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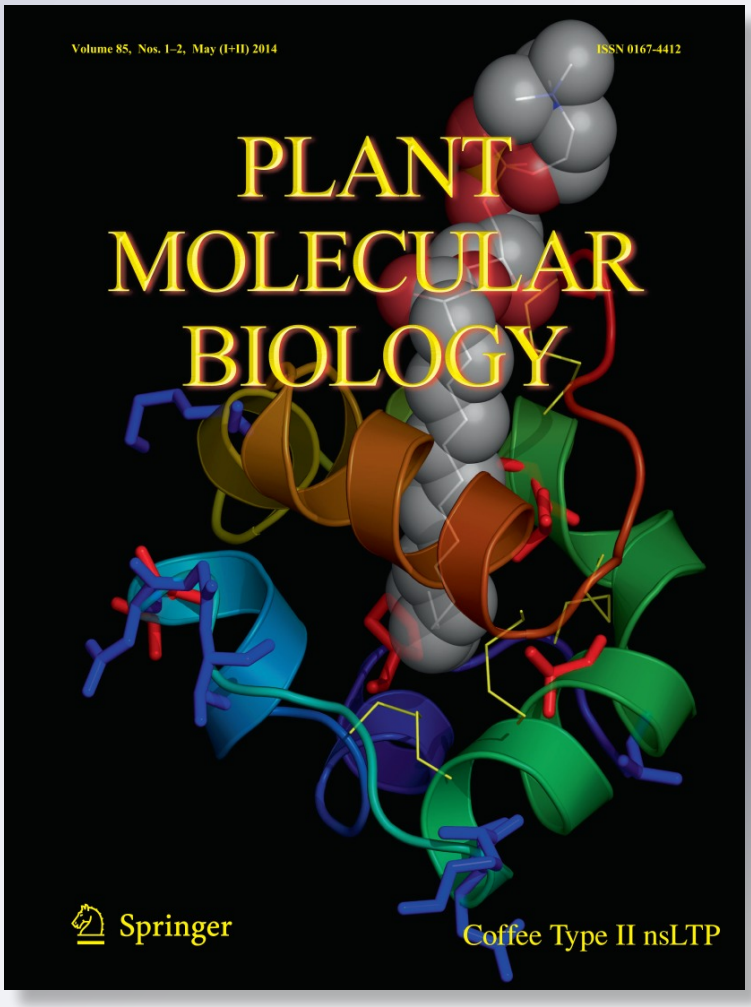

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Characteristic alatoid 'cineole cassette' monoterpene synthase present in *Nicotiana noctiflora*

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Abstract *Nicotiana* species of the section *Alatae* emit a characteristic floral scent comprising the, cineole cassette' monoterpenes 1,8-cineole, limonene, myrcene, β-pinene, α-pinene, sabinene and α-terpineol. All previously isolated 'cineole cassette'-monoterpene synthase genes are multi product enzymes that synthesize the seven compounds of the 'cineole cassette'. Interestingly, so far this 'alatoid' trait was only shared with the eponymous species *Nicotiana suaveolens* of the sister section *Suaveolentes*. To determine the origin of the 'cineole cassette' monoterpene phenotype other potential parent species of section *Noctiflorae* or *Petunoides* as well as of the distantly related section *Trigonophyllae* were analysed. A monoterpene synthase producing the set of 'cineole cassette' compounds was isolated from *N. noctiflorae*. *N. obtusifolia* emitted solely 1,8-cineole and no monoterpenes were found in floral scents of *N. petunoides* and *N. palmeri*. Interestingly, the phylogenetic analysis clustered the new gene of *N. noctiflora* closely to the terpineol synthase genes of e.g. *N. alata* rather than to cineole synthase genes of e.g. *N. forgetiana*.

Keywords *Nicotiana* · *Alatae* · *Suaveolentes* · *N. noctiflora* · 'Cineole cassette' monoterpenes

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Introduction

The genus *Nicotiana* is the sixth largest group within the Solanaceae and comprises 76 naturally occuring species including the important crop plant *N. tabacum* (Knapp et al. 2004). Goodspeed (1954) provided detailed informations of the taxonomy, cytology and biogeography. Phylogenetic studies of the genus *Nicotiana* classified thirteen sections: *Alatae, Nicotiana, Noctiflorae, Paniculatae, Petunioides, Polydicliae, Repandae, Rusticae, Suaveolentes, Sylvestres, Tomentosae, Trigonophyllae* and *Undulatae* (Knapp et al. 2004; Clarkson et al. 2004). The species are distributed worldwide, approximately 75 % of species occur in America and 25 % in Australia, only one species was so far found in Africa (*N. africana*) (Aoki and Ito 2000; Merxmüller and Buttler 1975). Based on ancient chromosome hybridizations and the present chromosome number (amphidiploidy) Goodspeed (1954) hypothesized that the progenitors of the section *Suaveolentes* originated in South America and dispersed to Australia via an Antarctic land bridge in the Cenozoic, while Ladiges et al. (2011) discussed two dispersal events. GISH results revealed a participation of an 'alatoid' genome in the amphidiploid ancestor of N. section *Suaveolentes* (Chase et al. 2003) supporting Goodspeed's hypothesis that section *Suaveolentes* derived from one ancestor of the present-day member of section *Alatae* and another ancestor either from a member of section *Noctiflorae* or *Petunioides*. Although gene sequence based analysis were performed to clarify the relationships within the genus *Nicotiana* several open questions remained. Figure 1 shows in a very simplified version the controversial results e.g. obtained with the internal transcribed sequences (ITS) or matK gene sequence analysis (Chase et al. 2003). One example of a controversial results is that with the analysis of ITS *Alatae* is a sister section to

