (*E*)-β-ocimene synthase (Figure 12 A). A comparative sequence analysis of deduced amino acid sequences of the isolated *A. lyrata* genes and published TPS sequences from *A. thaliana* was done with ClustalW (Figure 14).

## Induced *TPS* enzymes of *A. lyrata* with high similarity to At4g16730 and At5g23960 from *A. thaliana* make (*E*)-β-ocimene and (*E*)-β-caryophyllene

Terpene synthases with different enzymatic function still show a high sequence similarity. Therefore we investigated the catalytic activity of the proteins encoded by the full-length sequences of the homologues At4g16730 and At5g23960 from *A. lyrata*. A cDNA amplified by RT-PCR was cloned into an expression vector and overexpressed in *E. coli* BL21 (Codon Plus) cells. Proteins were extracted after 2 days from bacterial culture. Enzyme assays with the At4g16730 homologue from 10 µl crude extract in 1 ml total assay volume and 10 µM geranyl diphosphate as substrate yielded *Z*-ocimene and (*E*)-β-ocimene (Figure 12 A).

Enzyme assays with the At5g23960 homologue from 10 µl crude extract in 1 ml total assay volume and 10 µM farnesyl diphosphate as substrate yielded (*E*)-β-caryophyllene and α-humulene (Figure 12 B). The mass spectra of (*E*)-β-caryophyllene obtained from volatile collections after herbivory, from the *in vitro* enzymatic forma-

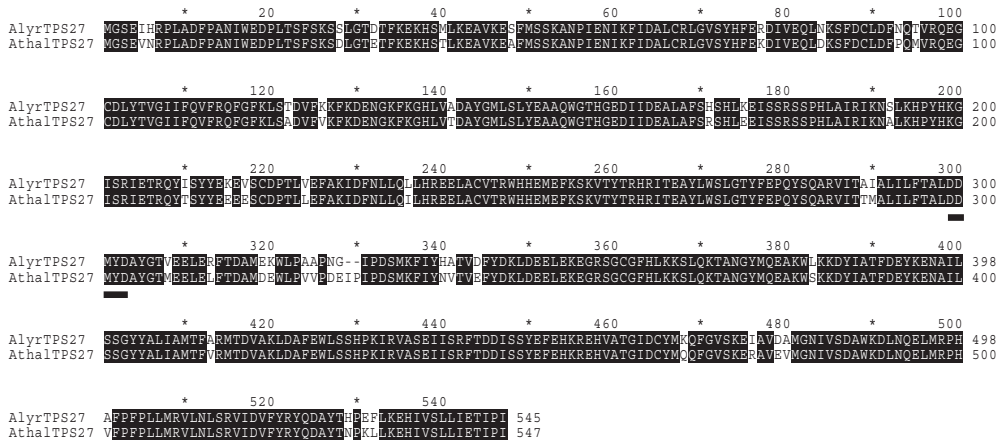


Figure 11: Protein sequence comparison of the full-length *A. lyrata* sequence of the At5g23960 homologue of *A. thaliana*. Sequence similarity was 95%, and sequence identity was 92% (shaded black). The DDxxD motif which is mandatory for the catalytic activity of TPS enzymes is underlined.

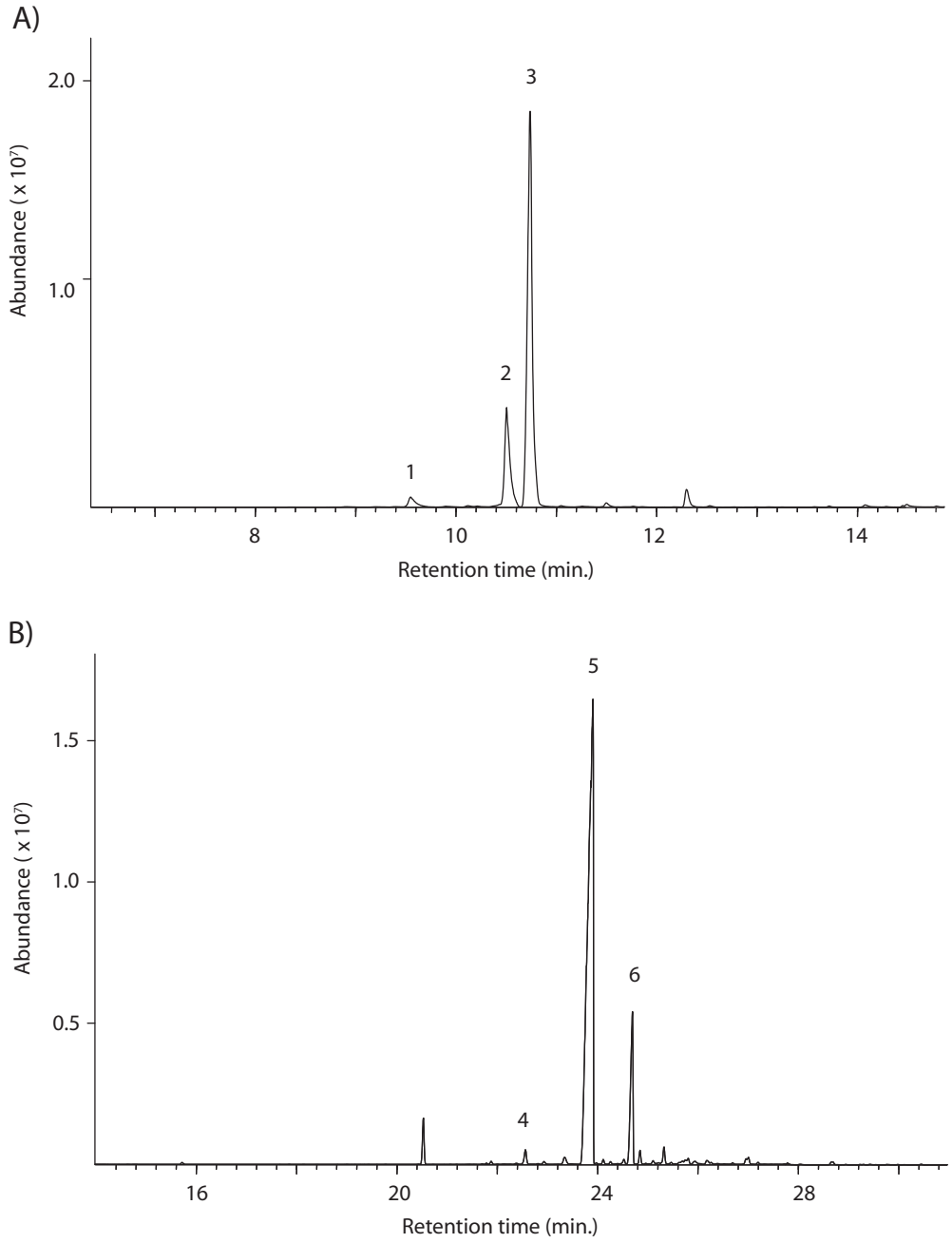


Figure 12: Separation of volatile products formed by *in vitro* activity of the *A. lyrata* proteins homologous to At4g16730 (A) and At5g23960 (B) from *A. thaliana*. Shown are total ion current GC-MS chromatograms after headspace sampling with SPME. The assays were performed for 60 min with crude bacterial extract and 10 $\mu$M GPP (A) and 10 $\mu$M FPP (B). 1: β-myrcene, 2: (*Z*)-ocimene, 3: (*E*)-β-ocimene, 4: α-copaene, 5: (*E*)-β-caryophyllene, 6: α-humulene

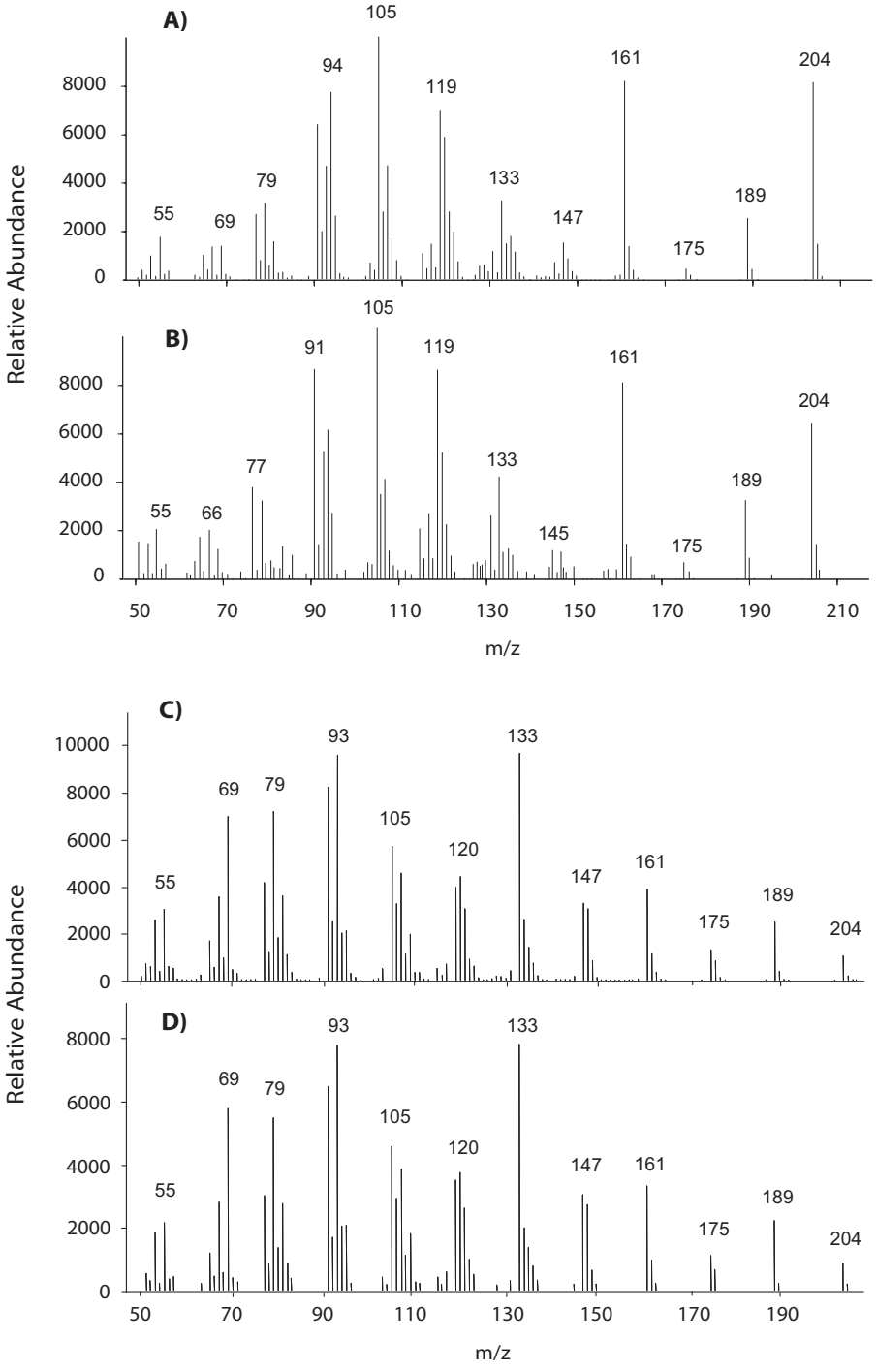


Figure 13: The EI mass spectra of the detected sesquiterpenes (+)-cyclosativene and (*E*)-β-caryophyllene are identical to authentic standards. A: authentic standard for (+)-cyclosativene, B: sample spectrum of (+)-cyclosativene, C: authentic standard for (*E*)-β-caryophyllene, D: sample spectrum of (*E*)-β-caryophyllene. Spectra were obtained by ionisation with 70 eV.

tion and from an authentic standard all gave the same fragment pattern after 70 eV ionization (Figure 13).

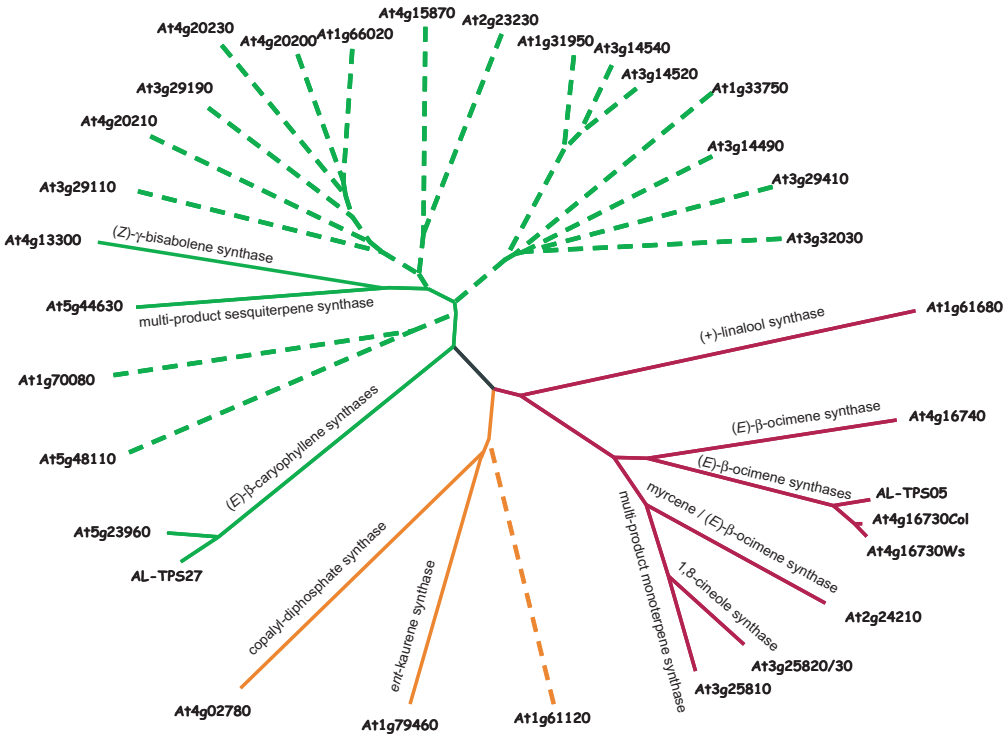


Figure 14: Amino acid sequence similarities of terpene synthases from *A. thaliana* and *A. lyrata* obtained by the ClustalW algorithm with standard parameters. Deduced amino acid sequences from databases (dashed lines) were included along with sequences of known enzymatic functions (solid lines). The sequences cluster in a monoterpene synthase group, a diterpene synthase group and a large group of sesquiterpene synthases which may contain pseudogenes as well as diterpene synthases.

## 2.4 Discussion

By analysing *A. lyrata* ssp. *petraea*, a close relative to *A. thaliana*, we found striking differences in floral volatiles and those emitted from vegetative organs. Nevertheless, the *TPS* genes responsible for the formation of terpenoid compounds were very similar.

Our analysis of floral scent of *A. lyrata* ssp. *petraea* populations in Germany showed no floral specific terpene emission. The most abundant volatiles were the benzenoid metabolites, benzaldehyde and phenylacetaldehyde. Studies of floral volatile emission of some members of the Brassicaceae family have been described [88]. From cultivars of *Brassica napus* it is known that the flowers emit several mono- and sesquiterpenes, but no benzenoids [78]. Floral monoterpene emission of myrcene and *cis*-ocimene from the North-American subspecies *A. lyrata* ssp. *lyrata* is reported [126]. The authors also report benzaldehyde and phenylacetaldehyde as major floral volatiles from *A. lyrata* ssp. *lyrata*.

Molecular and chemical analyses of floral volatiles from *A. thaliana* were described recently [30, 160]. *A. thaliana* produces low amounts of floral volatiles. The floral volatile blend consists mainly of a group of sesquiterpenes which are produced by the activity of only two terpene synthase genes [160]. Myrcene, limonene and linalool were detected as well. Benzaldehyde was a minor compound of the floral scent.

Diurnal as well as nocturnal rhythms of floral volatile emission are known from several members of different angiosperm families [109, 8, 69, 95, 132]. Benzenoids that peak during the night are known from *Hoya carnosa* (Rutaceae) [8], several species of *Nicotiana* (Solanaceae) [132], and *Antirrhinum majus* (Scrophulariaceae) [48]. For crucifers, nocturnal emission of benzenoids like phenylacetaldehyde is described from *Hesperis matronalis* [116], while cultivars of *Brassica napus* emit monoterpenes diurnally [78].

Floral emission patterns from *Nicotiana sylvestris* were studied as well. Flowers emit large amounts of benzaldehyde and benzyl alcohol during the night, but nearly none during the day [109]. For methylbenzoate and methylsalicylate, a similar oscillation was found, but not for (*E*)-β-caryophyllene [108].

Using *A. lyrata* we could show for a member of the *Arabidopsis* clade that floral specific volatiles were diurnally emitted. *A. lyrata* has a peak emission of benzenoids during daytime. The emission decreases several hours before the onset of darkness, and reach background levels at the onset of darkness. Emission rises again at the end of darkness. This pattern could be shown by two different methods; first with a dynamic volatile collection with solid phase trapping followed by GC-MS analysis (Figure 8) and second by PTR-MS analysis with a higher temporal resolution (Figure 9).

To attract pollinators, it may be a good strategy to release volatiles at times of

highest pollinator foraging activity. At times of low activity, volatile signals may be counter-productive, attracting herbivores or non-pollinating floral visitors, or at the very least a waste of resources. The benzenoids benzaldehyde and phenylacetaldehyde are known to attract nocturnal pollinating moths. For *Silene latifolia* (Caryophyllaceae), the nocturnal emission of phenylacetaldehyde is correlated with the flight activity of moths that act as pollinators [45]. We could demonstrate that the benzenoids from *A. lyrata* flowers were emitted in a distinct diurnal pattern. The peak of emission at mid-day suggests pollination by day active insects and not nocturnal ones. Foraging solitary bees (members of Andrenidae and Halictidae) have been observed on *A. lyrata* flowers (personal observations). Bees, known to be effective pollinators of other species [52, 71], were most abundant as flower visitors of *A. lyrata* between 11 am and 1 pm. That correlates positively with the peak emission of benzaldehyde and phenylacetaldehyde observed under laboratory conditions.

One reward for flower visiting insects is the carbohydrate-rich nectar produced by the floral nectaries. Nectaries are often found at the base of petals. Morphological analyses in the 1940s showed that among Brassicaceae, *A. thaliana* has nectaries [117]. For *A. lyrata*, the possible production of nectar was not investigated.

The spatial distribution of floral scent emission of *A. lyrata* revealed exclusive emission of benzenoids via the petals (Figure 7), which is characteristic for floral scent acting as an attractive pollinating cue. The restricted emission of floral volatiles to the petals is also known from *Petunia hybrida* [166]. In contrast to that, in *A. thaliana* other parts of the flower like sepals, stigma and the base of the flowers are the likely origin of scent emission. These findings are based on analysis of transgenic *A. thaliana* with promoter-GUS fusion constructs of the genes responsible for scent production in *A. thaliana* [30, 160], but not on direct analysis of scent emission. The spatial and temporal restricted emission of volatiles is a strong hint that floral scent in *A. lyrata* indeed was developed to attract pollinating insects.

We detected the monoterpene (*E*)-β-ocimene being emitted from leaves of *A. lyrata* after herbivory by larvae of *Plutella xylostella*. A large number of taxonomically distantly related plant species emit (*E*)-β-ocimene from flowers [47, 88]. Induced emission of (*E*)-β-ocimene upon biotic stress is only known from a small number of experiments. Members of the Solanaceae, Brassicaceae, and Fabaceae show an inducible emission of (*E*)-β-ocimene either after pathogen attack [75], wounding, application of jasmonic acid [56], or herbivory [12]. From *A. thaliana*, analyses of different ecotypes showed a high variability of induced (*E*)-β-ocimene (chapter 3). Here we could show that (*E*)-β-ocimene is emitted from lines of *A. lyrata* and *Arabis hirsuta* (unpublished results) upon herbivory by *Plutella xylostella*.

*A. thaliana* and the closely related perennial *A. lyrata* both show intraspecific vari-

ation in volatiles. When both species are subjected to herbivory, only *A. lyrata* emits the sesquiterpene (*E*)-β-caryophyllene. The results so far suggest that regulation of the (*E*)-β-caryophyllene synthase gene evolved significantly different during the last 5 million years, the time since *A. thaliana* and *A. lyrata* diverged [92]. From the *A. thaliana* ecotype Col-0, (*E*)-β-caryophyllene is only known as a floral volatile, but not from vegetative tissue [30, 165]. Reports from other plant systems are known which state (*E*)-β-caryophyllene to be important for the attraction of herbivore enemies [133, 158]. It can therefore be speculated that in the perennial *A. lyrata* also some yet undiscovered tritrophic interactions exist, which are not present in the annual *A. thaliana* because of its shorter life cycle or presence in habitats without the reliable presence of herbivore enemies. However, other as yet undiscovered functions of terpenes in interactions with other organisms are not excluded.

The presence or absence of (*E*)-β-caryophyllene emission from *A. lyrata* is dependent on specific genotypes, because not all investigated lines were able to emit (*E*)-β-caryophyllene. There are several possibilities for a no-emission phenotype: single nucleotide polymorphisms in the coding regions that lead to a non functional protein; differences in the promoter region leading to a lack of activation by induction signals; chromosomal rearrangement with gene loss; or changes in substrate availability. Further investigations are needed to select from among these and other explanations.

Other volatiles which are frequently detected from *A. lyrata* during herbivory are the C-11 and C-16 homoterpenes, 4,8-dimethyl-1,3,7-nonatriene (DMNT) and 4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), respectively. Both volatiles are known to play a role as signal molecules in insect-plant interactions [10, 25, 154, 162]. The correlated emission of nerolidol and DMNT was recently described from maize [41].

Herbivore-induced volatiles have been demonstrated to have beneficial effects for the emitting plants in tritrophic interactions. A transgenic approach with *A. thaliana* expressing nerolidol and DMNT showed the attraction of predatory mites to the plants in a laboratory study [82]. Still, it is not known whether the induced emission of terpenes in the Brassicaceae is of any direct benefit for the plants in nature.

The coding sequences of At5g23960 from *A. thaliana* and the homologue from *A. lyrata* have 85% identity on the nucleotide level and 92% similarity on the protein sequence. Similar values can be calculated between At4g16730 and the *A. lyrata* homologue. Terpene synthases from *A. thaliana* can be clustered based on sequence analysis in different functional groups [14]. The terpene synthases from *A. lyrata* group very closely with terpene synthases from *A. thaliana* (Figure 14).

The results presented show an interesting divergence in the volatile profile for the two *Arabidopsis* species. Floral scent of the outcrossing *A. lyrata* is different from the inbreeding *A. thaliana*, and the terpene-dominated volatile blends emitted after

herbivory are also clearly different. Still, the *TPS* structural genes involved in the biosynthesis of the terpene blends are very similar. This implies a rapid evolution of differences in gene expression during the last 5 mya, the time since the two species separated from a common ancestor. Future research should therefore be focused on investigations of the promoter regions and the determination of regulatory factors responsible for the observed difference in volatile terpene phenotypes.

# 3 Chapter 2: Floral Scent Variation of *Arabidopsis lyrata* in Populations from Central Europe

**Abstract**

In the Brassicaceae family, the phenomenon of floral chemotypes has rarely been studied outside of *Arabidopsis thaliana*. Here we investigated a close relative of *A. thaliana*, the perennial *A. lyrata* ssp. *petraea*. The habitat, the breeding system, and the range of these two species are completely different. We investigated the floral scent of three populations in central Europe which are separated from each other by more than 200 km. For each of the populations, a distinct floral chemotype could be assigned to, determined by the abundance of various benzenoids and terpenes.

## 3.1 Introduction

The phenomenon of chemotypes, which is the existence of genetically-based variation in a specific chemical trait, has been described for species of several different plant families. In the Brassicaceae, studies of *Arabidopsis thaliana* or cultivars of *Brassica oleracea* and relatives have shown differences in glucosinolate composition [170, 20, 164], but there are no reports of variation in volatiles. However, variation in floral volatiles can be expected among species of *Arabidopsis*.

*A. thaliana* and *A. lyrata* diverged from each other ca. 5 million years ago [93]. Whereas *A. thaliana* is an annual species and seedset is mainly by selfing, *A. lyrata* is a perennial and strictly self-incompatible [145]. One terminal flower per shoot is sequentially available for pollination. Main pollinators are insects like solitary bees and hover flies. Since the flowers of *A. lyrata* are likely to have been selected to attract pollinating insects, while those of *A. thaliana* may not have been, this might lead to a difference among these species. Previous work has shown that the flowers of *A. thaliana* emit volatiles in low amounts, and all belong to the mono- and sesquiterpenoids [30], while the flowers of *A. lyrata* emit benzenoids (chapter 1).

Both of these species have extensive geographical ranges. An insect-pollinated species distributed over a large area may face the challenge of attracting different species as pollinators in different regions and so have geographical variation in floral volatiles. We investigated whether the floral scent of *A. lyrata* is different in scattered European populations, or if the scent quality is constant.

## 3.2 Materials and Methods

### Plant Material

*A. lyrat*a ssp. *petraea* from populations in central Europe were investigated (Table 5, Figure 15). Seeds as well as complete plants were collected from a population in southern Germany (Germany, northern Bavaria, Fränkische Schweiz, Plech), from lower Austria (Austria, Lilienfeld), and from northern Bohemia (Czech Republic, České Středohoří, Boreč). Seeds were collected from two further populations in the southern Harz region (Germany, Südharz, Mühlberg and Stolberg). For a geographical overview of the sampled populations see the map in Figure 15.

As *A. lyrata* can reproduce vegetatively from roots, seeds were collected from plants that were separated from each other by at least 50 cm to prevent collecting from the same mother plant. Only plants with fully ripened capsules were harvested.

Seeds from these collections and from a controlled crossing experiment performed with seeds from the Plech population (Table 5) were treated for germination as follows: seeds were placed on wet filter paper in petri dishes and vernalized in the dark at 4°C for 2 – 3 weeks. Plant lines were established from germinated seeds and placed in plant climate chambers on wet substrate. To preserve high humidity conditions, the trays with plants were covered with a translucent plastic dome. Substrate for growing plants was an autoclaved mixture of vermiculite, fertilizer, and regular Arabidopsis soil. Plants were cultivated in plant climate chambers at 22°C, rel. H. 55%, 10-h light/14-h dark cycles, and light intensity of 160 $\mu$mol $m^{-2}$ $s^{-1}$ PAR.

Table 5: Designation of *A. lyrata* lines investigated in natural habitats. Except for Lilienfeld where only mature plants were available, all lines are derived from seeds of different mother plants.

| Population | Geographic Coordinates | Established lines |
|---|---|---|
| Alter Stolberg, Germany | 51°31'N10°55'E | S28-1, S28-2, S28-3, S28-4, S28-5, S32-1, S32-2, S36-1, S36-2, S36-3, S36-4, S36-5, S42-1, S42-2, S42-3, S42-4, S42-5 |
| Mühlberg, Germany | 51°33'N10°45'E | M59-1, M59-2, M59-3, M67-1, M67-2, M67-3, M67-4, M67-5, M67-6, M69-1, M69-2, M71-1, M71-2, M71-3, M71-4, M71-5, M71-6 |
| Plech, Germany | 49°37'N11°30'E | Gr4A2, 6A4, 11A2, 22A1, 24A1, 2B1, 16B3, 23B1 |
| Boreč, Czech Republic | 50°31'N13°59'E | BC1.1, BC1.2, BC1.3, BC1.5, BC1.6, BC2.3, BC2.7 |
| Lilienfeld, Austria | 47°59'N15°36'E | LF10, LF12, LF14 |

### Volatile Collection

Floral volatiles were collected with a closed loop stripping method [44]. Four to 15 individual flowers were cut and transferred to 10 mL glass beakers filled with tap water and placed in 2 L glass dessicators in a plant climate chamber. Air circulated continuously with a flowrate of ca. 1 L/min, passing over a trap with activated charcoal. Floral volatiles were collected over a period of 8 h during the light phase.

Another method used for the detection of flower specific volatile emission was headspace solid-phase micro extraction [176] with PDMS-100 (100 $\mu$m polydimethylsiloxane) and CW/DVB-65 (65 $\mu$m Carbowax/Divinylbenzene) fibers (Supelco, Bellefonte PA, USA). Single flowers were enclosed in a 1 mL glass vial closed with a septum cap. The septum was punctured and the SPME needle inserted without touching any sample material. The complete system was incubated at 25°C and the fiber exposed for 30 min to the headspace of samples. Following exposure, the fiber was inserted into a GC-MS system as described below.

Adsorbed volatiles from charcoal traps were eluted with 50 $\mu$L $CH_2Cl_2$ with nonylacetate at a concentration of 2 ng/$\mu$L as internal standard (IS). Samples were analyzed with a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 quadrupole mass selective detector. Separation was performed on (5%-phenyl)-methylpolysiloxane columns (J&W Scientific, Folstom, CA, USA) of 30 m x 0.25 mm i. d. x 0.25 $\mu$m film thickness. Helium was the carrier gas (flow rate of 2 mL/min). A splitless injection of 2 $\mu$L was used, and a temperature program starting at 45°C with a ramp of 5°C/min to 180°C followed by a ramp of 60°C/min to 300°C was applied.

Mass spectrometry was performed with a transfer line temperature of 230°C, source temperature of 230°C, quadrupole temperature of 150°C, ionization potential of 70 eV, and scan range of 35 to 350 atomic mass units. Compounds were identified using mass spectral libraries NIST98 (Agilent Technologies, Palo Alto, CA, USA) and Wiley275 and confirmed with authentic standards where available. For quantification, selected ion peaks of each compound were integrated and related to the response of the internal standard (SIM, selected ion monitoring).

## 3.3 Results and Discussion

### Distribution of *A. lyrata* populations in central Europe

*A. lyrata* ssp. *petraea* is most abundant in boreal areas like Iceland, the northern British Isles, southwestern Norway, Sweden and boreal Russia. Some occurences of *A. lyrata* are described for the mainland of central Europe, but the determination of some of them need to be verified. It soon became clear, that some locations of *A. lyrata* mentioned in the literature are not reliable. While initial field searches to verify individual locations of *A. lyrata* in Germany were successful, because detailed information of geographic coordinates was made available from local botanists, field visits outside of Germany were difficult because of missing detailed geographic data on putative habitats. Published data from flora distribution atlases usually have a very low geographic resolution (for example [68, 152]), because the intention of these volumes is to inform

about the occurence in a certain geographic area and not to provide exact information on habitats.

Locations of *A. lyrata* on the European mainland that could be retrieved from the literature, including the sampled populations, are depicted in Figure 15. The sampled populations are all within a distance of 200 km to each other.

For Germany, a detailed dataset exists for the geographic distribution of plant species. Information is available online [1] and in flora atlas books [96, 147]. Occurence of *A. lyrata* in the Harz region was confirmed by the author's visits to the populations.

For the Czech Republic the Czech Flora [68] and the Czech floral distribution atlas [152] were consulted. Besides the sampled population Boreč in the České Středohoří, two other locations were researched. The occurence of *A. lyrata* on Černý Vrch near Chomutov in Northern Bohemia had been previously noted [68], but searches in possible habitats of this area were unsuccessful. Approximately 100 km eastwards,

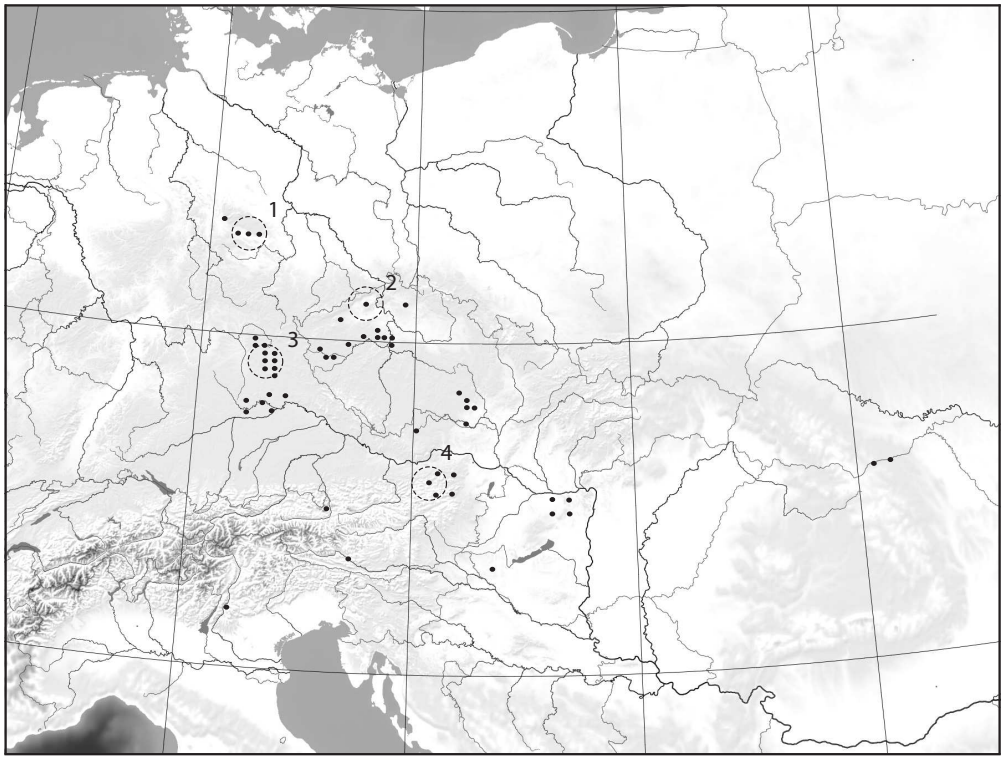


Figure 15: Distribution of *A. lyrata* ssp. *petraea* in central Europe. Data points were compiled for the different countries from diverse resources (see text). Indicated with numbers are the sampled populations. 1: southern Harz region, Germany, 2: České Středohoří, Czech Republic, 3: Plech, Fränkische Schweiz, Germany, 4: Lilienfeld, lower Austria. For details about the locations see text. The map was created with MapCreator.

*A. lyrata* is reported to be present on the Bezděz mountains. Here, some specimens were found which were phenotypically identified as *A. lyrata*. However, at this location, the closely related *A. arenosa* (L.) Lawalrée *s. l.* is growing in close vicinity and therefore hybrids among the two species may be possible and cannot be ruled out without further genetic analysis. From *A. arenosa* in this region, the occurence of diploid, tetraploid or aneuploid members are known [115]. This area may belong to the hybridization zone among these species proposed for eastern Austria (M. Koch and M. Matschinger, unpubl.).

The occurence of *A. lyrata* in western Bohemia and Moravia [152] were not confirmed by site visits, but these locations are indicated in Figure 15. Data points from Austria, Italy and Hungary were obtained from other sources [33, 54]. The occurence in Italy, southern Tirol, is doubtful as well as that in Austria, in eastern Tirol. For upper Austria the species is reported to be extinct [3]. For Hungary, only one source of information was available [135]. In general, the identity and occurence of *A. lyrata* in Hungary needs to be verified and revised. The occurence of *A. lyrata* in the northern border region of Romania may not be correct due to possible misidentification [118].

## The floral volatile blend of *A. lyrata* varies among selected European populations sampled

Headspace collections of flowering *A. lyrata* were analyzed by GC-MS and compounds identified by comparison with authentic standards. Benzenoid derivatives were prominent, including benzaldehyde, phenylacetaldehyde and methylbenzoate. One monoterpene was found, the alcohol linalool. Other compounds like ketones, dodecene, tetradecene, hexadecene, octanal, nonanal, or decanal were also present in minor amounts. These compounds varied significantly among the different populations.

All compounds detected are known constituents of floral scent from a wide variety of unrelated plant families [88]. The benzenoid compounds from *A. lyrata* ssp. *petraea* are similar to the ones previously reported from the North-American subspecies *A. lyrata* ssp. lyrata [126]. Benzaldehyde and phenylacetaldehyde were described to be the most abundant volatiles in that subspecies. However, compared to the floral scent of the close relative *A. thaliana*, the volatiles detected are strikingly different. *A. thaliana* emits very low amounts of a blend containing mono- and sesquiterpenes and minor amounts of benzaldehyde. Whereas two genes are responsible for the terpene synthases producing the sesquiterpene blend [160], the monoterpenes originate from a multi-product monoterpene synthase and a linalool synthase [30].

The qualitative composition of the floral scent bouquets from the individual plants of German populations were similar. Benzaldehyde was the main volatile compound,

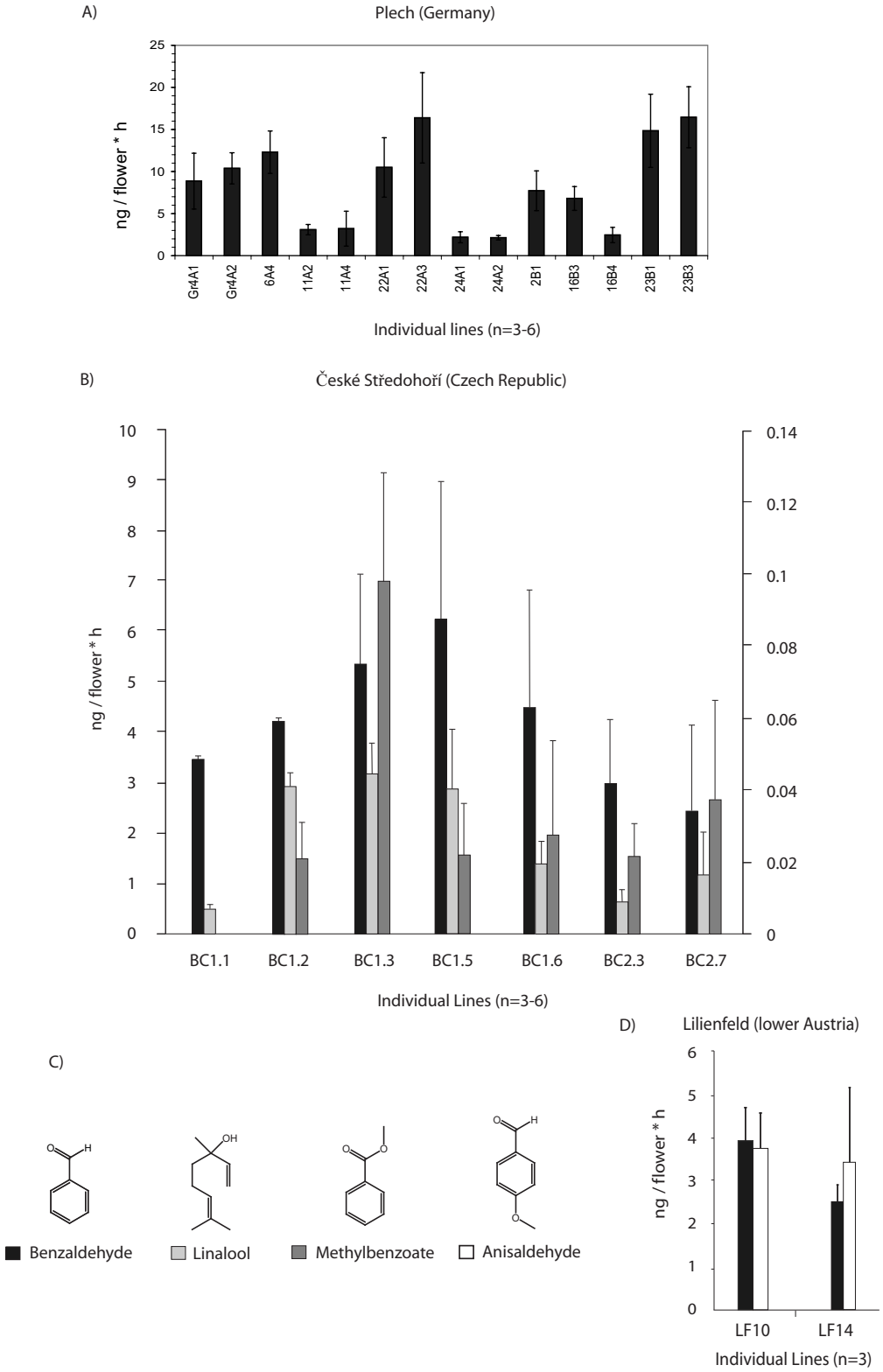


Figure 16: Major floral volatiles detected from *A. lyrata* populations in central Europe. From the Plech population (A) only benzaldehyde could be detected whereas from the Czech population (B) linalool was also abundant accompanied by minor amounts of methylbenzoate (right, different scale). From the Lilienfeld population in Austria (D) benzaldehyde and anisaldehyde could be quantified. The structures of these compounds are shown in the lower left (C).

but the total amount of volatiles showed intra- and inter population differences. From the populations of the southern Harz region, the total amount of volatiles was remarkably low in comparison to the other German populations (data not shown).

From the population in the Czech Republic (České Středohoří, Boreč), in addition to benzaldehyde, methylbenzoate and the terpene alcohol linalool were detected regularly (Figure 16). The amounts of methylbenzoate were app. 10 times less than benzaldehyde. The total amounts of linalool were slightly lower than benzaldehyde.

The samples from Austria (Lilienfeld) emitted benzaldehyde and anisaldehyde (4-methoxy-benzaldehyde) as the main floral scent compounds (Figure 16). Samples taken from another population in Austria (Vöslauer Hütte, [33]) showed the same emission profile as the ones from Lilienfeld (data not shown). Both benzenoids were emitted in similar amounts, and the results were also confirmed by single flower headspace SPME. Other compounds detected from the Lilienfeld population, including methylbenzoate and linalool, were present in lower amounts.

With the closed-loop stripping method used for volatile collection, none of the samples was found to contain phenylacetaldehyde. This is in contrast to the findings for the German Plech population made with dynamic headspace collection (chapter 1), which showed both benzaldehyde and phenylacetaldehyde as main volatile compounds. Analysis of these additional populations with headspace SPME also did not reveal any phenylacetaldehyde, which suggests that the differences reflect the natural situation.

## Benzaldehyde is consistently a major floral volatile while other compounds are variable

The sweet fragrance of benzaldehyde is best known from the oil of bitter almond, *Prunus amygdalus*. Benzaldehyde is widespread as a plant volatile, not only as part of floral scent [88]. It is also known from flowers of *A. lyrata* ssp. *lyrata* [126]. As we could demonstrate, all samples of *A. lyrata* ssp. *petraea* investigated from populations of central Europe emit benzaldehyde. The emission rates varied from 2 to 16 ng flower$^{-1}$hour$^{-1}$, while the samples from Czech and Austrian plants showed emission rates from 2 to 7 ng flower$^{-1}$hour$^{-1}$ (Figure 16). Although benzaldehyde is a relatively simple molecule and is widely used in the flavour and fragrance industry, its biosynthesis *in planta* is still unknown.

Phenylacetaldehyde is a benzenoid metabolite that is common as a component of floral scent. Several species of different plant families, monocots as well as dicots, emit phenylacetaldehyde as a main floral scent compound [88]. From data based on flowers of *Petunia hybrida* cv. Mitchell, a possible biosynthetic pathway for phenylacetaldehyde has been proposed [81].

Anisaldehyde is a volatile compound with a heavy sweet note. As a constituent of floral scent, several species from diverse plant families emit anisaldehyde [88]. A selection of species where anisaldehyde was found by floral headspace analysis include subspecies of *Cypripedium calceolus* in North America [21], *Fragaria ananassa* [65], *Syringa oblata* [102], *Crataegus monogyna* [137], *Brassica napus* [55], and some species of *Hypecoum* [36]. The latter case is interesting, because the absence and presence of anisaldehyde between subspecies and between populations of one subspecies is described. Behavioural experiments showed an attraction of certain thrips species to the scent of pure anisaldehyde [87, 157]. Thus the compound may be an attractant to pest insects as well as to pollinators. This interesting evolutionary problem is discussed elsewhere [77, 86]. Future experiments to investigate the ecological relevance of the compound for *A. lyrata* should be of interest.

Anisaldehyde is also found in a variety of essential oils from species like *Illicium verum* [174], *Thalictrum minus* [17], or *Foeniculum vulgare*, but it can also be emitted from mushrooms like *Pleurotus ostreatus* [120]. Results that show anisaldehyde as a constituent of the essential oil should be considered with care, in particular when (*Z*)-anethole is also present. Since anisaldehyde can be a byproduct of anethole oxidation *in planta* or during the process of oil extraction [9, 97]. If anisaldehyde from flowers of *A. lyrata* was derived from anethole, some of this alcohol should have also been detected. As anethole was not detected under any circumstances, we assume that *A. lyrata* is able to produce and emit anisaldehyde as a natural component of its floral scent.

Linalool, a highly volatile oxidized monoterpene, is mainly known from flowers but not from vegetative parts. However, after herbivory linalool is also emitted by some species from leaf tissue [85].

Unfortunately, for the profiling of some populations, we were restricted to limited numbers of samples because of the low abundance of plants and limits in flower production, making it difficult to perform statistical analysis. Nevertheless, there were very strong differences among these populations in floral volatiles, suggesting significant genetic differences among them. The investigations support a high variability as expected from studies investigating genetic variability among central European populations of *A. lyrata* [31, 33].

The analysis of floral scent variability of *A. lyrata* is a prerequisite for further studies on floral biology. As we could show that the scent is indeed different among certain European populations, it would be interesting to investigate the pollinators of these populations.

# 4 Chapter 3: The emission of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene from leaves of different ecotypes of *A. thaliana* is regulated by two different terpene synthases

**Abstract**

*Arabidopsis thaliana* has become a valuable model system to investigate the biochemistry and function of terpene volatile biosynthesis. So far, little is known about the variation of stress-induced volatile terpene biosynthesis and the mechanisms controlling these differences among *A. thaliana* ecotypes. Here, we investigated induced terpene volatile emissions from vegetative tissue of the two ecotypes, Wassilewskija (Ws) and Columbia (Col-0) in response to elicitor treatment and herbivory. While the Ws ecotype emits (*E*)-β-ocimene upon insect feeding and elicitor treatment, the Col-0 ecotype almost entirely lacks the emission of (*E*)-β-ocimene. RNAi- and antisense analysis as well as GFP hybrid constructs enabled us to pinpoint the differences in observed chemical phenotypes of these two ecotypes to two closely related terpene synthase genes, At4g16730 and At4g16740. Expression of both genes is induced in leaves of the Col-0 ecotype while in Ws only At4g16730 is transcribed under induced conditions. *In vitro* analysis of the At4g16730 (Ws) and At4g16740 (Col-0) recombinant proteins showed catalytic activity as (*E*)-β-ocimene / (*E*,*E*)-α-farnesene synthase for both enzymes. At4g16730 (Col-0) yielded an inactive protein due to a double nucleotide insertion. After reverting the sequence to a fully closed ORF, the protein was still inactive but could be turned into a functional enzyme by site-directed mutagenesis of selected amino acids. Studies using C-terminal GFP hybrid constructs with the signal peptide (SP) from At4g16730 (Ws) showed a plastidial localization whereas the same approach with the putative SP from At4g16740 (Col-0) showed no plastidial targeting. The differential subcellular location of both enzymes suggests *in vivo* activities of At4g16730 in Ws primarily as (*E*)-β-ocimene synthase and of At4g16740 in Col-0 as an (*E*,*E*)-α-farnesene synthase.

## 4.1 Introduction

Plants are known to emit volatile organic compounds (VOCs) [84]. A large group of VOCs belongs to the chemical class of terpenoids (synonymous to terpenes) which include low molecular weight hydrocarbon olefins and alcohols of the monoterpene ($C_{10}$), and sesquiterpene ($C_{15}$) classes. Diterpenes ($C_{20}$) are less or not volatile due to their higher mass, an example for a plant volatile diterpene is *ent*-kaurene [173]. The emission of terpenes occurs either constitutively during a plant's life cycle or after induction by external factors. These may be abiotic stress factors like elevated

ozone [67], water stress [123], temperature or light stress [66]. Biotic factors include bacterial and fungal pathogen attack, damage by oviposition [168] or herbivory by feeding insects [124].

Induced terpenes are usually emitted as a complex mixture of mono-, sesqui-, and homoterpenes. The monoterpene (*E*)-β-ocimene is frequently found in terpene blends emitted by plant species in response to different stress factors [12, 107, 142, 167]. Sesquiterpenes show a more diverse pattern of induction. Several distantly related plant species emit sesquiterpenes after herbivore attack [63, 142]. Our knowledge of specific functions of induced volatile terpenes is still limited. However, some investigations point towards their importance for plant fitness. Potential functions include protection against microbes due to the antibiotic effects of some terpenes (*e. g.* [28, 98]) and a role as indirect defense compounds in tritrophic interactions [42, 85]. Nevertheless, emitted volatiles can also have non-beneficial effects on the emitting plant by attracting even more herbivores [74].

*Arabidopsis thaliana* has been established as a model plant for the study of volatile terpene biosynthesis, and herbivory on *A. thaliana* leads to the induced production of volatile terpenes [165]. In the genomic sequence of *A. thaliana* (Columbia ecotype) more than 30 putative terpene synthase genes have been annotated [14]. Functional analyses have been performed with four putative monoterpene synthases and three sesquiterpene synthases. Two of the sesquiterpene synthases are a florally expressed (*E*)-β-caryophyllene synthase and a multi-product sesquiterpene synthase [160], while one other is a root expressed (*Z*)-γ-bisabolene synthase [136]. However, for some terpenes emitted under induced conditions like (*E*)-β-ocimene or (*E,E*)-α-farnesene, the corresponding terpene synthases are yet not characterized. The reason why *A. thaliana* contains such a large family of terpene synthase genes is unclear. One explanation may be that only one ecotype of *A. thaliana* (Col-0) has been investigated, but the species is able to express other terpene synthases in ecotypes from other locations. To gain a deeper insight into the activity and function of the terpene synthase gene family, the natural variability of terpene formation in the ecotypes needs to be considered. Recently, it was shown that several *A. thaliana* ecotypes exhibit considerable quantitative and to some extent qualitative variation in the emission of floral volatiles [160]. Variation of induced terpene emission has not yet been investigated.

Aside from differences in gene transcription, a possible cause for the variation in volatile terpene formation can be the location of terpene synthases in different cellular compartments. Proteins are targeted to different compartments by transit peptide sequences [22], which are clipped off during the trans-membrane transport generating fully functional proteins inside the compartment. For terpene synthases, substrate availability may be restricted in different compartments. According to subcellular lo-

calization studies and the prediction of targeting sequences for prenyltransferases, it is generally believed that geranylpyrophosphate (GPP), the substrate for monoterpene synthases, is present almost exclusively in plastids [24]. On the other hand, farnesylpyrophosphate (FPP), the substrate for sesquiterpene synthases, is primarily synthesized in the cytosol [35]. However, GPP and FPP can also be produced in other compartments.

In *A. thaliana* two isoforms of an FPP synthase derived from the same gene are active. One isoform is targeted to mitochondria, whereas the other remains in the cytosol [35]. The targeting of isoforms to different cellular compartments is not uncommon (reviewed by [37]). Similarly, in *A. thaliana* a cytosolic as well as a plastidic isoform of GPP synthase is also known [24]. Thus the question remains where particular terpene synthases perform their activity. From genome sequence information all putative monoterpene synthases of *A. thaliana* posess a conserved transit peptide, whereas the annotated sesquiterpene synthases do not bear any distinct targeting signal [14]. However, experimental verification of the hypothesized localization of most terpene synthases in *A. thaliana* has not been achieved. In other species, a more detailed picture is available which helps to understand the functions of terpenes. For example, in fruits of cultivated strawberry (*Fragaria* x *ananassa*) the monoterpene alcohol linalool and the sesquiterpene alcohol nerolidol are formed in the cytosol whereas in the presumed ancestor of cultivars *F. vesca* these compounds are not produced, but instead terpenes are formed in plastids [4]. The breeding process presumably resulted in a change of protein targeting from plastids to the cytosol, thereby enabling the production of these new products, which are responsible for the aroma of cultivated strawberry.

To study the range of inducible terpene emission in *A. thaliana,* we determined the variability of emission using an array of ecotypes. Then, two ecotypes (Col-0 and Ws) were chosen for a more in-depth analysis of the genes encoding the synthesis of variable compounds, (*E*)-β-ocimene and *(E,E)*-α-farnesene. *In vitro* and *in vivo* experimental methods were used to show the function of two synthases that are most likely responsible for the observed phenotypes. Expression analysis, knock-out studies and subcellular localization investigations with signal peptide-GFP hybrid proteins were also performed.

## 4.2 Materials and Methods

### Plants and Treatments

An array of 27 ecotypes of *Arabidopsis thaliana* (L.) Heynh. was used for comparative analyses of induced leaf volatiles. The two ecotypes Ws (Wassilewskija) and Col-0 (Columbia) were chosen for cloning of genes and RT-PCR analysis. Plants were grown from seeds in plant climate chambers either under long-day (16-h light/8-h dark) or short-day (10-h light/14-h dark) conditions. Hydroponic plants were raised from seeds with an aqueous mineral solution [61], with the following modification: instead of $(NH_4)_6Mo_7O_{24}$ we used $Na_2MoO_4$, the final concentration of 0.075 $\mu$M $Mo^{6+}$ remained the same. Induction of volatile emission was achieved with detached leaves or via treatment of roots of hydroponically grown plants of various *A. thaliana* ecotypes. For induction, aqueous solutions of 2.5 $\mu$M alamethicin or 100 $\mu$M of the jasmonic acid mimic coronalon (O-methyl-L-isoleucine-(2*S*;3*S*)-ethyl-indanoylcarboxylamide) [148] were applied. Controls contained 0.1% ethanol or 0.1% DMSO. For inhibitor experiments, one day prior to induction the inhibitors fosmidomycin (50 $\mu$M) and lovastatin (100 $\mu$M) were applied.

### Insects

Insect feeding experiments were performed with larvae of a *Plutella xylostella* G88 colony. Larvae were reared on artificial diet on wheat germ base at 27°C with a 18-h light/6-h dark cycle. For feeding experiments, approximately 20 larvae were used in their third or fourth instar with a size of ca. 8 – 10 mm.

### Volatile Collection

A closed-loop stripping method was employed for the detection of induced volatiles from cut leaves and intact rosettes of hydroponically grown plants of wild-type Col-0 and Ws ecotypes as well as for the screening of RNAi/antisense plants. Approximately 1 g leaves from 5-week old plants was transferred to a small glass beaker filled with 10 mL water with or without the elicitor. The beaker was placed in a 2 L desiccator and emitted volatiles were collected under continuous air circulation on activated charcoal traps. Volatiles from hydroponically grown plants were collected in a similar way with the exception that plants were placed with their roots into a shallow beaker containing 30 ml aqueous solution. Trapped volatiles were eluted with 50 $\mu$L $CH_2Cl_2$ containing 1.8 ng 1-bromodecane or 2.0 ng nonylacetate per $\mu$L as internal standard (IS). Samples from volatile collection or from headspace solid-phase microextraction were analyzed by gas chromatography-mass spectrometry (GC-MS). Conditions were used as described previously [30] with the following modifications: the temperature program for all samples started at 45°C with a ramp of 5°C/min to 180°C followed by a ramp of 60°C/min to 300°C. For quantification, one main fragment and two further fragments together with their specific relative intensities were selected for the identification of each compound, and peaks of each compound were integrated and related

to the response of authentic standards and the IS for quantification (SIM, selected ion monitoring).

### Isolation and cloning of At4g16730 and At4g16740 cDNAs and heterologous protein expression in *E. coli*

Total RNA of *A. thaliana* leaves was isolated with the TRIzol reagent and 4 $\mu$g RNA were reverse transcribed into first strand cDNA as described previously [160]. After amplification of full length *TPS* cDNAs, the PCR products were inserted into the cloning vector pCR-TOPO4 (Invitrogen, Carlsbad, CA, USA). Gene sequences were verified for correct amplification with an ABI3100 capillary sequencer (ABI Applied Biosystems, Foster City, CA, USA). For *A. thaliana* ecotype Ws, sequences of products from three independent amplification reactions were analyzed to obtain a consensus sequence for the ecotype-specific gene sequence, since no reference sequence was available. The full-length cDNAs of At4g16730 and At4g16740 in the sequencing vector pCR-TOPO4 were then amplified by PCR and cloned into the expression vector pCR-T7/CT (Invitrogen, Carlsbad, CA, USA). Gene sequences in this expression vector and sequences modified by site-directed mutagenesis carrying a C-terminal tag for 6xHis were expressed in *E. coli* BL21(DE3) under ampicillin selection (50 $\mu$g/ml). Proteins were purified from crude bacterial extract and desalted as described previously [160].

### Site-directed mutagenesis

Site-directed mutagenesis was employed to alter specific nucleotides of the full-length transcript of At4g16730 (Col) in the expression vector pCR-T7/CT. Specific primers with up to 36 nucleotides, the proofreading polymerase *Pfu*Turbo (Stratagene, La Jolla CA, USA), and the restriction enzyme *Dpn*I (New England Biolabs, Ipswich MA, USA) were used according to the manufacturer's protocol of the "QuikChange Site-Directed Mutagenesis Kit" (Stratagene, La Jolla CA, USA).

### Terpene synthase enzyme assays

Terpene synthase enzyme assays were performed as described [30] with the following modifications: The reaction volume was reduced to 500 $\mu$L containing 100 $\mu$L of protein extract, and assays were performed in glass vials of 2 mL volume. The reaction mixture was incubated for 1 h at 30°C. Volatiles were trapped with a solid-phase microextraction (SPME) fiber exposed to the headspace of the assay and analyzed by GC-MS as described above.

### Construction of antisense and RNA interference (RNAi) lines

As no knock-out lines for the genes of interest were available through the seed stock centres or other sources, we attempted to silence At4g16730 and At4g16740 by anti-

sense and RNAi approaches. For At4g16740 antisense lines, the coding sequence of At4g16740 (Col-0) was amplified by PCR with the primers 5'-ataggtacccattatataatttgagtagag-3' and 5'-atagagctcattagcgagaggcgacac-3' introducing the restriction sites *Kpn*I (5') and *Sac*I (3'), respectively. The fragment was cloned into the binary vector pBIN420 to be expressed as an antisense fragment under control of a CaMV 35S-promoter. RNAi lines for At4g16740 were created with the vector pFGC5941 (ABRC stock CD3-447, Basta resistance) in a two-step procedure. First a sense fragment amplified by PCR with the primers 5'-aatctagaggcgcgccaggcaaagtggtataaaagtggt-3' and 5'-aaggatccatttaaatagagggtggacgagcaaggac-3' was introduced in pFGC5941 after restriction digestion with *Asc*I and *Swa*I, then an antisense fragment was introduced with *Bam*HI and *Xba*I restriction sites. In the vector the two fragments are separated by a chalcone synthase gene of 1365 nucleotides. The antisense as well as the RNAi constructs were transformed into *A. tumefaciens* GV1301 and *A. thaliana* ecotypes Col-0 and Ws were subsequently transformed with resistant clones via vacuum infiltration [18]. Transformed seeds carrying the pBIN420 derived fragment were selected on agar plates for kanamycin resistance. Transformed seeds carrying the pFGC5941 derived fragment were selected on soil for glufosinate resistance.

## GFP Localization Experiments

Fragments encoding putative signal peptides were cloned in the sequencing vector pCR-TOPO4 (Invitrogen, Carlsbad, CA, USA). A sequence fragment of At4g16730 (Ws) was amplified by PCR with the primers 5'-taaccatggctgctcataatctatgcttc-3' and 5'-taactagtggtcgaggtcgttttggagacagc-3' introducing the restriction sites *Nco*I (5') and *Spe*I (3') and fused upstream and in-frame with the GFP variant mGFP5 [151], downstream of a 35S-CaMV promoter in the vector pCAMBIA1302 (Access. Nr AF234298,-[62]). The same procedure was used for the fragment of At4g16740 (Col) with the primers 5'-taaccatggctaaacgacaggctcaacggcg-3' and 5'-taactagttttcacatatgtattaccgagcg-3'. Following confirmation of error free constructs by sequencing, constructs were transformed in *Agrobacterium tumefaciens* GV1301 and clones selected against kanamycin resistance (50 $\mu$g/mL). Subsequently, *A. thaliana* ecotypes Col-0 and Ws were transformed by vacuum infiltration [18]. Transgenic seeds were screened on agar plates for hygromycin resistance (30 $\mu$g/mL) and genomic insertion of the corresponding fusion constructs was confirmed by PCR.

Fresh, two week old plantlets were mounted on water and subsequently used for microscopy. The microscope used was a confocal laser scanning microscope LSM 510 (Carl Zeiss, Jena, Germany) equipped with a HeNe laser. Tissue autofluorescence was excited with 458 nm and GFP fluorescence was excited with 488 nm. Band pass was set to 500 – 550 nm (GFP fluorescence), long pass was set to 560 nm (chlorophyll fluorescence). 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. The final images were aligned with the image software Adobe Photoshop 7.0 (Adobe Systems, San José, CA, USA).

### Determination of At4g16730 and At4g16740 gene expression by RT-PCR

For expression analysis of At4g16730 in tissues of Col-0 and Ws, RT-PCR reactions were performed with the Col-0 specific primers 5'-ctccgtcctatccgaccac-3' and 5'-ctgggcttccgtggc-3'. For expression analysis of At4g16740 in Col-0, RT-PCR reactions were set up with the Col-0 specific primers 5'-ctacacttgttctgtctcttatc-3' and 5'-gggtggacgagcaaggac-3'. cDNA was produced with reverse transcriptase as described for cDNA cloning. PCR reactions and analysis of reaction products were done as described [160].

## 4.3 Results

### (*E*)-β-Ocimene and (*E,E*)-α-farnesene biosynthesis and emission are induced in *Arabidopsis thaliana* leaves but vary among different *A. thaliana* ecotypes

A survey of 29 hydroponically grown ecotypes of *A. thaliana* was done to investigate the qualitative and quantitative natural variation of induced terpene emission from rosette leaves. Induction was achieved with the jasmonic acid / coronatine mimic coronalon. The experimental setup allowed the uptake of the inducer into the leaves via the roots. Detected terpenes were 4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT), *(E,E)*-α-farnesene and (*E*)-β-ocimene. Control samples had very little or no terpene emission. The total amount of terpenes emitted at day 2 after induction was highly variable among different ecotypes (Table 6). Although (*E*)-β-ocimene was detected in the largest amounts, some ecotypes emitted none of this compound and minor amounts of TMTT or *(E,E)*-α-farnesene instead. Roughly more than two thirds of all ecotypes showed induced emission of either (*E*)-β-ocimene and (*E,E*)-α-farnesene. From three

Table 6: Terpene emission of 27 ecotypes of *A. thaliana* after induction with the elicitor coronalon. Volatiles were collected between 22–30 h after induction. Values are expressed as average of three replicates (ng $gFW^{-1}$ $h^{-1}$) along with the standard deviation. The ecotypes are sorted in ascending order for the emitted amount of (*E*)-β-ocimene.

| | Compound | | | | | |
|---|---|---|---|---|---|---|
| Ecotype | (*E*)-β-ocimene | | (*E,E*)-α-farnesene | | TMTT | |
| | Induced | Control | Induced | Control | Induced | Control |
| AA-0 | n.d. | n.d. | 1,3± 0,6 | 0,0± 0,0 | 3,9± 1,9 | 0,0± 0,0 |
| Bl-1 | n.d. | n.d. | 0,1± 0,0 | 0,0± 0,0 | 1,8± 0,4 | 0,0± 0,0 |
| Lip-0 | n.d. | n.d. | 1,8± 1,0 | n.d. | 3,1± 1,7 | 0,1± 0,1 |
| Pi-0 | n.d. | n.d. | 0,8± 0,5 | 0,1± 0,1 | 1,1± 0,3 | 0,0± 0,0 |
| Est-H1 | 0,1± 0,0 | n.d. | n.d. | n.d. | 0,0± 0,0 | n.d. |
| Tsu-1 | 0,1± 0,1 | n.d. | 0,0± 0,0 | 0,0± 0,0 | 0,4± 0,6 | n.d. |
| Col-0 | 0,1± 0,1 | n.d. | 1,8± 1,7 | 0,1± 0,0 | 2,5± 0,7 | 0,1± 0,1 |
| Can-0 | 0,2± 0,1 | n.d. | n.d. | n.d. | 6,4± 4,4 | n.d. |
| Bla 1-10 | 0,2± 0,1 | n.d. | n.d. | 0,0± 0,0 | 0,0± 0,0 | n.d. |
| Di-g | 2,6± 1,4 | n.d. | 0,2± 0,1 | n.d. | 0,4± 0,2 | n.d. |
| Ri-0 | 9,4± 7,7 | n.d. | 0,7± 0,7 | n.d. | 0,6± 0,3 | 0,0± 0,0 |
| Stw-0 | 9,7± 2,2 | 0,3± 0,6 | 0,2± 0,2 | n.d. | 1,2± 0,2 | n.d. |
| Lu-1 | 11,1± 4,8 | 0,3± 0,3 | 0,4± 0,3 | n.d. | 1,9± 1,2 | 0,1± 0,0 |
| Est-0 | 18,6± 15,3 | n.d. | 1,3± 1,0 | n.d. | 0,7± 0,2 | n.d. |
| Sei-0 | 19,9± 10,1 | 0,1± 0,0 | 0,3± 0,1 | n.d. | 0,2± 0,1 | n.d. |
| Chi-0 | 20,0± 16,3 | 0,3± 0,1 | 0,4± 0,3 | n.d. | 0,5± 0,3 | 0,0± 0,0 |
| Hodja | 20,8± 11,5 | 0,2± 0,2 | 0,6± 0,3 | 0,0± 0,0 | 0,6± 0,3 | n.d. |
| Tul-0 | 32,8± 13,4 | n.d. | 3,2± 1,3 | n.d. | 0,0± 0,0 | n.d. |
| Pog-0 | 36,2± 18,8 | n.d. | 2,9± 1,6 | 0,3± 0,2 | 0,1± 0,0 | 0,0± 0,0 |
| An-1 | 37,5± 7,8 | 0,3± 0,2 | 1,2± 0,1 | 0,1± 0,1 | 0,5± 0,1 | 0,0± 0,0 |
| Mt-0 | 45,9± 33,8 | 0,1± 0,0 | 5,9± 4,3 | 0,1± 0,1 | 2,2± 0,2 | 0,2± 0,1 |
| Kil-0 | 46,9± 30,2 | 0,0± 0,0 | 1,1± 0,8 | n.d. | 0,3± 0,2 | n.d. |
| Jl-3 | 51,2± 34,8 | n.d. | 2,8± 2,1 | 0,1± 0,1 | 1,3± 0,6 | 0,0± 0,0 |
| Ang-0 | 64,4± 37,8 | 0,8± 0,1 | 2,1± 1,5 | 0,1± 0,1 | 5,1± 2,5 | 0,2± 0,1 |
| Condara | 80,4± 33,6 | 0,3± 0,1 | 3,6± 1,4 | 0,3± 0,3 | 3,5± 1,4 | 0,1± 0,1 |
| Bla-1 | 89,2± 15,7 | 0,1± 0,1 | 3,6± 1,5 | 0,0± 0,0 | 0,2± 0,2 | n.d. |
| Ws | 121,2± 15,3 | 0,1± 0,1 | 2,7± 0,5 | 0,0± 0,0 | 4,4± 1,2 | n.d. |
| Ty-0 | 141,5± 50,9 | 0,1± 0,1 | 10,8± 4,7 | 0,1± 0,0 | 0,5± 0,2 | 0,1± 0,1 |
| Kas-1 | 160,6± 20,9 | 0,2± 0,1 | 2,7± 0,3 | 0,0± 0,0 | 5,4± 1,5 | n.d. |

ecotypes (Ty-0, Kas-1, and Ws) very high emission rates of (*E*)-β-ocimene were observed, but these did not correlate with high emission of either (*E*,*E*)-α-farnesene or TMTT.

The two ecotypes Col-0 and Ws were selected for further studies because of their different inducible terpenoid emission patterns (Table 6). Induced terpene emission from these ecotypes was not only observed upon treatment with coronalon but also in response to treatment with the peptaibol elicitor alamethicin from the fungus *Trichoderma viride* (Figure 17). The Col-0 ecotype emitted small amounts of (*E*,*E*)-α-farnesene, but no (*E*)-β-ocimene, whereas the Ws ecotype emitted a small amount of (*E*,*E*)-α-farnesene, and large amounts of (*E*)-β-ocimene.

## Two closely related terpene synthases At4g16730 and At4g16740 can catalyze the formation of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene, but are differentially expressed in Col and Ws ecotypes

Based on previous findings that terpene biosynthesis is, to a large extent, regulated at the transcription level of terpene synthases, we wanted to investigate whether the presence and absence of (*E*)-β-ocimene correlated with the transcription pattern of putative monoterpene synthase genes in the two ecotypes Col-0 and Ws. Detached leaf material treated with alamethicin was harvested and RNA extracted. RT-PCR was done with gene specific primers for all monoterpene synthases [30]: Expression patterns showed an upregulation of At4g16730 in both ecotypes, although in the Ws ecotype some expression of At4g16730 was also detected from the control samples. By contrast, At4g16740 was upregulated in the Col-0 ecotype but not in the Ws ecotype (Figure 18). The DNA sequences and deduced amino acid sequences from At4g16730 and

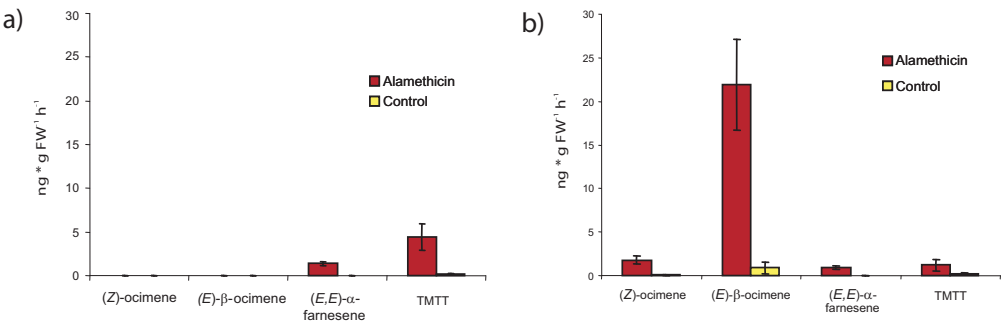


Figure 17: Induced emission of volatile terpenes from detached leaves of the *A. thaliana* ecotypes Col-0 (a) and Ws (b). Emission of *(E)*-β-ocimene occured in Ws but not in Col-0. Leaves were cut and placed for 30 h in a solution with the fungal derived peptaibol alamethicin. Volatiles were collected over 8 h on day 1 after induction.

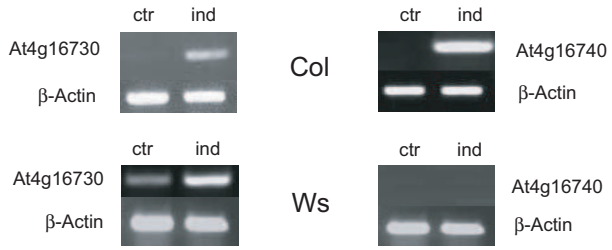


Figure 18: Transcript analysis of At4g16730 and At4g16740 of Col and Ws plants in response to treatment with alamethicin for 30 h.

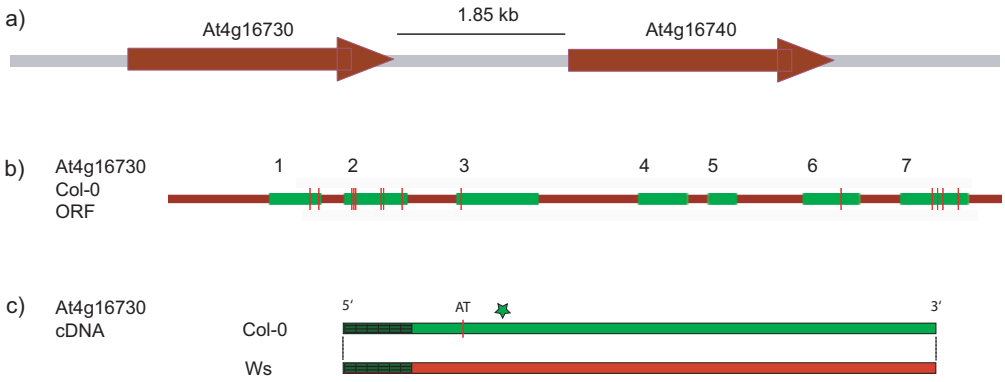


Figure 19: Scheme of At4g16730 and At4g16740 in the two ecotypes Col-0 and Ws. The genes in the two ecotypes are arranged as tandem copies (a) and assumed to be orthologous. The open reading frame of At4g16730 spans around 3.1 kb; exons are numbered from 1 – 7 (b). Non-synonymous nucleotide substitutions in At4g16730 of the Col-0 ecotype in comparison to the orthologous gene in Ws are in the exons 1, 2, 3, 6, and 7. A frame-shift mutation in the Col-0 ecotype is caused by insertion of an AT in the first exon (c). The insertion creates a premature translation termination codon downstream which results in a shortened protein (asterisk). Putative subcellular N-terminal targeting sequences are shaded dark (c). Complete cDNAs of At4g16730 (ca. 1.7 kb) were detected in both ecotypes after induction.

At4g16740 of both ecotypes were compared. In the Col-0 ecotype the two genes are arranged in tandem as deduced from the genomic sequence (Figure 19 a). Genomic sequencing of that region in the ecotype Ws revealed that the two genes were also present in tandem, and thus can be considered as orthologues. However, in the Ws-At4g16730 promoter region, around 1 kb upstream of the start codon, ca. 300 nucleotides were found to be deleted. After induction with alamethicin, complete cDNAs of At4g16730 in the Col-0 and Ws ecotypes and At4g16740 in the Col-0 ecotype could be retrieved. Sequence analysis of At4g16730 revealed 13 non-synonymous nucleotide substitutions and one double insertion in the Col-0 ecotype as compared to the corresponding sequence in Ws. The insertion at the end of the first exon caused a frame-shift that leads to a short protein due to a premature termination codon downstream of the start codon (Figure 19 c).

To determine whether the isolated cDNAs encoded terpene synthases whose products correlated with the induced volatile profiles, full-length cDNAs of At4g16730 (Ws) and At4g16740 (Col) were expressed in *E. coli* and purified via a fused 6xHis-tag. Enzyme assays with crude as well as partially purified protein revealed enzyme activities with the $C_{10}$ substrate geranylpyrophosphate (GPP) and the $C_{15}$ substrate farnesylpyrophosphate (FPP). Both enzymes converted GPP to (*E*)-β-ocimene as the predominant product together with small amounts of (*Z*)-ocimene and β-myrcene. With FPP, (*E*,*E*)-α-farnesene was the main volatile product (Figure 20).

Since At4g16730 from Col-0 appeared to be a pseudogene due to a shift in the reading frame [2], expression of the corresponding cDNA resulted in a non-functional

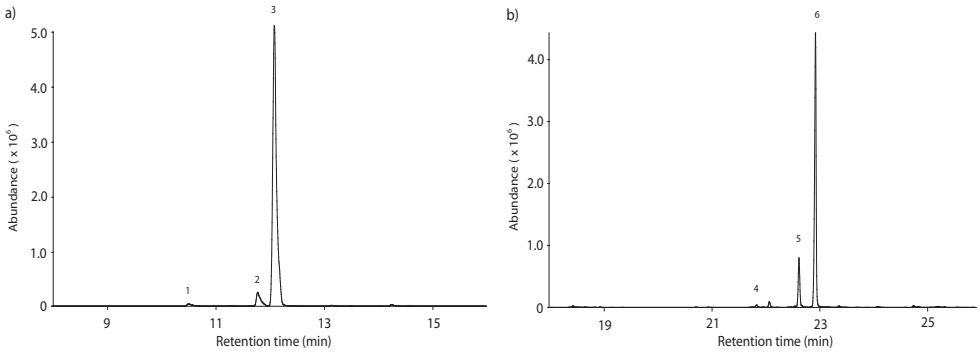


Figure 20: Volatile products formed by *in vitro* activity of Ws-At4g16730. Volatiles were sampled in the headspace of the enzyme assay and analyzed by GC-MS; total ion chromatograms are shown. (a) The substrate geranylpyrophosphate was transformed to (*E*)-β-ocimene and (Z)-ocimene. (b) With farnesylpyrophosphate, the main volatile products were (*Z*,*E*)- and (*E*,*E*)-α-farnesene. Monoterpenes: 1: β-myrcene, 2: (*Z*)-ocimene, 3: (*E*)-β-ocimene; sesquiterpenes: 4: β-farnesene, 5: (*Z*,*E*)-α-farnesene, 6: (*E*,*E*)-α-farnesene. Col-At4g16740 gave a similar product spectrum with both substrates.

protein. We further investigated if it was possible to convert the protein into a functionally active enzyme by selected mutations based on comparison of the Col-0 and Ws amino acid sequence. First, using site-directed mutagenesis, we reverted the disrupted open reading frame of At4g16730 (Col-0) by removing the AT pair responsible for the frame-shift. However, the resulting protein was still not active as shown by *in vitro* enzyme assays. Next, the remaining non-synonymous nucleotide polymorphisms responsible for 13 different amino acids in At4g16730 as compared to the corresponding gene in Ws were subjected to step-by-step site-directed mutagenesis. The resulting sequences were overexpressed in *E. coli* and proteins analysed for enzymatic function. Full enzymatic function was achieved when all sites in exon 1 and 2 (encode part of the N-terminal domain) were changed to the sequence of At4g16730 from Ws. The single change of I103F (exon 2), supposedly a structurally critical amino acid, resulted in an active protein. In contrast, several changes in the C-terminal domain alone did yield in proteins with no or only marginal activity. Taken together, our studies indicated that the Col-0 and the Ws ecotype each express one of two closely related functional monoterpene synthases which both form (*E*)-β-ocimene and (*E,E*)-α-farnesene *in vitro*. The difference in the emission of (*E*)-β-ocimene between both ecotypes suggested that the enzymes might reside in different cellular compartments and either primarily convert GPP to (*E*)-β-ocimene (plastids) or only FPP to (*E,E*)-α-farnesene (cytosol).

### At4g16740 (Col) is responsible for the formation of (*E,E*)-α-farnesene while At4g16730 (Ws) might be responsible for the formation of (*E*)-β-ocimene

By silencing of At4g16730 (Ws) and At4g16740 (Col-0) we hoped to show the function of the respective genes *in planta*. However, only for At4g16740 (Col-0) could antisense as well as RNAi lines be established. Analyses of these transformants showed, that no (*E,E*)-α-farnesene was emitted after induction with coronalon. The emission of TMTT was not inhibited in the transformants and was similar to that in the Col-0 wild type (Figure 21).

Due to the high sequence similarity of TPS sequences, a broad silencing of TPS genes with one specific antisense fragment can be imagined. We therefore investigated whether the induced emission of (*E*)-β-ocimene in the Ws ecotype could be also inhibited with the RNAi construct for At4g16740 (Col-0). Analyses of 20 independent transformant lines harboring the RNAi constructs in the Ws background showed no reduced emission of (*E*)-β-ocimene after induction. Induced control samples (Ws wild-type) could not be distinguished from the transformants (Figure 22). Our results demonstrate that At4g16740 functions as an (*E,E*)-α-farnesene synthase in Col-0 and that its homologous enzyme At4g16730 might be responsible for (*E*)-β-ocimene formation in the Ws ecotype.

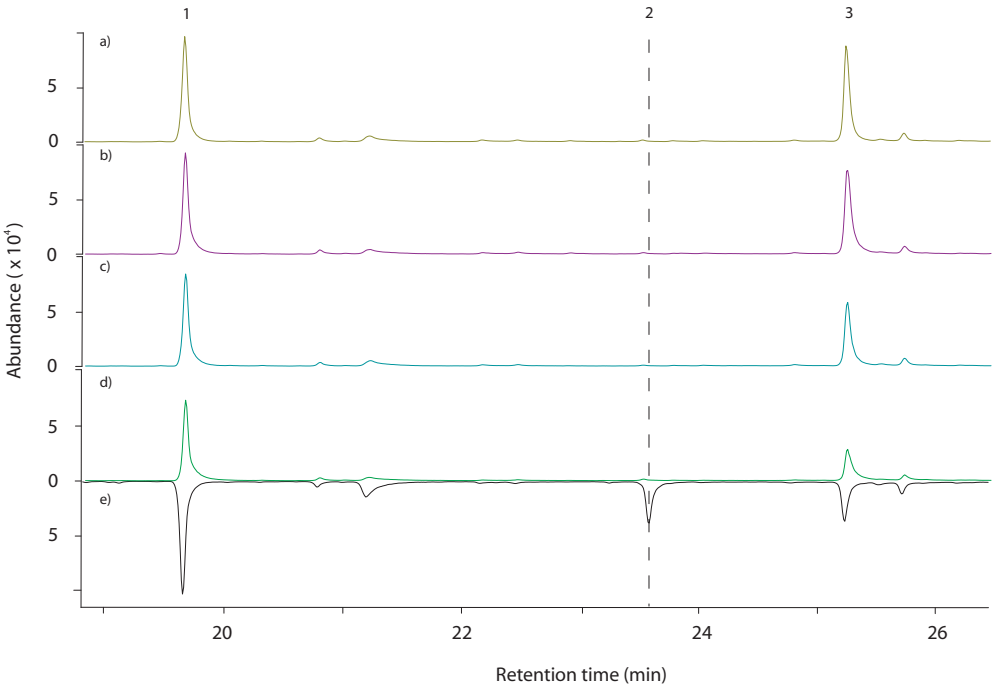


Figure 21: GC-MS chromatograms of induced volatiles from leaves of At4g16740 RNAi lines in the Col-0 background. Induction was achieved by placing cut leaves in an aqueous solution of 100 $\mu$M coronalon. Selected ion monitoring of 4 independent RNAi lines (a-d) and a wild-type Col-0 as control (e) revealed the absence of (*E*,*E*)-α-farnesene in the transformants but not in wild-type. The trace signal at rt=23.6 min represents background contamination and has no m/z signals characteristic for (*E*,*E*)-α-farnesene. 1: internal standard, 2: (*E*,*E*)-α-farnesene, 3: TMTT

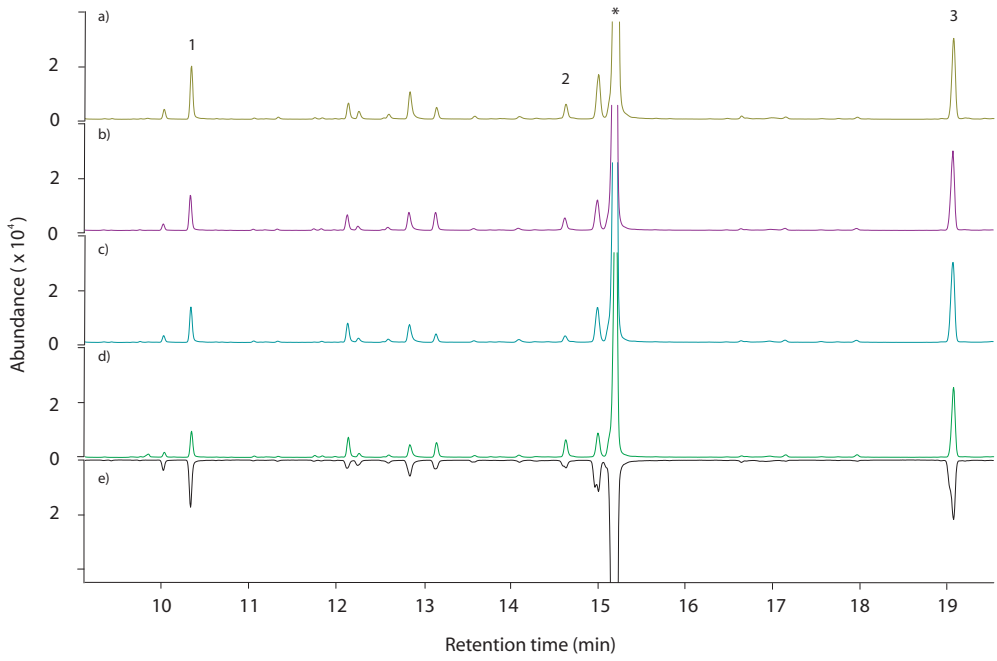


Figure 22: GC-MS chromatograms of induced volatiles from leaves of At4g16740 RNAi lines in Ws background. Selected ion monitoring of 4 independent lines (a-d) compared with wild-type Ws as control (e) revealed no differences between wild-type and transformants. 1: (*E*)-β-ocimene, 2: methyl salicylate, 3: internal standard, asterisk: impurity

## At4g16740 (Col) is located in the cytosol while At4g16730 (Ws) is targeted to the chloroplast

According to what is known about intracellular targeting of terpene synthases in plants, mono- and sesquiterpene synthases are thought to be located in plastids and the cytoplasm, respectively. In order to investigate whether the difference in emission of (*E*)-β-ocimene in Ws and Col-0 ecotypes is caused by differential compartmentation of the At4g16730 and At4g16740 enzymes in plastids and the cytosol, respectively, we intended to determine the subcellular location of both *TPS* proteins. To investigate the subcellular targeting of the *TPS* proteins encoded by At4g16730 and At4g16740, we subjected the sequences of At4g16740 (Col-0) and At4g16730 (Ws) to *in silico* analysis [50]. The algorithm TargetP which integrates the predictions from the common algorithms ChloroP and SignalP, predicted a chloroplast transit peptide for Ws-At4g16730 and, interestingly, a putative mitochondrial transit peptide for Col-At4g16740 (Table 7). To prove the subcellular targeting of the respective terpene synthases, we produced GFP hybrid fusion proteins with the putative signal peptide sequences of At4g16730 (the putative (*E*)-β-ocimene synthase from Ws ecotype) and the (*E,E*)-α-farnesene At4g16740 from the Col-0 ecotype. The binary vector pCAMBIA-1302 with mGFP5 resulted in C-terminal fused protein-mGFP5 hybrids. As a control for plastidial localization, we used a construct with EGFP attached to the C-terminus of the signal peptide (SP) of ferredoxin N-reductase (FNR). Transformed plantlets in the four leaf stage were examined under UV light with a binocular microscope, but no GFP fluorescence could be visualized. Therefore screens for GFP excitation were conducted directly with a laser scanning microscope LSM510 (Zeiss, Jena, Germany). Pictures acquired from the different plant lines are shown in Figure 23. The plastid localized control (SP-FNR-EGFP) showed distinct GFP signals that colocalized with the chlorophyll fluorescence of chloroplasts. Whereas a similar picture could be obtained from the SP-At4g16730-(Ws)-GFP transformants, the plants with SP-At4g16740-(Col-0)-GFP did not show any clear subcellular localization. GFP signals

Table 7: Prediction results of the TargetP algorithm to test for subcellular targeting of Col-At4g16740 and Ws-At4g16730. cTP: chloroplast targeted, mTP: mitochondrial targeted, SP: signal peptide for secretion, Loc: subcellular compartment (chloroplast or mitochondria), RC: reliability class, TPlen: predicted length of transit peptide. http://www.cbs.dtu.dk/services/TargetP/

| Name | Length | cTP | mTP | SP | other | Loc | RC | TPlen |
|---|---|---|---|---|---|---|---|---|
| Ws-At4g16730 | 589 | 0.652 | 0.122 | 0.111 | 0.076 | C | 3 | 25 |
| Col-At4g16740 | 565 | 0.205 | 0.722 | 0.008 | 0.126 | M | 3 | 9 |
| cutoff | | 0.000 | 0.000 | 0.000 | 0.000 | | | |

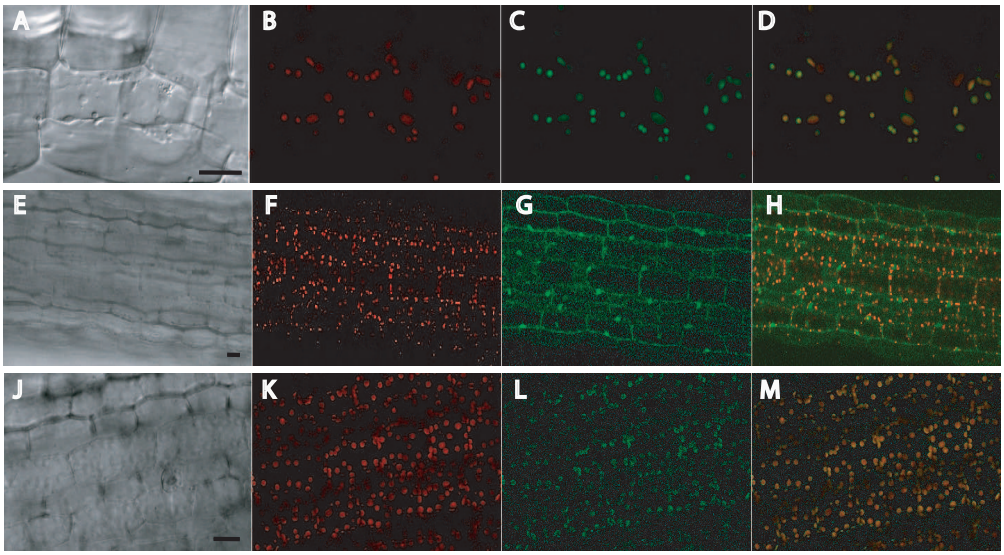


Figure 23: Confocal laser scanning microscopy of *A. thaliana* seedlings transformed with signal peptide-green fluorescent protein (SP-GFP) hybrid constructs. Pictures were taken from hypocotyl regions. A-D: plastid localized control with the SP of ferredoxin N-reductase and EGFP; E-H: SP of At4g16740 (Col) and mGFP5; J-M: SP of At4g16730 (Ws) with mGFP5. In H no colocalization of GFP signal and chloroplasts can be seen. The plastid-localized control (D) showed colocalization of GFP signals and chloroplasts as did At4g16730 (M). 1$^{st}$ column: images acquired by differential interference contrast; 2$^{nd}$ column: autofluorescence of chlorophyll; 3$^{rd}$ column: fluorescence signals of GFP; 4$^{th}$ column: overlay of GFP and chlorophyll fluorescence. Band pass for GFP fluorescence was 500 – 550 nm, long pass of 560 nm for autofluorescence. Laser excitation was with 458 nm and 488 nm. Scale bars: 20 $\mu$m

may possibly be attributed to the nucleus and the cellwall, but did clearly not colocalize with chloroplasts.

In addition to subcellular localization of the two terpene synthases, we studied the contribution of the plastidial MEP pathway and the cytosolic MVA pathway in the formation of (*E*)-β-ocimene and (*E,E*)-α-farnesene using pathway-specific inhibitors. We hypothesized that the respective terpenes should be produced by $C_5$ units from one of the terpene biosynthetic pathways or the other, and not both. As the cytosolic MVA pathway can be specifically blocked by inhibiting the key enzyme HMGR with statins, and the plastid localized MEP pathway can be inhibited by fosmidomycin, we applied lovastatin and fosmidomycin separately to the ecotypes Col-0 and Ws and monitored volatile emission after induction with coronalon. We could show that the emission of TMTT was completely inhibited in both ecotypes by fosmidomycin but not with lovastatin. In the Ws ecotype, induced emission of (*E*)-β-ocimene was roughly eight-fold reduced by the application of fosmidomycin and approximately two-fold by lovastatin treatment. Interestingly, fosmidomycin also led to a four-fold reduction in the formation of (*E,E*)-α-farnesene, whereas lovastatin showed no effect. The results suggest, that formation of (*E,E*)-α-farnesene could occur in plastids – in contrast to the localization of (*E,E*)-α-farnesene synthase outside of plastids, – but might rather indicate cross-talk between terpene biosynthesis pathways in both compartments as observed in other plant species.

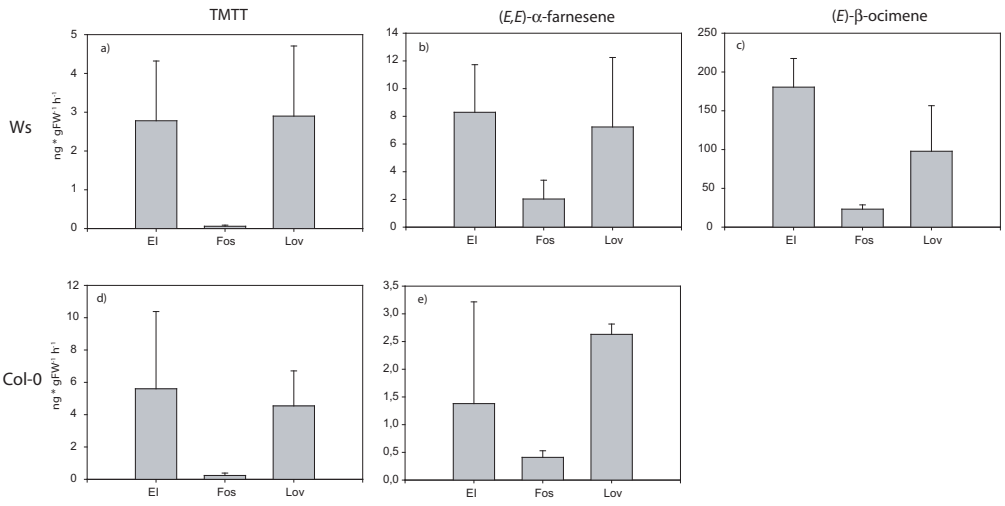


Figure 24: Volatile emission after inhibitor application and 24 h after induction with ethylindanone on cut leaves of *A. thaliana* Col-0 and Ws (ng $gFW^{-1}$ $h^{-1}$, standard deviation for n=3). Twenty four h after application of the inhibitors the samples were induced. First row (a-c): ecotype Ws, second row (d,e): ecotype Col-0. EI: ethylindanone alone, Fos: EI plus fosmidomycin, Lov: EI plus lovastatin.

## 4.4 Discussion

*A. thaliana* ecotypes were shown previously to have different profiles of terpene floral volatiles [160], but variation in induced terpene emissions from rosette leaves has not been studied until now. Terpene emission from different ecotypes of *A. thaliana* was induced using different elicitors. As these inducers mimic herbivore attack by eliciting defence pathways [51, 57], this induction can be used as a reliable method to investigate plant stress responses. Each ecotype showed the same volatile profile with the fungal-derived elicitor alamethicin or with the coronatine analog coronalon when applied to cut leaves or to intact plants grown in hydroponic culture.

A survey of 29 hydroponically grown ecotypes with coronalon as inducer revealed a broad spectrum of (*E*)-β-ocimene emission rates from no emission to nearly 200 ng $gFW^{-1}$ $h^{-1}$. For example, the ecotype Ws emitted ca. 120 ng $gFW^{-1}$ $h^{-1}$ (*E*)-β-ocimene under these conditions, which represents a high emission rate in the spectrum obtained from all ecotypes (Table 6). However, the ecotype Col-0 emitted only trace amounts of (*E*)-β-ocimene. The amount of emitted (*E,E*)-α-farnesene was similarly low in both Col-0 and Ws ecotypes (ca. 2 ng $gFW^{-1}$ $h^{-1}$). To explain the underlying molecular and biochemical mechanisms responsible for these differences, a closer investigation with the ecotypes Ws and Col-0 was carried out.

Genomic sequence analysis and previous characterization of terpene synthases in *A. thaliana* suggested that two terpene synthases, At4g16730 and At4g16740, may be responsible for the observed differences in the Col-0 and Ws ecotypes. At4g16740 was described recently as a monoterpene synthase (94% (*E*)-β-ocimene, 4% (*Z*)-β-ocimene) [56]. The authors reported an upregulation of this gene by mechanical wounding and with jasmonic acid. By sequence comparison, At4g16730 was also assigned as a monoterpene synthase [14]. Another monoterpene synthase, At2g24210, was previously reported to synthesize (*E*)-β-ocimene (20%) as well as β-myrcene (56%) [23]. But, since we did not find any significant amounts of β-myrcene after induction, At2g24210 was not further considered as a candidate for induced ocimene emission.

As terpene synthesis is often regulated at the transcript level of terpene synthases [26, 172], it was assumed that the observed differences in terpene blends could be reflected in different *TPS* gene transcript patterns. After induction of the ecotype Col-0, we observed an upregulation of both At4g16730 and At4g16740. However, in the ecotype Ws, only At4g16730 was upregulated (Figure 18). In all cases, the induced transcripts were full-length. While (*E*)-β-ocimene emission from the Ws ecotype was positively correlated with the observed transcript pattern of At4g16730, no emission of (*E*)-β-ocimene was observed in the Col-0 ecotype despite the induced expression of both genes. We therefore investigated the coding sequences of the respective *TPS*

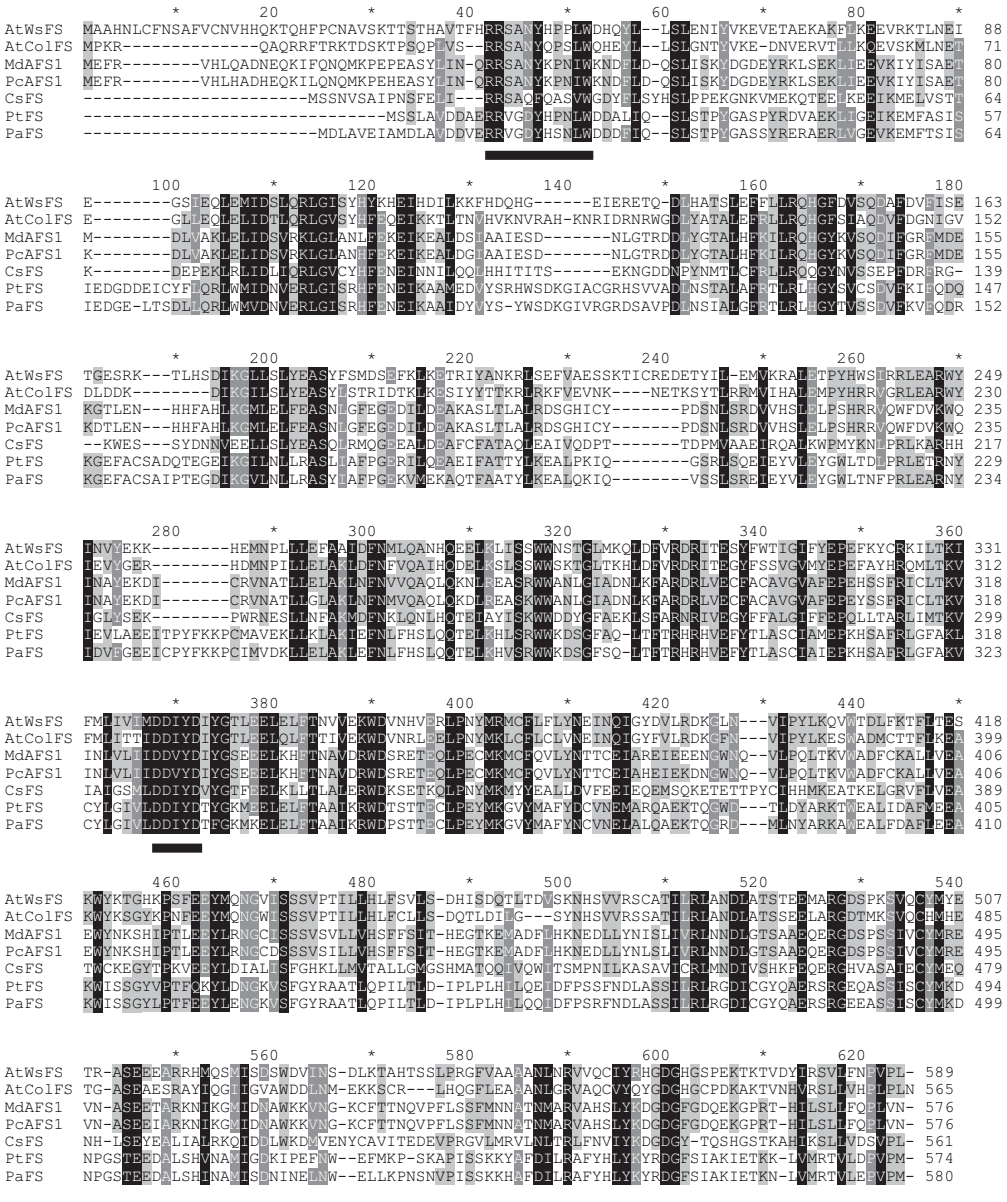

Figure 25: Alignment of deduced amino acid sequences of proteins with known (*E,E*)-α-farnesene synthase activity generated with ClustalX and GeneDoc. AtWsFS: At4g16730 from *A. thaliana* ecotype Ws; AtColFS: At4g16740 from *A. thaliana* ecotype Col-0; MdAFS1: AAO22848 from *Malus domestica*; PcAFS1: DQ309034 from *Pyrus communis*; CsFS: AY640154 from *Cucumis sativus*; PtFS: AAO61226 from *Pinus taeda*; PaFS: AY473627 from *Picea abies.* Underlined motifs: The DDxxD motif which is characteristic for terpene synthases is at position 370, the $RRX_8W$ motif is at position 42.

genes and the *in vitro* activities of their recombinant enzymes in greater detail.

In exon 1 of At4g16730 (Col-0) an insertion of an AT nucleotide pair results in a shift of the reading-frame which leads to a premature termination codon (PTC) (Figure 19). In plants as well as in yeast and animals there are quality control systems like nonsense-mediated mRNA decay (NMD) to eliminate erroneous transcripts like those with PTCs [73, 83, 111]. Interestingly, we still detected complete transcripts of At4g16730 in Col-0 with the PTC in high amounts after induction. However, it is unclear whether the mRNA is targeted for NMD or not because we did not measure transcript stability in detail. Nevertheless, even if the transcript is not targeted for NMD, and a shortened protein or even a longer nonsense version is translated, it would likely not have enzymatic activity because of the large missing portion of the catalytic domain. To verify the *in vitro* function of the other expressed *TPS* genes, At4g16730 from Ws and At4g16740 from Col-0, we expressed both genes in *E. coli* and analysed the proteins for enzyme activity. Both enzymes were able to convert the substrates geranyl pyrophosphate (GPP, $C_{10}$) and farnesyl pyrophosphate (FPP, $C_{15}$) into olefinic products. Whereas with GPP the monoterpenes (*E*)-β-ocimene (75%), (*Z*)-ocimene (22%), and β-myrcene (3%) were produced, the main volatile product with FPP was the sesquiterpene (*E*,*E*)-α-farnesene (plus minor amounts of (*Z*,*E*)-α-farnesene) (Figure 20). The results of At4g16740 are in contrast to a previous analysis by Fäldt *et al.* [56] who note the inability to produce farnesene from the substrate FPP. The reason for that may rely on sequence differences because they used the ecotype C24 and not Col-0. As the wild type version of At4g16730 from Col-0 was not active due to the frame-shift mutation (see above), we asked whether only this specific mutation was responsible for the loss of enzyme activity or if other observed polymorphisms also contribute to enzyme function. We used site-directed mutagenesis with a mutated primer-based polymerase chain reaction to try to convert the sequence to one encoding an active protein. Enzyme assays following overexpression of the construct without the frame-shift mutation still yielded an inactive protein. We then conducted a step-by-step mutagenesis of the 7 non-synonymous nucleotide polymorphisms different to the Ws gene in the N-terminal domain of the deduced protein sequence. After all 7 different sites in the N-terminal domain were changed to fit the sequence of the Ws gene, the result was an active protein. One of these sites, Ile143 (position 160 in Figure 25), is located in the protein's periphery and not close to the active site, and can therefore be regarded as a residue required for stability of the conformation of the enzyme. Changes of non-synonymous nucleotide polymorphisms in the translated C-terminal domain did not seem to have any effect on enzymatic activity.

The amino acid sequences of the investigated *A. thaliana* genes were compared to published sequences with significant similarity (Figure 25). All compared sequences

are reported to be (*E*,*E*)-α-farnesene synthases. Four of them, AtWsFS, AtColFS, MdAFS1 [125], and CsFS [114], also convert GPP to (*E*)-β-ocimene as shown by *in vitro* assays. From PcAFS1 which differs from MdAFS1 in only 13 amino acids, no data on activity with GPP are available [59]. From the two gymnosperm (*E*,*E*)-α-farnesene synthases, PtFS [127] and PaFS [113], only (*E*,*E*)-α-farnesene synthase activity is reported. A high degree of conserved residues was found between the sequences, even though they are members of the Brassicaceae, Rosaceae, Cucurbitaceae, and Pinaceae plant families which are only distantly related. The protein alignment (Figure 25) showed two conserved motifs characteristic for terpene synthases: The DDxxD motif is essential for the binding of the cation $Mg^{2+}$ in the central cavity of the protein. The other conserved motif in the N-terminal domain, the $RRX_8W$ motif, is predicted to be important for monoterpene formation [169]. Bohlmann [113] suggested it to be a conserved plastid targeting motif. Dudareva *et al*. [47] point out that this motif is characteristic for the *tps*-b and *tps*-d gene families of monoterpene synthases, but is absent in the *tps*-g group. The latter is a family of terpene synthases that produce acyclic monoterpenes. However, these statements are deduced from sequence comparisons only and lack proof of function in targeting.

In addition to the analysis of recombinant proteins, we intended to determine the function of the targeted *A. thaliana* genes *in vivo.* For most of the genes of *A. thaliana,* multiple gene knock-out lines have been generated via T-DNA insertion or transposon mutagenesis. However, for neither At4g16730 nor for At4g16740 are such mutants available in the Col-0 or Ws backgrounds. Because of these restrictions we attempted to generate mutants by using antisense and RNAi-silencing approaches. No antisense or RNAi lines could be established for At4g16730 (Ws) in the Ws background due to problems generating the correct sequence constructs. However, an antisense approach with the full coding sequence of Col-At4g16740 resulted in a complete depletion of induced (*E*,*E*)-α-farnesene emission. To verify this result by an alternative method, we generated an RNAi construct. Transformed plants showed a loss of (*E*,*E*)-α-farnesene emission (Figure 21). Since At4g16730 and At4g16740 have 74% sequence identity over 650 nucleotides in the middle of their transcripts, we examined whether the At4g16740 (Col-0) RNAi construct might affect expression of At4g16730 in the Ws background by inhibiting induced emission of (*E*)-β-ocimene and (*E*,*E*)-α-farnesene. No difference between Ws transformants and wild-type was observed after induction indicating no loss of At4g16730 expression. We therefore conclude that *TPS* gene expression can be specifically altered by RNAi approaches despite the high sequence similarity of *TPS* genes (Figure 22). The combination of *in vitro* analysis and the gene silencing experiments strengthen the hypothesis, that At4g16740 in Col-0 is indeed responsible for the production of (*E*,*E*)-α-farnesene whereas At4g16730 in Ws may

be responsible for (*E*)-β-ocimene formation in this ecotype.

To further analyze why the Col-0 enzyme functions as an (*E,E*)-α-farnesene *in vivo* without any production of (*E*)-β-ocimene, we investigated the subcellular location of this protein in comparison to its homolog At4g16730 in Ws. In *A. thaliana*, the enzyme that produces the substrate FPP, the FPP synthase, is located in the cytoplasm and mitochondria [35], while the GPP synthase may be present in the cytosol or the plastids [24]. The subcellular targeting of signal sequences can be estimated by algorithms [50]. The *in silico* localization prediction by the TargetP algorithm (which integrates the algorithms of ChloroP and SignalP), suggested targeting of Col-At4g16740 to mitochondria and Ws-At4g16730 to plastids. The generation of GFP-protein fusion constructs was attempted to confirm the predictions of the TargetP algorithm.

A green fluorescent protein (GFP) was fused with the putative transit peptide sequences of the respective proteins. We used mGFP5 [151] available through the vector pCAMBIA-1302 (Accession Nr AF234298, [62]). The sequence of mGFP5 was optimized for plant codon usage, but the modified version produced a less bright fluorescence signal compared to other versions like the enhanced GFP (eGFP). The latter version was used as positive control fused to the chloroplast targeting signal of the ferredoxin NADP reductase (FNR) [112]. Compared to the eGFP version, the background intensities with mGFP5 were much higher with confocal microscopy due to lower signal specificity. However, a fusion product of the signal peptide of At4g16730 (Ws) was localized in the chloroplasts, confirming the prediction of TargetP. For At4g16740 (Col-0), no chloroplast transit peptide localization could be assigned, and confocal microscopical analysis could neither confirm nor exclude targeting of the protein to mitochondria, as suggested by *in silico* analysis. Mitochondria might be a possible compartment because the substrate FPP is present and used for the formation of ubiquinone and heme A. As recent studies indicate, sesquiterpene synthases can be directed to mitochondria and hence efficiently convert FPP into volatiles without diverting too much of the substrate [82]. Another case of metabolite change caused by the breeding of cultivated strawberry was discovered recently [4]. Whereas ripe fruits of the cultivated form's ancestor *F. vesca* produce monoterpenes as their main terpene volatiles, the cultivar *F.* x *ananassa* makes linalool and the sesquiterpene nerolidol. Localization experiments showed that the *F.* x *ananassa* mono- and sesquiterpene synthases both were in the cytosol, but the *F. vesca* monoterpene synthase is localized to the mitochondrion. The flavor change may have occured by selection for mutated varieties with the more pleasant linalool-nerolidol flavor due to a change in enzyme location from the mitochondria to the cytosol.

An alternative to compartmentation different from the cytosol would be a compartment exchange of intermediates of terpene biosynthesis. The plastidially local-

ized MEP pathway could provide $C_5$-units transported into the cytosol and further be converted to sesquiterpenes, a scenario which is suggested by other studies [16, 46]. Therefore we further investigated the compartmentation of (*E*)-β-ocimene and (*E,E*)-α-farnesene formation via inhibition of the plastidial MEP and the cytosolic MVA pathway that provide the IPP precursor for terpene biosynthesis. The overall picture of biosynthetic pathways responsible for the production of terpenes in plants was only recently completed. Until the mid-90s, the MVA pathway operating in the cytoplasm was considered to be the only pathway for the production of the $C_5$units of terpenes, although doubts about that unique role were already pronounced. The rate-limiting step of the MVA pathway, which supplies substrates sterols and sesquiterpenes among other compounds, is HMG-CoA reductase (HMGR) [104] which can be specifically inhibited by statins. In plastids, only recently a separate pathway, the methyl-erythritol phosphate (MEP) pathway, was found to operate that produces substrates for mono- and diterpenes and carotenoids [103]. The regulation of that pathway seems to be more complex than that of the MVA pathway, but a limiting step is the enzyme DXS [53], which produces DXP and can be inhibited by fosmidomycin [80]. The application of this inhibitor in *A. thaliana* is well established to block the formation of carotenoids, isoprene and the chlorophyll phytyl side chain [175]. Induction of *A. thaliana* ecotype Col-0 by herbivory of *Pieris rapae* resulted in emission of TMTT [165]. Our inhibition experiments with fosmidomycin on cut leaves of *A. thaliana* strengthen the finding that TMTT in *A. thaliana* is derived from a diterpene precursor [11, 70]. Nearly no TMTT was emitted after fosmidomycin was added to the ecotypes Col-0 and Ws. As expected, (*E*)-β-ocimene emission in Ws was reduced by inhibition of the MEP pathway although some reduction was also observed with lovastatin. Interestingly, *(E,E)*-α-farnesene emission was also reduced in both ecotypes by fosmidomycin but not by lovastatin. These results possibly indicate a cross-talk between the cytosolic and plastidial compartment as has been described in other cases [16, 46]. However, since lovastatin was not determined to inhibit the production of any terpenoids in these experiments, its application may not have been effective.

# 5 Summary

Plants emit a variety of volatile organic compounds into the atmosphere, but the formation and function of many of these substances is not yet known. The biological variation of volatiles apparent in ecotypes or populational differences in terms of biochemical and molecular regulation is also widely unknown. This thesis employed molecular biological methods as well as chemical analyses to elucidate the emission profiles of two closely related crucifer species. Model organisms serve as a basis for solving important scientific questions, but each model suffers from certain limitations because of its physiological and ecological niche. We used the model plant *Arabidopsis thaliana* and the close relative *A. lyrata* ssp. *petraea*. The variability between these two species is likely caused by differential selective pressure since the divergence of the two species 5 million years ago. *A. thaliana* is a predominantly selfing annual of disturbed sites while *A. lyrata* is an obligate outcrossing perennial of rocky habitats. Temporal and spatial variability of volatile emission was investigated including different organs, ecotypes and populations.

### Variability of floral scent in *A. lyrata* ssp. *petraea* populations

Using *A. thaliana* and *A. lyrata* as a model system, we demonstrated significant intra- and interspecific differences in floral and vegetative volatiles of *A. thaliana* and *A. lyrata*. To estimate the variability of floral scent in central European *A. lyrata* ssp. *petraea* populations, plants from geographically isolated populations were investigated. Floral volatiles of *A. lyrata* ssp. *petraea* from populations from Germany, the Czech Republic and Austria were collected by headspace volatile trapping and analysed and identified by coupled gas chromatography-mass spectrometry (GC-MS). Besides single timepoint analysis of flowers, continuous whole plant measurements were done with proton-transfer-reaction mass spectrometry (PTR-MS) to test for temporal changes of scent emission over 24 hours. Furthermore, the organ-specificity of floral scent emission was determined by dissection of *A. lyrata* flowers. The results were compared with the known volatile spectrum of *A. thaliana*.

While the low amounts of floral scent of *A. thaliana* mainly consist of sesquiterpenes, no such compounds were found in the floral scent of *A. lyrata* ssp. *petraea*. The scent of this species is instead dominated by benzenoid compounds for which a distinct diurnally regulated emission was shown by different methods. The emission peaked between 10 a.m. and 1 p.m., which correlates positively with the high flight activity of important pollinating hymenoptera observed in the field. Dissection of floral organs showed that the floral volatiles were exclusively emitted from the petals; neither the gynoecium nor the androecium contributed to the emission. This finding is

in contrast to promoter-GUS studies of floral volatile biosynthetic genes in *A. thaliana* but in agreement with other studies on insect pollinated species like *Silene latifolia* or *Nicotiana suaveolens*.

The emission of floral scent from *A. lyrata* ssp. *petraea* was different within and between central European populations. In three investigated populations, distinct floral chemotypes were found (chapter 2). Compounds that dominated a particular chemotype were absent or minor compounds in other chemotypes (phenylacetaldehyde, 4-methoxy-benzaldehyde, methylbenzoate, methyl salicylate, linalool), with the exception of benzaldehyde which was detected in all chemotypes. The observed interpopulational differences in floral scent profiles of *A. lyrata* ssp. *petraea* might be a consequence of coevolution with a different local pollinating fauna.

### Variation of vegetative volatile terpenes

We observed qualitative differences in insect-induced volatiles between the annual *A. thaliana* and the closely related perennial *A. lyrata* ssp. *petraea*. When both species are subjected to herbivory by the Brassicaceae specialist *Plutella xylostella*, only *A. lyrata* emits the sesquiterpene (*E*)-β-caryophyllene (chapter 1). Considerable variation of (*E*)-β-caryophyllene emission together with other volatiles including DMNT, TMTT and nerolidol was found among individuals of a single *A. lyrata* population. A broad range from no emission of (*E*)-β-caryophyllene to emission of (*E*)-β-caryophyllene as the predominant induced volatile compound was found. In *A. thaliana*, (*E*)-β-caryophyllene is only emitted from floral tissue [30, 165] but it is not a constituent of floral scent in *A. lyrata* (chapters 1 and 2). Therefore, it can be assumed that the regulation of (*E*)-β-caryophyllene synthesis has evolved significantly different since the divergence of *A. thaliana* and *A. lyrata* 5 million years ago. While *A. thaliana* as an annual species may use (*E*)-β-caryophyllene as an antimicrobial agent to protect flowers and seed formation, *A. lyrata* may use other volatiles with antimicrobial activity such as benzaldehyde for floral defense. Herbivore-induced emission of (*E*)-β-caryophyllene in maize was shown to be involved in a putatively beneficial tritrophic interaction [133], but whether a similar function exists for *A. lyrata* is as yet unknown.

In order to characterize (*E*)-β-caryophyllene formation in *A. lyrata* at the molecular level, a gene encoding a (*E*)-β-caryophyllene synthase was isolated by RT-PCR using primers derived from the previously identified (*E*)-β-caryophyllene synthase from *A. thaliana* [160]. The function of the gene was confirmed by heterologous expression in *E. coli* and analysis of terpene synthase activity. The coding sequences of the (*E*)-β-caryophyllene synthases from *A. thaliana* and *A. lyrata* have the same degree of similarity as the housekeeping genes *mat*K and *Chs* [93], with 85% identity at the

nucleotide level and 92% similarity at the protein sequence level. Similar values were obtained for other *A. thaliana* terpene synthase genes and their putative homologues in *A. lyrata*. In contrast to the exclusive expression of the *A. thaliana* (*E*)-β-caryophyllene synthase in floral tissues, the homologous gene from *A. lyrata* is not expressed in flowers. Instead, transcription of this gene is upregulated in the foliage of *A. lyrata* which is in correlation with the induced (*E*)-β-caryophyllene emission from this tissue. The results suggest that tissue-specific differences in the formation of (*E*)-β-caryophyllene in *A. thaliana* and *A. lyrata* evolved by differential expression of the respective homologous terpene synthases in both species.

## Two differently regulated terpene synthase genes are responsible for induced formation of (*E*)-β-ocimene and (*E,E*)-α-farnesene in *A. thaliana*

We investigated the emission of volatiles among 29 *A. thaliana* ecotypes in response to treatment with the elicitor coronalon. The monoterpene (*E*)-β-ocimene, the sesquiterpene (*E,E*)-α-farnesene and the C16-homoterpene TMTT were observed in most ecotypes. Major quantitative differences were found for the emission of (*E*)-β-ocimene; for example, the Col-0 ecotype almost completely lacks (*E*)-β-ocimene emission, while the ecotype Ws shows release of (*E*)-β-ocimene as the major volatile compound upon coronalon treatment. These two ecotypes were selected for a detailed molecular analysis of the difference in (*E*)-β-ocimene emission.

A treatment with coronalon caused an upregulation of transcripts of the closely related terpene synthases At4g16730 and At4g16740 in the ecotype Col-0, while only At4g16730 was upregulated in the ecotype Ws. Both ecotypes produced full-length transcripts of the respective genes after induction. At2g24210, another monoterpene synthase gene of *A. thaliana*, was previously shown to function as a β-myrcene (56%) and (*E*)-β-ocimene (20%) synthase [23]. As we did not find any β-myrcene in considerable amounts, At2g24210 was not considered to be responsible for the polymorphism in (*E*)-β-ocimene / (*E,E*)-α-farnesene emission.

Although At4g16730 and At4g16740 are annotated as monoterpene synthases by sequence comparisons with other organisms [14, 47, 113], we demonstrated by *in vitro* assays with the recombinant enzymes of At4g16730 from Ws and of At4g16740 from Col-0 that each enzyme can function as a mono- and sesquiterpene synthase. Whereas with geranyl pyrophosphate the monoterpenes (*E*)-β-ocimene (75%), (*Z*)-ocimene (22%), and β-myrcene (3%) were produced, the main volatile product with farnesyl pyrophosphate was the sesquiterpene (*E,E*)-α-farnesene (plus minor amounts of (*Z,E*)-α-farnesene). No enzyme activity was found for the recombinant protein encoded by At4g16730 from Col-0 because of a mutation introducing a premature stop codon. Reestablishment of the full-length open reading frame and site-directed mu-

tagenesis of selected amino acids in the N-terminal region based on nucleotide polymorphisms in the gene of the Ws ecotype restored (*E*)-β-ocimene / (*E*,*E*)-α-farnesene activity of the Col-0 enzyme.

Silencing of At4g16740 in Col-0 via RNAi and antisense approaches resulted in the lack of induced (*E*,*E*)-α-farnesene emission, concluding that this terpene synthase is responsible for the formation of (*E*,*E*)-α-farnesene in the Col-0 ecotype. The function of At4g16740 from Col-0 as a sesquiterpene synthase was supported by a putative localization of a GFP-fusion protein in the cytosol, which is the primary compartment for the synthesis of FPP. In contrast, GFP-fusion of the N-terminal sequence of the At4g16730 protein from Ws resulted in targeting of this protein to the chloroplast, suggesting an *in vivo* function of At4g16730 from Ws as a (*E*)-β-ocimene synthase. This function remains to be further confirmed via RNAi-analysis. Together, the results indicate that differences in induced (*E*)-β-ocimene / (*E*,*E*)-α-farnesene emissions of two *A. thaliana* ecotypes are determined at the levels of gene mutation, differential gene expression and subcellular location of two closely related terpene synthases.

# 6 Zusammenfassung

Obgleich schon lange bekannt ist, daß Pflanzen flüchtige Stoffe in die Umgebung abgeben, so ist bis heute von vielen dieser Duftstoffe wenig über ihre Biosynthese und Funktion bekannt. Wie die biologische Variabilität dieser Stoffe auf der Ebene von Ökotypen oder Populationen biochemisch und molekularbiologisch erklärt werden kann, ist ebenso weitgehend ungeklärt. Mit Hilfe chemischer Analytik und molekularbiologischen Methoden behandelt die vorliegende Arbeit die Frage, inwieweit die induzierte pflanzliche Emission von Volatilen zwischen nahe verwandten Arten erklärt werden kann. An Modellorganismen können viele grundlegende und wichtige Fragen geklärt werden, mit ihrer Hilfe allein können jedoch nur bestimmte Probleme geklärt werden, da jede Art phylogenetisch in ihrer Ausprägung begrenzt ist. Hier wurde die Modellpflanze *Arabidopsis thaliana* verwendet sowie vergleichend dazu die eng verwandte Felsen-Schaumkresse, *A. lyrata* ssp. *petraea*. Die rezenten Arten haben sich in den vergangenen fünf Millionen Jahren aus einer gemeinsamen Ursprungsart durch unterschiedliche Ausleseprozesse ausreichend ökologisch diversifiziert. *A. thaliana* ist eine vorwiegend selbstbefruchtende Annuelle mit Pioniercharakter, *A. lyrata* dagegen ist ausdauernd, auf Fremdbestäubung angewiesen und eher auf felsigem Terrain zu finden. Die räumliche und zeitliche Variabilität der Abgabe von flüchtigen Stoffen wurde auf der Ebene verschiedener Pflanzenorgane, Ökotypen und Populationen untersucht.

### Variabilität im Blütenduft von *A. lyrata* ssp. *petraea*

Mit dem vergleichenden System *A. thaliana* und *A. lyrata* konnte gezeigt werden, daß sowohl Blütenduft als auch die Emission über vegetative Organe in beiden Arten intra- und interspezifisch erhebliche Schwankungen aufweist. Die qualitative und quantitative Bandbreite an Blütenduft von *A. lyrata* in Mitteleuropa wurde anhand von geographisch weit voneinander isolierten Populationen untersucht. Blütenduft von *A. lyrata* aus Populationen in Deutschland, Tschechien und Österreich wurde gesammelt und die Komponenten durch gekoppelte Gas-Chromatographie-Massenspektrometrie (GC-MS) analysiert und identifiziert. Es wurden sowohl punktuelle Blütenmessungen als auch kontinuierliche Messungen von gesamten Pflanzen durchgeführt. Dafür wurde u. a. die neue Technik der Protonentransfer-Massenspektrometrie (PTR-MS) benutzt, um zeitliche Veränderungen der Duftemission über mehr als 24 Stunden aufzunehmen. Die Ergebnisse wurden in den vergleichenden Kontext zu *A. thaliana* gestellt.

Der Blütenduft von *A. thaliana* ist schwach ausgeprägt und besteht hauptsächlich aus verschiedenen Sesquiterpenen. Blüten von *A. lyrata* emittieren dagegen große

Mengen an Benzenderivaten in einem ausgeprägten diurnalen Rhythmus, was durch verschiedene Methoden gezeigt werden konnte. Die Hauptemission im Labor war zwischen 10 und 13 Uhr. Zur gleichen Tageszeit war die Flugaktivität von wichtigen bestäubenden Hymenopteren im Feld am höchsten. Durch die getrennte Analyse einzelner Blütenorgane wurden die Blütenblätter als alleinige Duftquelle innerhalb der Blüte identifiziert, weder Gynoeceum noch Androeceum trugen zur Emission bei. Diese Ergebnisse stehen im Gegensatz zu Promoter-GUS Untersuchungen von Genen zur Biosynthese von Blütenduftstoffen in *A. thaliana*, stimmen aber überein mit Ergebnissen von Arten wie *Silene latifolia* oder *Nicotiana suaveolens*, die durch Insekten bestäubt werden.

Die Duftemission von *A. lyrata* Blüten war sowohl zwischen als auch innerhalb der untersuchten Populationen verschieden. Von drei untersuchten Populationen wurden spezifische Duftmuster ausgemacht (Kapitel 2). Substanzen die einen bestimmten Chemotyp ausmachten waren nicht oder nur in geringen Mengen in anderen Chemotypen zu finden (Phenylacetaldehyd, 4-Methoxybenzaldehyd, Benzoësäuremethylester, Salicylsäuremethylester und Linalool). Die Ausnahme dazu bildet Benzaldehyd, das in allen Chemotypen gefunden wurde. Die gefundenen Unterschiede im Blütenduft zwischen den Populationen sind möglicherweise das Ergebnis einer Anpassung an eine unterschiedliche lokale Bestäuberfauna.

### Unterschiede in der vegetativen Abgabe von flüchtigen Terpenen

Durch Insektenfraß induziert abgegebene Duftstoffe waren qualitativ unterschiedlich zwischen der ausdauernden Art *A. lyrata* ssp. *petraea* und der Annuellen *A. thaliana*. Nach Fraß durch Raupen der auf Kreuzblütler spezialisierten Kohlmotte *Plutella xylostella* gab nur *A. lyrata* das Sesquiterpen (*E*)-β-Caryophyllen ab (Kapitel 1). Allerdings war die Abgabe flüchtiger Stoffe innerhalb einer Population von *A. lyrata* variabel, einige Individuen emittierten die Homoterpene DMNT, TMTT oder den Sesquiterpenalkohol Nerolidol. (*E*)-β-Caryophyllen war teilweise der einzige abgegebene Stoff, z. T. war es überhaupt nicht vorhanden. Von Blüten wurde kein (*E*)-β-Caryophyllen abgegeben, bei *A. thaliana* dagegen ist es Teil des Blütendufts [30, 165]. Aus den Ergebnissen kann geschlossen werden, daß die Regulation der Biosynthese von (*E*)-β-Caryophyllen seit der Trennung in die beiden Arten *A. thaliana* und *A. lyrata* vor ca. fünf Millionen Jahren eine unterschiedliche Evolution erfahren hat. Während bei der Annuellen *A. thaliana* (*E*)-β-Caryophyllen vielleicht als präventives Antibiotikum von Vorteil ist, um Blüten und Samenansatz vor einem Befall durch Pathogene zu schützen, so übernehmen in Blüten von *A. lyrata* die Benzenderivate neben einer Signalfunktion auf Bestäuber möglicherweise eine Schutzfunktion gegen Pathogene. In Mais wurde eine Emission von (*E*)-β-Caryophyllen nach Raupenfraß gezeigt, wo es an

einer für Mais möglicherweise nützlichen tritrophischen Interaktion beteiligt ist [133]. Es ist jedoch nicht bekannt, ob eine ähnliche Funktion für *A. lyrata* gegeben ist.

Die Bildung von (*E*)-β-Caryophyllen in *A. lyrata* sollte auf molekularer Ebene untersucht werden. Dafür wurde durch einen RT-PCR Ansatz mit Primern, die homolog zur kürzlich identifizierten (*E*)-β-Caryophyllensynthase aus *A. thaliana* sind, ein Gen identifiziert, das ebenfalls für eine (*E*)-β-Caryophyllensynthase kodiert [160]. Die Genfunktion wurde durch Analyse der Terpensynthaseaktivität nach heterologer Expression in *E. coli* bestätigt. Die zueinander homologen (*E*)-β-Caryophyllensynthasen aus *A. thaliana* und *A. lyrata* haben mit 85% identischer Nukleotidsequenz und einer zu 92% ähnlichen Proteinsequenz den gleichen Grad der Ähnlichkeit wie *mat*K und *Chs* [93]. Ähnliche Werte wurden für andere Terpensynthasegene und ihre möglichen Homologe in *A. lyrata* gefunden. Im Gegensatz zur ausschließlichen Expression der (*E*)-β-Caryophyllensynthase in Blüten von *A. thaliana* war das homologe Gen von *A. lyrata* nicht in Blüten exprimiert. Die Transkription dieses Gens war dagegen in Blättern von *A. lyrata* nach Raupenfraß hochreguliert, was mit der induzierten Abgabe von (*E*)-β-Caryophyllen von Blättern korreliert war. Die Ergebnisse lassen vermuten, daß gewebespezifische Unterschiede in der Bildung von (*E*)-β-Caryophyllen in *A. thaliana* und *A. lyrata* durch differentielle Expression der jeweiligen homologen Terpensynthase in beiden Arten entstanden sind.

## Zwei unterschiedlich regulierte Terpensynthasegene sind für die induzierte Bildung von (*E*)-β-Ocimen und (*E,E*)-α-Farnesen in *A. thaliana* verantwortlich

Die Bandbreite der Emission von Volatilen nach Induktion durch das Induktionsmittel Coronalon sollte bei *A. thaliana* anhand von 29 Ökotypen untersucht werden. Die meisten Ökotypen gaben das Monoterpen (*E*)-β-Ocimen, das Sesquiterpen (*E,E*)-α-Farnesen sowie das C16 Homoterpen TMTT ab. Große quantitative Unterschiede wurden für (*E*)-β-Ocimen gefunden. Während der Ökotyp Col-0 z. B. fast kein (*E*)-β-Ocimen abgab, ist bei dem Ökotyp Ws (*E*)-β-Ocimen die Hauptkomponente. Diese zwei Ökotypen wurden für eingehende molekulare Untersuchungen in Bezug auf die unterschiedliche (*E*)-β-Ocimenabgabe verwendet.

Coronalon bewirkte eine Hochregulation von Transkripten der nahe verwandten Terpensynthasegene At4g16730 und At4g16740 in dem Ökotyp Col-0, wohingegen At4g16730 nur in dem Ökotyp Ws transkribiert wurde. In beiden Ökotypen wurden vollständige Gentranskripte nach Induktion gefunden. At2g24210, eine weitere Monoterpensynthase von *A. thaliana*, wurde vor kurzem als β-Myrcen- (56%) und (*E*)-β-Ocimen (20%) Synthase beschrieben [23]. Bei unseren Untersuchungen wurden keine wesentlichen Mengen an β-Myrcen gefunden, At2g24210 wurde daher für

den (*E*)-β-Ocimen / (*E,E*)-α-Farnesen Polymorphismus nicht in Betracht gezogen.

Durch Sequenzvergleiche mit anderen Organismen waren die Gene At4g16730 und At4g16740 als Monoterpensynthasen annotiert [14, 47, 113]. Unsere *in vitro* Untersuchungen mit den rekombinanten Enzymen At4g16730 aus Ws und At4g16740 aus Col-0 ergaben jedoch, daß die Enzyme sowohl als Mono- als auch als Sesquiterpensynthasen fungieren können. Während mit dem $C_{10}$-Substrat Geranyldiphosphat die Monoterpene (*E*)-β-Ocimen (75%), *Z*-Ocimen (22%) und β-Myrcen (3%) produziert wurden, so wurde das $C_{15}$-Substrat Farnesyldiphosphat neben geringen Mengen an (*Z,E*)-α-Farnesen zu (*E,E*)-α-Farnesen umgesetzt. Keine Enzymaktivität wurde dagegen mit At4g16730 von Col-0 erhalten, da eine Mutation ein vorzeitiges Translationsterminationscodon verursachte, das ein stark verkürztes Protein zur Folge hatte. Nukleotidspezifische Mutagenese wurde eingesetzt, um *in vitro* einen geschlossenen Leserahmen zu erstellen und gezielt einzelne Aminosäuren im N-Terminus im Vergleich zur Sequenz aus Ws zu revertieren. Durch diesen Ansatz wurde die Aktivität von At4g16730 von Col-0 als (*E*)-β-Ocimen / (*E,E*)-α-Farnesensynthase künstlich hergestellt.

Mittels *RNAi silencing* und einem *antisense* Ansatz von At4g16740 in Col-0 wurde die induzierte (*E,E*)-α-Farnesen Emission vollständig unterdrückt. Das lässt schließen, daß At4g16740 in Col-0 für die Bildung von (*E,E*)-α-Farnesen verantwortlich ist. Die Funktion von At4g16740 aus Col-0 als Sesquiterpensynthase wurde unterstützt durch die Lokalisierung von GFP-Hybridproteinen im Cytosol, dem Ort, an dem am meisten Farnesyldiphosphat hergestellt wird. Im Gegensatz dazu zeigten Hybridproteinversuche mit der N-terminalen Region von At4g16730 aus Ws und GFP eine Lokalisierung in Chloroplasten, was die *in vivo* Funktion von At4g16730 in Ws als (*E*)-β-Ocimensynthase unterstützt. Die Funktion von At4g16730 als (*E*)-β-Ocimensynthase harrt weiterer Bestätigung durch *RNAi* Experimente. Zusammengefasst zeigen die Ergebnisse, daß die Unterschiede in der induzierten (*E*)-β-Ocimen / (*E,E*)-α-Farnesen Emission von *A. thaliana* Ökotypen auf verschiedenen Ebenen reguliert werden: Genmutationen, differentielle Genexpression und subzelluläre Lokalisierung von zwei ähnlichen Terpensynthasegenen sind dafür verantworlich.

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# List of Tables



# List of Figures

# References

[1] *http://www.floraweb.de*.

[2] *http://www.ncbi.nlm.nih.gov*.

[3] D. AESCHIMANN, K. LAUBER, D. M. MOSER, AND J.-P. THEURILLAT, *Flora alpina*, vol. 1, Haupt Verlag, Bern, 2004.

[4] A. AHARONI, A. P. GIRI, F. W. A. VERSTAPPEN, C. M. BERTEA, R. SEVENIER, Z. SUN, M. A. JONGSMA, W. SCHWAB, AND H. J. BOUWMEESTER, *Gain and loss of fruit flavor compounds produced by wild and cultivated strawberry species.*, Plant Cell, 16 (2004), pp. 3110–3131.

[5] I. AL-SHEHBAZ AND S. O'KANE, *Taxonomy and Phylogeny of Arabidopsis (Brassicaceae)*, in The Arabidopsis Book, C. R. Somerville and E. M. Meyerowitz, eds., American Society of Plant Biologists, 2002, pp. 1–22.

[6] I. A. AL-SHEHBAZ, *Transfer of most North American species of Arabis to Boechera (Brassicaceae)*, Novon, 13 (2003), pp. 381–391.

[7] I. A. AL-SHEHBAZ, M. A. BEILSTEIN, AND E. A. KELLOGG, *Systematics and phylogeny of the Brassicaceae (Cruciferae): an overview*, Plant Sys. Evol., 259 (2006), pp. 89–120.

[8] R. ALTENBURGER AND P. MATILE, *Circadian rhythmicity of fragrance emission in flowers of Hoya carnosa R. Br.*, Planta, V174 (1988), pp. 248–252.

[9] H. M. ALVAREZ, D. P. BARBOSA, A. T. FRICKS, D. A. G. ARANDA, R. H. VALDES, AND O. A. C. ANTUNES, *Production of piperonal, vanillin, and p-anisaldehyde via solventless supported iodobenzene diacetate oxidation of isosafrol, isoeugenol, and anethol under microwave irradiation*, Org. Process Res. Dev., 10 (2006), pp. 941–943.

[10] K. AMENT, M. R. KANT, M. W. SABELIS, M. A. HARING, AND R. C. SCHUURINK, *Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato*, Plant Physiol., 135 (2004), pp. 2025–2037.

[11] K. AMENT, C. C. VAN SCHIE, H. J. BOUWMEESTER, M. A. HARING, AND R. C. SCHUURINK, *Induction of a leaf specific geranylgeranyl pyrophosphate synthase and emission of (E,E)-4,8,12-trimethyltrideca-1,3,7,11-tetraene in tomato are dependent on both jasmonic acid and salicylic acid signaling pathways*, Planta, 224 (2006), pp. 1197–1208.

[12] G. ARIMURA, R. OZAWA, S. KUGIMIYA, J. TAKABAYASHI, AND J. BOHLMANN, *Herbivore-induced defense response in a model legume. Two-spotted spider mites induce emission of (E)-β-ocimene and transcript accumulation of (E)-β-ocimene synthase in Lotus japonicus*, Plant Physiol., 135 (2004), pp. 1976–1983.

[13] G. ARIMURA, R. OZAWA, T. SHIMODA, T. NISHIOKA, W. BOLAND, AND J. TAKABYASHI, *Herbivory-induced volatiles elicit defence genes in lima bean leaves*, Nature, 406 (2000), pp. 512–515.

[14] S. AUBOURG, A. LECHARNY, AND J. BOHLMANN, *Genomic analysis of the terpenoid synthase (AtTPS) gene family of Arabidopsis thaliana*, Mol. Genet. Genomics, 267 (2002), pp. 730–745.

[15] I. T. BALDWIN, R. HALITSCHKE, A. PASCHOLD, C. C. VON DAHL, AND C. A. PRESTON, *Volatile signaling in plant-plant interactions: "talking trees" in the genomics era.*, Science, 311 (2006), pp. 812–815.

[16] S. BARTRAM, A. JUX, G. GLEIXNER, AND W. BOLAND, *Dynamic pathway allocation in early terpenoid biosynthesis of stress-induced lima bean leaves*, Phytochemistry, 67 (2006), pp. 1661–1672.

[17] K. H. C. BAŞER, *Isolation and identification of anisaldehyde and 3 alkaloids from leaves of Thalictrum minus var. microphyllum*, J. Nat. Prod., 45 (1982), pp. 704–706.

[18] N. BECHTOLD, J. ELLIS, AND G. PELLETIER, *In planta Agrobacterium mediated gene transfer by infiltration of adult Arabidopsis thaliana plants*, C. R. Acad. Sci. Paris, Sciences de la vie/Life sciences, 316 (1993), pp. 1194–1199.

[19] M. A. BEILSTEIN, I. A. AL-SHEHBAZ, AND E. A. KELLOGG, *Brassicaceae phylogeny and trichome evolution*, Am. J. Bot., 93 (2006), pp. 607–619.

[20] M. BENDEROTH, S. TEXTOR, A. J. WINDSOR, T. MITCHELL-OLDS, J. GERSHENZON, AND J. KROYMANN, *Positive selection driving diversification in plant secondary metabolism.*, Proc. Natl. Acad. Sci. U.S.A., 103 (2006), pp. 9118–9123.

[21] G. BERGSTRÖM, G. BIRGERSSON, I. GROTH, AND L. ANDERS NILSSON, *Floral fragrance disparity between three taxa of lady's slipper Cypripedium calceolus (Orchidaceae)*, Phytochemistry, 31 (1992), pp. 2315–2319.

[22] G. BLOBEL AND B. DOBBERSTEIN, *Transfer of Proteins across Membranes. II. Reconstitution of Functional Rough Microsomes from Heterologous Components*, J. Cell Biol., 67 (1975), pp. 852–862.

[23] J. BOHLMANN, D. MARTIN, N. OLDHAM, AND J. GERSHENZON, *Terpenoid secondary metabolism in Arabidopsis thaliana: cDNA cloning, characterization, and functional expression of a myrcene/(E)-β-ocimene synthase*, Arch. Biochem. Biophys., 375 (2000), pp. 261–269.

[24] F. BOUVIER, C. SUIRE, A. D'HARLINGUE, R. BACKHAUS, AND B. CAMARA, *Molecular cloning of geranyl diphosphate synthase and compartmentation of monoterpene synthesis in plant cells*, Plant J., 24 (2000), pp. 241–252.

[25] H. J. BOUWMEESTER, F. W. VERSTAPPEN, M. A. POSTHUMUS, AND M. DICKE, *Spider mite-induced (3S)-(E)-nerolidol synthase activity in cucumber and lima bean. The first dedicated step in acyclic C11-homoterpene biosynthesis*, Plant Physiol., 121 (1999), pp. 173–180.

[26] P. BROUN, Y. LIU, E. QUEEN, Y. SCHWARZ, M. ABENES, AND M. LEIBMAN, *Importance of transcription factors in the regulation of plant secondary metabolism and their relevance to the control of terpenoid accumulation*, Phytochem. Rev., 5 (2006), pp. 27–38.

[27] J. BUCKINGHAM, ed., *Dictionary of Natural Products*, Chapman and Hall, London, 1994.

[28] C. CARSON AND T. RILEY, *Antimicrobial activity of the major components of the essential oil of Melaleuca alternifolia*, J. Appl. Bacteriol., 78 (1995), pp. 264–269.

[29] J. CHAPPELL, *Biochemistry and molecular biology of the isoprenoid biosynthetic pathway in plants*, Annu. Rev. Plant Physiol. Plant Mol. Biol., 46 (1995), pp. 521–547.

[30] F. CHEN, D. THOLL, J. D'AURIA, A. FAROOQ, E. PICHERSKY, AND J. GERSHENZON, *Biosynthesis and emission of terpenoid volatiles from Arabidopsis flowers*, Plant Cell, 15 (2003), pp. 1–14.

[31] M. CLAUSS AND T. MITCHELL-OLDS, *Functional divergence in tandemly duplicated Arabidopsis thaliana trypsin inhibitor genes*, Genetics, 166 (2004), pp. 1419–1436.

[32] M. J. CLAUSS AND M. A. KOCH, *Poorly known relatives of Arabidopsis thaliana*, Trends Plant Sci., 11 (2006), pp. 449–459.

[33] M. J. CLAUSS AND T. MITCHELL-OLDS, *Population genetic structure of Arabidopsis lyrata in europe*, Mol. Ecol., 15 (2006), pp. 2753–2766.

[34] D. N. CROWELL, B. J. BIERMANN, AND S. K. RANDALL, *Identification of cdnas encoding isoprenylated proteins*, Mol. Biotechnol., 5 (1996), pp. 253–258.

[35] N. CUNILLERA, A. BORONAT, AND A. FERRER, *The Arabidopsis thaliana FPS1 Gene Generates a Novel mRNA That Encodes a Mitochondrial Farnesyldiphosphate Synthase Isoform*, J. Biol. Chem., 272 (1997), pp. 15381–15388.

[36] A. E. DAHL, A.-B. WASSGREN, AND G. BERGSTRÖM, *Floral scents in Hypecoum sect. Hypecoum (Papaveraceae): Chemical composition and relevance to taxonomy and mating system*, Biochem. Syst. Ecol., 18 (1990), pp. 157–168.

[37] C. J. DANPURE, *How can the products of a single gene be localized to more than one intracellular compartment?*, Trends Cell Biol., 5 (1995), pp. 230–238.

[38] A. P. DE CANDOLLE, *Cruciferae*, Systema Naturale, 2 (1821), pp. 139–700.

[39] J. DE GOUW, C. WARNEKE, T. KARL, G. EERDEKENS, C. VAN DER VEEN, AND R. FALL, *Sensitivity and specificity of atmospheric trace gas detection by proton-transfer-reaction mass spectrometry*, Int. J. Mass Spectrom., 223-224 (2003), pp. 365–382.

[40] C. M. DE MORAES, W. J. LEWIS, P. W. PARE, H. T. ALBORN, AND J. H. TUMLINSON, *Herbivore-infested plants selectively attract parasitoids*, Nature, 393 (1998), pp. 570–573.

[41] J. DEGENHARDT AND J. GERSHENZON, *Demonstration and characterization of (E)-nerolidol synthase from maize: a herbivore-inducible terpene synthase participating in (3E)-4,8-dimethyl-1,3,7-nonatriene biosynthesis*, Planta, 210 (2000), pp. 815–822.

[42] M. DICKE, *Evolution of induced indirect defense of plants*, in The Ecology and Evolution of Inducible Defenses, R. Tollrian and C. J. Harvell, eds., Princeton University Press, Princeton, New Jersey, 1999, pp. 62–88.

[43] H. E. M. DOBSON, *Floral volatiles in insect biology*, in Insect-plant Interactions, E. A. Bernays, ed., CRC Press, London, 1994, pp. 47–81.

[44] J. DONATH AND W. BOLAND, *Biosynthesis of acyclic homoterpenes - enzyme selectivity and absolute-configuration of the nerolidol precursor*, Phytochemistry, 39 (1995), pp. 785–790.

[45] S. DÖTTERL, *Importance of floral scent compounds for the interaction between Silene latifolia (Caryophyllaceae) and the nursery pollinator Hadena bicruris (Lepidoptera: Noctuidae)*, PhD thesis, Universität Bayreuth, 2004.

[46] N. DUDAREVA, S. ANDERSSON, I. ORLOVA, N. GATTO, M. REICHELT, D. RHODES, W. BOLAND, AND J. GERSHENZON, *The nonmevalonate pathway supports both monoterpene and sesquiterpene formation in snapdragon flowers.*, Proc. Natl. Acad. Sci. U. S. A., 102 (2005), pp. 933–938.

[47] N. DUDAREVA, D. MARTIN, C. KISH, N. KOLOSOVA, N. GORENSTEIN, J. FALDT, B. MILLER, AND J. BOHLMANN, *(E)-β-Ocimene and Myrcene Synthase Genes of Floral Scent Biosynthesis in Snapdragon: Function and Expression of Three Terpene Synthase Genes of a New Terpene Synthase Subfamily*, Plant Cell, 15 (2003), pp. 1227–1241.

[48] N. DUDAREVA, L. MURFITT, C. MANN, N. GORENSTEIN, N. KOLOSOVA, C. KISH, C. BONHAM, AND K. WOOD, *Developmental regulation of methyl benzoate biosynthesis and emission in snapdragon flowers*, Plant Cell, 12 (2000), pp. 949–961.

[49] W. EISENREICH, A. BACHER, D. ARIGONI, AND F. ROHDICH, *Biosynthesis of isoprenoids via the non-mevalonate pathway*, Cell. Mol. Life Sci., 61 (2004), pp. 1401–1426.

[50] O. EMANUELSSON, H. NIELSEN, S. BRUNAK, AND G. VON HEIJNE, *Predicting Subcellular Localization of Proteins Based on their N-terminal Amino Acid Sequence*, J. Mol. Biol., 300 (2000), pp. 1005–1016.

[51] J. ENGELBERTH, T. KOCH, G. SCHÜLER, N. BACHMANN, J. RECHTENBACH, AND W. BOLAND, *Ion channel-forming alamethicin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between jasmonate and salicylate signaling in lima bean*, Plant Physiol., 125 (2001), pp. 369–377.

[52] N. ESCARAVAGE AND J. WAGNER, *Pollination effectiveness and pollen dispersal in a Rhododendron ferrugineum (Ericaceae) population*, Plant Biol., 6 (2004), pp. 606–615.

[53] J. M. ESTEVEZ, A. CANTERO, A. REINDL, S. REICHLER, AND P. LEON, *1-Deoxy-D-xylulose-5-phosphate Synthase, a Limiting Enzyme for Plastidic Isoprenoid Biosynthesis in Plants*, J. Biol. Chem., 276 (2001), pp. 22901–22909.

[54] F. EUROPAEA, *Flora Europaea on CD-ROM*, Cambrigde University Press, 2001.

[55] K. A. EVANS AND L. J. ALLEN-WILLIAMS, *Electroantennogram responses of the cabbage seed weevil, Ceutorhynchus assimilis, to oilseed rape, Brassica napus ssp.oleifera, volatiles,* J. Chem. Ecol., V18 (1992), pp. 1641–1659.

[56] J. FÄLDT, G. ARIMURA, J. GERSHENZON, J. TAKABAYASHI, AND J. BOHLMANN, *Functional identification of AtTPS03 as (E)-β-ocimene synthase: a monoterpene synthase catalyzing jasmonate- and wound-induced volatile formation in Arabidopsis thaliana,* Planta, 216 (2003), pp. 745–751.

[57] J. FLIEGMANN, G. SCHÜLER, W. BOLAND, J. EBEL, AND A. MITHÖFER, *The role of octadecanoids and functional mimics in soybean defense responses*, Biol. Chem., 384 (2003), pp. 437–446.

[58] G. GALLOWAY, R. MALMBERG, AND R. PRICE, *Phylogenetic utility of the nuclear gene arginine decarboxylase: an example from Brassicaceae*, Mol. Biol. Evol., 15 (1998), pp. 1312–1320.

[59] N. E. GAPPER, J. BAI, AND B. D. WHITAKER, *Inhibition of ethylene-induced α-farnesene synthase gene PcAFS1 expression in 'd'Anjou' pears with 1-MCP reduces synthesis and oxidation of α-farnesene and delays development of superficial scald*, Postharvest Biol. Tech., 41 (2006), pp. 225–233.

[60] J. GERSHENZON AND N. DUDAREVA, *The function of terpene natural products in the natural world*, Nat. Chem. Biol., 3 (2007), pp. 408–414.

[61] D. M. GIBEAUT, J. HULETT, G. R. CRAMER, AND J. R. SEEMANN, *Maximal Biomass of Arabidopsis thaliana Using a Simple, Low-Maintenance Hydroponic Method and Favorable Environmental Conditions*, Plant Physiol., 115 (1997), pp. 317–319.

[62] P. HAJDUKIEWICZ, Z. SVAB, AND P. MALIGA, *The small, versatile pPZP family of Agrobacterium binary vectors for plant transformation*, Plant Mol. Biol., 25 (1994), pp. 989–994.

[63] R. HALITSCHKE, A. KESSLER, J. KAHL, A. LORENZ, AND I. T. BALDWIN, *Ecophysiological comparison of direct and indirect defenses in Nicotiana attenuata*, Oecologia, 124 (2000), pp. 408–417.

[64] A. HALL, A. FIEBIG, AND D. PREUSS, *Beyond the Arabidopsis genome: Opportunities for comparative genomics*, Plant Physiol., 129 (2002), pp. 1439–1447.

[65] T. R. HAMILTON-KEMP, J. H. LOUGHRIN, AND R. A. ANDERSEN, *Identification of some volatile compounds from strawberry flowers*, Phytochemistry, 29 (1990), pp. 2847–2848.

[66] U. HANSEN AND G. SEUFERT, *Temperature and light dependence of* β*-caryophyllene emission rates*, J. Geophys. Res., D, 108 (2003), p. 4801.

[67] A. C. HEIDEN, T. HOFFMANN, J. KAHL, D. KLEY, D. KLOCKOW, C. LANGEBARTELS, H. MEHLHORN, J. SANDERMANN, H., M. SCHRAUDNER, G. SCHUH, AND J. WILDT, *Emission of Volatile Organic Compounds from Ozone-Exposed Plants*, Ecol. Appl., 9 (1999), pp. 1160–1167.

[68] S. HEJNÝ AND B. SLAVÍK, *Kvetena Ceske republiky 3*, Academia, Praha, 1992.

[69] J. P. F. G. HELSPER, J. A. DAVIES, H. J. BOUWMEESTER, A. F. KROL, AND M. H. VAN KAMPEN, *Circadian rhythmicity in emission of volatile compounds by flowers of Rosa hybrida L. cv. Honesty*, Planta, V207 (1998), pp. 88–95.

[70] M. HERDE, *Identifikation und Regulation einer durch Insektenfrass induzierbaren Geranyllinalool-Synthase in Arabidopsis thaliana*, PhD thesis, Georg-August-Universität zu Göttingen, 2006.

[71] C. M. HERRERA, *Components of Pollinator "Quality": Comparative Analysis of a Diverse Insect Assemblage*, Oikos, 50 (1987), pp. 79–90.

[72] M. HOFFMANN, M. BREMER, K. SCHNEIDER, F. BURGER, E. STOLLE, AND G. MORITZ, *Flower Visitors in a Natural Population of Arabidopsis thaliana*, Plant Biol., 5 (2003), pp. 491–494.

[73] K. HORI AND Y. WATANABE, *UPF3 suppresses aberrant spliced mRNA in Arabidopsis*, Plant J., 43 (2005), pp. 530–540.

[74] J.-I. HORIUCHI, G.-I. ARIMURA, R. OZAWA, T. SHIMODA, M. DICKE, J. TAKABAYASHI, AND T. NISHIOKA, *Lima bean leaves exposed to herbivore-induced conspecific plant volatiles attract herbivores in addition to carnivores*, Appl. Entomol. Zool., 38 (2003), pp. 365–368.

[75] J. HUANG, Y. J. CARDOZA, E. A. SCHMELZ, R. RAINA, J. ENGELBERTH, AND J. H. TUMLINSON, *Differential volatile emissions and salicylic acid levels from tobacco plants in response to different strains of Pseudomonas syringae*, Planta, 217 (2003), pp. 767–775.

[76] A. G. INITIATIVE, *Analysis of the genome sequence of the flowering plant Arabidopsis thaliana*, Nature, 408 (2000), pp. 796–815.

[77] R. IRWIN, L. ADLER, AND A. BRODY, *The dual role of floral traits: Pollinator attraction and plant defense*, Ecology, 85 (2004), pp. 1503–1511.

[78] H. B. JAKOBSEN, P. FRIIS, J. K. NIELSEN, AND C. E. OLSEN, *Emission of volatiles from flowers and leaves of Brassica napus in situ*, Phytochemistry, 37 (1994), pp. 695–699.

[79] C. D. JOHNSON, S. N. CHARY, E. A. CHERNOFF, Q. ZENG, M. P. RUNNING, AND D. N. CROWELL, *Protein geranylgeranyltransferase I is involved in specific aspects of abscisic acid and auxin signaling in Arabidopsis*, Plant Physiol., 139 (2005), pp. 722–733.

[80] H. JOMAA, J. WIESNER, S. SANDERBRAND, B. ALTINCICEK, C. WEIDEMEYER, M. HINTZ, I. TURBACHOVA, M. EBERL, J. ZEIDLER, H. LICHTENTHALER, D. SOLDATI, AND E. BECK, *Inhibitors of the nonmevalonate pathway of isoprenoid biosynthesis as antimalarial drugs*, Science, 285 (1999), pp. 1573–1576.

[81] Y. KAMINAGA, J. SCHNEPP, G. PEEL, C. M. KISH, G. BEN-NISSAN, D. WEISS, I. ORLOVA, O. LAVIE, D. RHODES, K. WOOD, D. M. PORTERFIELD, A. J. L. COOPER, J. V. SCHLOSS, E. PICHERSKY, A. VAINSTEIN, AND N. DUDAREVA, *Plant phenylacetaldehyde synthase is a bifunctional homotetrameric enzyme that catalyzes phenylalanine decarboxylation and oxidation*, J. Biol. Chem., 281 (2006), pp. 23357–23366.

[82] I. F. KAPPERS, A. AHARONI, T. W. J. M. VAN HERPEN, L. L. P. LUCKERHOFF, M. DICKE, AND H. J. BOUWMEESTER, *Genetic engineering of terpenoid metabolism attracts bodyguards to Arabidopsis*, Science, 309 (2005), pp. 2070–2072.

[83] S. KERTESZ, Z. KERENYI, Z. MERAI, I. BARTOS, T. PALFY, E. BARTA, AND D. SILHAVY, *Both introns and long 3 '-UTRs operate as cis-acting elements to trigger nonsense-mediated decay in plants*, Nucleic Acids Res., 34 (2006), pp. 6147–6157.

[84] J. KESSELMEIER AND M. STAUDT, *Biogenic Volatile Organic Compounds (VOC): An Overview on Emission, Physiology and Ecology*, J. Atmos. Chem., V33 (1999), pp. 23–88.

[85] A. KESSLER AND I. T. BALDWIN, *Defensive function of herbivore-induced plant volatile emissions in nature.*, Science, 291 (2001), pp. 2141–2144.

[86] D. KESSLER AND I. T. BALDWIN, *Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of Nicotiana attenuata*, Plant J., 49 (2007), pp. 840–854.

[87] W. D. J. KIRK, *Effect of some floral scents on host finding by thrips (insecta: Thysanoptera)*, J. Chem. Ecol., V11 (1985), pp. 35–43.

[88] J. T. KNUDSEN, R. ERIKSSON, J. GERSHENZON, AND B. STAHL, *Diversity and distribution of floral scent*, Bot. Rev., 72 (2006), pp. 1–120.

[89] J. T. KNUDSEN AND J. GERSHENZON, *The chemistry diversity of floral scent*, in Biology of Floral Scent, N. Dudareva and E. Pichersky, eds., CRC Press, Boca Raton, 2006, pp. 27–52.

[90] M. KOCH, I. AL-SHEHBAZ, AND K. MUMMENHOFF, *Molecular Systematics, Evolution, and Population Biology in the Mustard Family (Brassicaceae)*, Ann. Missouri Bot. Gard., 90 (2003), pp. 151–171.

[91] M. KOCH, J. BISHOP, AND T. MITCHELL-OLDS, *Molecular systematics and evolution of Arabidopsis and Arabis*, Plant Biol., 1 (1999), pp. 529–537.

[92] M. KOCH, B. HAUBOLD, AND T. MITCHELL-OLDS, *Comparative evolutionary analysis of chalcone synthase and alcohol dehydrogenase loci in Arabidopsis, Arabis, and related genera (Brassicaceae)*, Mol. Biol. Evol., 17 (2000), pp. 1483–1498.

[93] M. KOCH, B. HAUBOLD, AND T. MITCHELL-OLDS, *Molecular systematics of the Brassicaceae: evidence from coding plastidic matK and nuclear Chs sequences*, Am. J. Bot., 88 (2001), pp. 534–544.

[94] T. KÖLLNER, *Molekulare und genetische Aspekte der Biosynthese von komplexen Sesquiterpengemischen in Mais*, PhD thesis, Friedrich-Schiller-Universität Jena, 2004.

[95] N. KOLOSOVA, N. GORENSTEIN, C. M. KISH, AND N. DUDAREVA, *Regulation of circadian methyl benzoate emission in diurnally and nocturnally emitting plants.*, Plant Cell, 13 (2001), pp. 2333–2347.

[96] H. KORSCH, W. WESTHUS, AND H.-J. ZÜNDORF, *Verbreitungsatlas der Farn- und Blütenpflanzen Thüringens*, Thüringische Botanische Gesellschaft, 2002.

[97] M. KRIZMAN, D. BARICEVIC, AND M. PROSEK, *Fast quantitative determination of volatile constituents in fennel by headspace-gas chromatography*, Anal. Chim. Acta, 557 (2006), pp. 267–271.

[98] I. KUBO, H. MUROI, AND M. HIMEJIMA, *Antibacterial activity against Streptococcus mutans of mate tea flavor components*, J. Agric. Food Chem., 41 (1993), pp. 107–111.

[99] T. KUZUYAMA, T. SHIMIZU, S. TAKAHASHI, AND H. SETO, *Fosmidomycin, a Specific Inhibitor of 1-Deoxy-D-Xylulose 5-Phosphate Reductoisomerase in the Nonmevalonate Pathway for Terpenoid Biosynthesis*, Tetrahedron Lett., 39 (1998), pp. 7913–7916.

[100] B. M. LANGE, M. R. WILDUNG, D. MCCASKILL, AND R. CROTEAU, *A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway.*, Proc. Natl. Acad. Sci. U. S. A., 95 (1998), pp. 2100–2104.

[101] J. H. LANGENHEIM, *Higher plant terpenoids: A phytocentric overview of their ecological roles*, J. Chem. Ecol., 20 (1994), pp. 1223–1280.

[102] Z. G. LI, M. R. LEE, AND D. L. SHEN, *Analysis of volatile compounds emitted from fresh Syringa oblata flowers in different florescence by headspace solid-phase microextraction-gas chromatography-mass spectrometry*, Anal. Chim. Acta, 576 (2006), pp. 43–49.

[103] H. LICHTENTHALER, *The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants*, Annu. Rev. Plant Physiol. Plant Mol. Biol., 50 (1999), pp. 47–65.

[104] H. K. LICHTENTHALER, M. ROHMER, AND J. SCHWENDER, *Two independent biochemical pathways for isopentenyl diphosphate and isoprenoid biosynthesis in higher plants*, Physiol. Plant., 101 (1997), pp. 643–652.

[105] H. K. LICHTENTHALER, J. SCHWENDER, A. DISCH, AND M. ROHMER, *Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathway*, FEBS Lett., 400 (1997), pp. 271–274.

[106] W. LINDINGER, A. HANSEL, AND A. JORDAN, *On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS) medical applications, food control and environmental research*, Int. J. Mass Spectrom. Ion Processes, 173 (1998), pp. 191–241.

[107] F. LORETO, P. NASCETTI, A. GRAVERINI, AND M. MANNOZZI, *Emission and content of monoterpenes in intact and wounded needles of the Mediterranean Pine, Pinus pinea*, Funct. Ecol., 14 (2000), pp. 589–595.

[108] J. H. LOUGHRIN, T. R. HAMILTON-KEMP, R. A. ANDERSEN, AND D. F. HILDEBRAND, *Circadian rhythm of volatile emission from flowers of Nicotiana sylvestris and N. suaveolens*, Physiol. Plant., 83 (1991), pp. 492–496.

[109] J. N. LOUGHRIN, T. R. HAMILTON-KEMP, R. A. ANDERSEN, AND D. F. HILDEBRAND, *Volatiles from flowers of Nicotiana sylvestris, N. otophora and Malus x domestica: headspace components and day/night changes in their relative concentrations*, Phytochemistry, 29 (1990), pp. 2473–2477.

[110] F. LYNEN AND U. HENNING, *Über den biologischen Weg zum Naturkautschuk*, Angew. Chem., 72 (1960), pp. 820–829.

[111] L. E. MAQUAT, *Nonsense-mediated mRNA decay: A comparative analysis of different species*, Curr. Genomics, 5 (2004), pp. 175–190.

[112] J. MARQUES, M. SCHATTAT, G. HAUSE, I. DUDECK, AND R. KLÖSGEN, *In vivo transport of folded EGFP by the ΔpH/TAT-dependent pathway in chloroplasts of Arabidopsis thaliana*, J. Exp. Bot., 55 (2004), pp. 1697–1706.

[113] D. MARTIN, J. FÄLDT, AND J. BOHLMANN, *Functional characterization of nine norway spruce tps genes and evolution of gymnosperm terpene synthases of the tps-d subfamily*, Plant Physiol., 135 (2004), pp. 1908–1927.

[114] P. MERCKE, I. F. KAPPERS, F. W. VERSTAPPEN, O. VORST, M. DICKE, AND H. J. BOUWMEESTER, *Combined transcript and metabolite analysis reveals genes involved in spider mite induced volatile formation in cucumber plants*, Plant Physiol., 135 (2004), pp. 2012–2024.

[115] J. MĚSÍČEK, *Chromosome Counts in Cardaminopsis arenosa Agg. Cruciferae*, Preslia, Praha, 42 (1970), pp. 225–248.

[116] J. K. NIELSEN, H. B. JAKOBSEN, P. FRIIS, K. HANSEN, J. MOLLER, AND C. E. OLSEN, *Asynchronous rhythms in the emission of volatiles from Hesperis matronalis flowers*, Phytochemistry, 38 (1995), pp. 847–851.

[117] T. NORRIS, *Torus Anatomy and Nectary Characteristics as Phylogenetic Criteria in the Rhoeadales*, Am. J. Bot., 28 (1941), pp. 101–113.

[118] E. I. NYÁRÁDY, *Flora Republicii Populare Romîne. Cruciferae.*, vol. 3, Editura Academiei Republicii Populare Romîne, Bucureşti, 1955.

[119] K. OKADA, T. SAITO, T. NAKAGAWA, M. KAWAMUKAI, AND Y. KAMIYA, *Five Geranylgeranyl Diphosphate Synthases Expressed in Different Organs Are Localized into Three Subcellular Compartments in Arabidopsis*, Plant Physiol., 122 (2000), pp. 1045–1056.

[120] K. OKAMOTO, S. NARAYAMA, A. KATSUO, I. SHIGEMATSU, AND H. YANASE, *Biosynthesis of p-anisaldehyde by the white-rot basidiomycete Pleurotus ostreatus*, J. Biosci. Bioeng., 93 (2002), pp. 207–210.

[121] S. L. O'KANE AND I. A. AL-SHEHBAZ, *A Synopsis of Arabidopsis (Brassicaceae)*, Novon, 7 (1997), pp. 323–327.

[122] S. L. O'KANE, B. A. SCHAAL, AND I. A. AL-SHEHBAZ, *The Origins of Arabidopsis suecica (Brassicaceae) as Indicated by Nuclear rDNA Sequences*, Syst. Bot., 21 (1996), pp. 559–566.

[123] E. ORMENO, J. P. MEVY, B. VILA, A. BOUSQUET-MELOU, S. GREFF, G. BONIN, AND C. FERNANDEZ, *Water deficit stress induces different monoterpene and sesquiterpene emission changes in mediterranean species. relationship between terpene emissions and plant water potential*, Chemosphere, 67 (2007), pp. 276–284.

[124] P. W. PARE AND J. H. TUMLINSON, *De novo biosynthesis of volatiles induced by insect herbivory in cotton plants*, Plant Physiol., 114 (1997), pp. 1161–1167.

[125] S. PECHOUS AND B. WHITAKER, *Cloning and functional expression of an (E,E)-α-farnesene synthase cDNA from peel tissue of apple fruit*, Planta, 219 (2004), pp. 84–94.

[126] W. PEER AND A. MURPHY, *Floral scent of Arabidopsis lyrata (brassicaceae)*, Biochem. Syst. Ecol., 31 (2003), pp. 1193–1195.

[127] M. A. PHILLIPS, M. R. WILDUNG, D. C. WILLIAMS, D. C. HYATT, AND R. CROTEAU, *cDNA isolation, functional expression, and characterization of (+)-α-pinene synthase and (-)-α-pinene synthase from loblolly pine (Pinus taeda): Stereocontrol in pinene biosynthesis*, Arch. Biochem. Biophys., 411 (2003), pp. 267–276.

[128] C. POULTER, *Farnesyl Diphosphate Synthase. A Paradigm for Understanding Structure and Function Relationships in E-polyprenyl Diphosphate Synthases*, Phytochem. Rev., 5 (2006), pp. 17–26.

[129] K. PRANTL, *Cruciferae*, in Die natürlichen Pflanzenfamilien, A. Engler and K. Prantl, eds., Wilhelm Engelmann, Leipzig, 1891, pp. 145–206.

[130] R. A. PRICE, J. D. PALMER, AND I. A. AL-SHEHBAZ, *Systematic relationships of Arabidopsis: A molecular and morphological perspective*, in Arabidopsis, C. R. Somerville and E. M. Meyerowitz, eds., New York, Cold Spring Harbor Laboratory Press, 1994, pp. 7–19.

[131] R. A. RAGUSO, *Floral scent, olfaction, and scent-driven foraging behaviour*, in Cognitive ecology of pollination, L. Chittka and J. D. Thompson, eds., Cambridge University Press, Cambridge, 2001, pp. 83–105.

[132] R. A. RAGUSO, R. A. LEVIN, S. E. FOOSE, M. W. HOLMBERG, AND L. A. MCDADE, *Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in Nicotiana*, Phytochemistry, 63 (2003), pp. 265–284.

[133] S. RASMANN, T. G. KÖLLNER, J. DEGENHARDT, I. HILTPOLD, S. TOEPFER, U. KUHLMANN, J. GERSHENZON, AND T. C. J. TURLINGS, *Recruitment of entomopathogenic nematodes by insect-damaged maize roots*, Nature, 434 (2005), pp. 732–737.

[134] A. RATZKA, H. VOGEL, D. J. KLIEBENSTEIN, T. MITCHELL-OLDS, AND J. KROYMANN, *Disarming the mustard oil bomb.*, Proc. Natl. Acad. Sci. U. S. A., 99 (2002), pp. 11223–11228.

[135] S. REZSÖ, *A Magyar Flóra és vegetáció rendszertani-növeényföldrajzi kézikönyve III. Synopsis systematico-geobotanica florae vegetationisque hungariae III.*, Akadémiai Kiadó, Budapest, 1968.

[136] D.-K. RO, J. EHLTING, C. I. KEELING, R. LIN, N. MATTHEUS, AND J. BOHLMANN, *Microarray expression profiling and functional characterization of AtTPS genes: duplicated Arabidopsis thaliana sesquiterpene synthase genes At4g13280 and At4g13300 encode root-specific and wound-inducible (Z)-γ-bisabolene synthases*, Arch. Biochem. Biophys., 448 (2006), pp. 104–116.

[137] G. W. ROBERTSON, D. W. GRIFFITHS, J. A. T. WOODFORD, A. N. E. BIRCH, J. A. PICKET, AND L. J. WADHAMS, *A comparison of the flower volatiles from hawthorn and four raspberry cultivars*, Phytochemistry, 33 (1993), pp. 1047–1053.

[138] M. RODRÍGUEZ-CONCEPCIÓN, *Early Steps in Isoprenoid Biosynthesis: Multilevel Regulation of the Supply of Common Precursors in Plant Cells*, Phytochem. Rev., 5 (2006), pp. 1–15.

[139] F. ROHDICH, F. ZEPECK, P. ADAM, S. HECHT, J. KAISER, R. LAUPITZ, T. GRAWERT, S. AMSLINGER, W. EISENREICH, A. BACHER, AND D. ARIGONI, *The deoxyxylulose phosphate pathway of isoprenoid biosynthesis: Studies on the mechanisms of the reactions catalyzed by IspG and IspH protein*, Proc. Natl. Acad. Sci. U.S.A., 100 (2003), pp. 1586–1591.

[140] M. ROHMER, M. KNANI, P. SIMONIN, B. SUTTER, AND H. SAHM, *Isoprenoid Biosynthesis In Bacteria - A Novel Pathway For The Early Steps Leading To Isopentenyl Diphosphate*, Biochem. J., 295 (1993), pp. 517–524.

[141] U. S. R. RÖSE, W. J. LEWIS, AND J. H. TUMLINSON, *Specificity of systemically released cotton volatiles as attractants for specialist and generalist parasitic wasps*, J. Chem. Ecol., 24 (1998), pp. 303–319.

[142] U. S. R. RÖSE AND J. H. TUMLINSON, *Volatiles released from cotton plants in response to Helicoverpa zea feeding damage on cotton flower buds*, Planta, 218 (2004), pp. 824–832.

[143] W. ROTHMALER, *Exkursionsflora von Deutschland. Gefäßpflanzen: Kritischer Band*, vol. 4, Spektrum Akademischer Verlag, Heidelberg, 9th ed., 2002.

[144] K. SANMIYA, O. UENO, M. MATSUOKA, AND N. YAMAMOTO, *Localization of farnesyl diphosphate synthase in chloroplasts*, Plant Cell Physiol., 40 (1999), pp. 348–354.

[145] M. H. SCHIERUP, *The Effect of Enzyme Heterozygosity on Growth in a Strictly Outcrossing Species, the Self-Incompatible Arabis petraea (Brassicaceae)*, Hereditas, 128 (1998), pp. 21–31.

[146] C. SCHNEE, T. G. KÖLLNER, M. HELD, T. C. J. TURLINGS, J. GERSHENZON, AND J. DEGENHARDT, *The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores*, Proc. Natl. Acad. Sci. U. S. A., 103 (2006), pp. 1129–1134.

[147] P. SCHÖNFELDER, A. BRESINSKY, E. GARNWEIDNER, E. KRACH, H. LINHARD, O. MERGENTHALER, W. NEZADAL, AND V. WIRTH, *Verbreitungsatlas der Farn- und Blütenpflanzen Bayerns*, Ulmer, Stuttgart, 1990.

[148] G. SCHÜLER, A. MITHÖFER, I. T. BALDWIN, S. BERGER, J. EBEL, J. G. SANTOS, G. HERRMANN, D. HÖLSCHER, R. KRAMELL, T. M. KUTCHAN, H. MAUCHER, B. SCHNEIDER, I. STENZEL, C. WASTERNACK, AND W. BOLAND, *Coronalon: a powerful tool in plant stress physiology*, FEBS Letters, 563 (2004), pp. 17–22.

[149] O. E. SCHULZ, *Cruciferae*, in Die natürlichen Pflanzenfamilien, A. Engler and H. Harms, eds., Wilhelm Engelmann, Leipzig, 1936, pp. 227–658.

[150] J. SCHWENDER, M. SEEMANN, H. K. LICHTENTHALER, AND M. ROHMER, *Biosynthesis of isoprenoids (carotenoids, sterols, prenyl side-chains of chlorophylls and plastoquinone) via a novel pyruvate/glyceraldehyde 3-phosphate*

*non-mevalonate pathway in the green alga Scenedesmus obliquus*, Biochem. J., 316 (1996), pp. 73–80.

[151] K. SIEMERING, R. GOLBIK, R. SEVER, AND J. HASELOFF, *Mutations that suppress the thermosensitivity of green fluorescent protein*, Curr. Biol., 6 (1996), pp. 1653–1663.

[152] B. SLAVÍK, *Fytokartografické syntézy CR. Phytocartographical syntheses of the CR.*, vol. 2, Botanical Institute of Czechoslovak Academy of Sciences, Prøuhonice, 1990.

[153] C. STANCU AND A. SIMA, *Statins: mechanism of action and effects*, J. Cell. Mol. Med., 5 (2001), pp. 378–387.

[154] M. STRANDEN, T. ROSTELIEN, I. LIBLIKAS, T. ALMAAS, A. BORG-KARLSON, AND H. MUSTAPARTA, *Receptor neurones in three heliothine moths responding to floral and inducible plant volatiles*, Chemoecology, 13 (2003), pp. 143–154.

[155] S. TAKAHASHI, T. KUZUYAMA, H. WATANABE, AND H. SETO, *A 1-deoxy-D-xylulose 5-phosphate reductoisomerase catalyzing the formation of 2-C-methyl-D-erythritol 4-phosphate in an alternative nonmevalonate pathway for terpenoid biosynthesis*, Proc. Natl. Acad. Sci. U. S. A., 95 (1998), pp. 9879–9884.

[156] M. TAMBASCO-STUDART, O. TITIZ, T. RASCHLE, G. FORSTER, N. AMRHEIN, AND T. B. FITZPATRICK, *Vitamin B6 biosynthesis in higher plants*, Proc. Natl. Acad. Sci. U.S.A., 102 (2005), pp. 13687–13692.

[157] D. A. J. TEULON, B. HOLLISTER, R. C. BUTLER, AND E. A. CAMERON, *Colour and odour responses of flying western flower thrips: wind tunnel and greenhouse experiments*, Entomol. Exp. Appl., 93 (1999), pp. 9–19.

[158] J. S. THALER, M. A. FARAG, P. W. PARE, AND M. DICKE, *Jasmonate-deficient plants have reduced direct and indirect defences against herbivores*, Ecol. Lett., 5 (2002), pp. 764–774.

[159] D. THOLL, W. BOLAND, A. HANSEL, F. LORETO, U. S. R. RÖSE, AND J.-P. SCHNITZLER, *Practical approaches to plant volatile analysis.*, Plant J., 45 (2006), pp. 540–560.

[160] D. THOLL, F. CHEN, J. PETRI, J. GERSHENZON, AND E. PICHERSKY, *Two sesquiterpene synthases are responsible for the complex mixture of sesquiterpenes emitted from Arabidopsis flowers*, Plant J., 42 (2005), pp. 757–771.

[161] D. THOLL AND E. PICHERSKY, *Biosynthesis of volatile terpenes in the flowers of the model plant Arabidopsis thaliana*, in Biology of Floral Scent, N. Dudareva and E. Pichersky, eds., CRC Press, Boca Raton, 2006, pp. 79–90.

[162] T. TSCHARNTKE, S. THIESSEN, R. DOLCH, AND W. BOLAND, *Herbivory, induced resistance, and interplant signal transfer in Alnus glutinosa*, Biochem. Syst. Ecol., 29 (2001), pp. 1025–1047.

[163] T. C. J. TURLINGS, U. B. LENGWILER, M. L. BERNASCONI, AND D. WECHSLER, *Timing of induced volatile emissions in maize seedlings*, Planta, 207 (1998), pp. 146–152.

[164] H. VAN LEUR, C. E. RAAIJMAKERS, AND N. M. VAN DAM, *A heritable glucosinolate polymorphism within natural populations of Barbarea vulgaris*, Phytochemistry, 67 (2006), pp. 1214–1223.

[165] R. VAN POECKE, M. POSTHUMUS, AND M. DICKE, *Herbivore-induced volatile production by Arabidopsis thaliana leads to attraction of the parasitoid Cotesia rubecula: Chemical, behavioral, and gene-expression analysis*, J. Chem. Ecol., 27 (2001), pp. 1911–1928.

[166] J. C. VERDONK, C. H. RIC DE VOS, H. A. VERHOEVEN, M. A. HARING, A. J. VAN TUNEN, AND R. C. SCHUURINK, *Regulation of floral scent production in petunia revealed by targeted metabolomics*, Phytochemistry, 62 (2003), pp. 997–1008.

[167] T. VUORINEN, A. M. NERG, E. VAPAAVUORI, AND J. K. HOLOPAINEN, *Emission of volatile organic compounds from two silver birch (Betula pendula Roth) clones grown under ambient and elevated CO2 and different O3 concentrations*, Atmos. Environ., 39 (2005), pp. 1185–1197.

[168] R. WEGENER, S. SCHULZ, T. MEINERS, K. HADWICH, AND M. HILKER, *Analysis of volatiles induced by oviposition of elm leaf beetle Xanthogaleruca luteola on Ulmus minor*, J. Chem. Ecol., 27 (2001), pp. 499–515.

[169] D. WILLIAMS, D. MCGARVEY, E. KATAHIRA, AND R. CROTEAU, *Truncation of limonene synthase preprotein provides a fully active 'pseudomature' form of this monoterpene cyclase and reveals the function of the amino-terminal arginine pair*, Biochemistry, 37 (1998), pp. 12213–12220.

[170] A. WINDSOR, M. REICHELT, A. FIGUTH, A. SVATOS, J. KROYMANN, D. KLIEBENSTEIN, J. GERSHENZON, AND T. MITCHELL-OLDS, *Geographic*

*and evolutionary diversification of glucosinolates among near relatives of Arabidopsis thaliana (brassicaceae)*, Phytochemistry, 66 (2005), pp. 1321–1333.

[171] G. A. WRIGHT, A. LUTMERDING, N. DUDAREVA, AND B. H. SMITH, *Intensity and the ratios of compounds in the scent of snapdragon flowers affect scent discrimination by honeybees (Apis mellifera)*, J. Comp. Physiol. A Neuroethol. Sens. Neural Behav. Physiol., 191 (2005), pp. 105–114.

[172] Y. H. XU, J. W. WANG, S. WANG, J. Y. WANG, AND X. Y. CHEN, *Characterization of GaWRKY1, a cotton transcription factor that regulates the sesquiterpene synthase gene (+)-δ-cadinene synthase-A*, Plant Physiol., 135 (2004), pp. 507–515.

[173] S. YAMAGUCHI, *Gibberellin biosynthesis in Arabidopsis*, Phytochem. Rev., 5 (2006), pp. 39–47.

[174] J. H. YAN, X. X. XIAO, AND K. L. HUANG, *Component analysis of volatile oil from Illicium verum Hook. f.*, J. Central South Uni. Technol., 9 (2002), pp. 173–176.

[175] J. ZEIDLER, J. SCHWENDER, C. MÜLLER, J. WIESNER, C. WEIDEMEYER, E. BECK, H. JOMAA, AND H. K. LICHTENTHALER, *Inhibition of the Non-Mevalonate 1-Deoxy-D.xylulose-5-phosphate Pathway of Plant Isoprenoid Biosynthesis by Fosmidomycin*, Z. Naturforsch., 53c (1998), pp. 980–986.

[176] Z. ZHANG AND J. PAWLISZYN, *Headspace solid-phase microextraction*, Anal. Chem., 65 (1993), pp. 1843–1852.

## Erklärung

Die z. Zt. gültige Promotionsordung der Biologisch-Pharmazeutischen Fakultät ist mir bekannt. Die vorliegende Arbeit wurde von mir selbst mit den angegeben Hilfsmitteln erstellt und alle benutzten Quellen angegeben. Es wurden keine Textabschnitte eines Dritten ohne Kennzeichnung übernommen. Alle Personen, die an der Gewinnung von Daten beteiligt, bei der Erstellung des Manuskripts hilfreich waren oder sonstige Hilfestellung gaben, sind benannt.

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Jena, im August 2007 Christian Abel