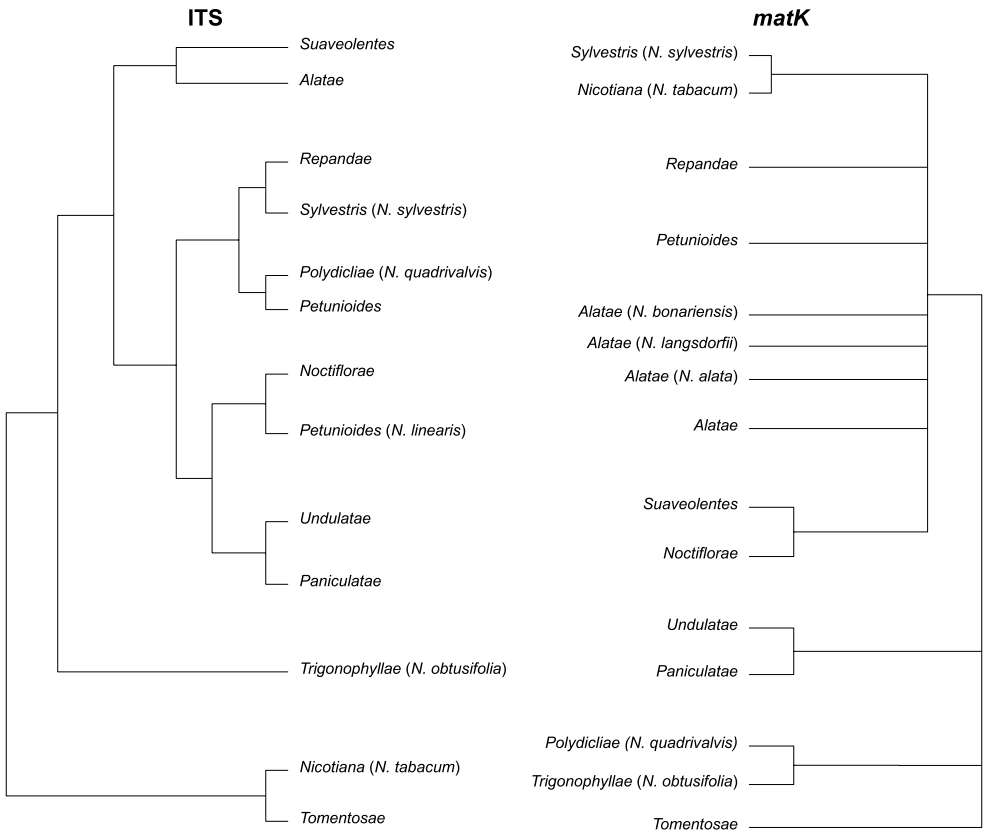

Fig. 1 Comparison of ITS and matK phylogenetic trees of the genus of *Nicotiana.* Simplified presentation of the phylogenetic trees based on internal transcribed spacer (ITS) and mat K sequence alignments (from Fig. 5 of Chase et al. 2003; Knapp et al. 2004) demonstrates the controversial results and unsolved relationships within the *Nicotiana* genus

Suaveolentes and the branch of *Noctiflorae* appears quite distantly, while the mat K tree locates *Suaveolentes* closer to *Noctiflorae* than to *Alatae*.

Despite genetic diversity also flower shapes and colors, and scent compositions are very different within the genus *Nicotiana*. Eleven species are examplarily summarized in Table 1. The dominating flower colors are pink, white and greenish and the corollas appear round or star shaped or shapes between these extremes. Flower opening occuring in the evening, at dusk or vespertine often correlates with moth being the pollinator. The floral scent emission is also very different in the species, different in the number of compounds as well as the composition. It is an interesting observation that all species of the section *Alatae* (*N. alata, N. langsdorfii, N. bonariensis, N. forgetiana, N. mutabilis, N. longiflora, N. plumbaginifolia,* except *N. azembique*) emit a characteristic volatile pattern comprising the seven monoterpenes of the 'cineole cassette': 1,8-cineole, β-myrcene, limonene, sabinene, α-/β-pinene, and α-terpineol (Raguso et al. 2003, 2006). Often 1,8-cineole is the major compound within these emission profiles. In the past, several monoterpene synthase genes were isolated from species of section *Alatae* synthesizing this characteristic set of monoterpenes (Fähnrich et al. 2011, 2012). Monoterpene synthases belong to a large group of genes that encode enzymes present in floral and vegetative tissues of angiosperms and gymnosperms (summarized in Degenhardt et al. 2009, Fähnrich et al. 2011). The isolated monoterpene synthases of the section *Alatae* turned out to be multi product enzymes, which simultaneously synthesize the 'cineole cassette'-monoterpenes (Fähnrich et al. 2011, 2012). According to the major compound these monoterpene synthases were either named cineole (CIN) or terpineol (TER) synthases (Table 1). Since the 'cineole cassette'-monoterpene emission is a typical feature of N. section *Alatae* it can be regarded as a characteristic trait locus. In contrast, the species of the sister section *Suaveolentes* do not emit the 'cineole cassette'-monoterpenes, except *N. suaveolens* (Raguso et al. 2003, 2006). Subsequently a CIN was isolated from *N. suaveolens,* which is also a multi product enzyme producing the 'cineole cassette'-monoterpenes (Roeder et al. 2007). Together, these results opened the discussion, whether the emission of the 'cineole cassette' monoterpenes and the presence

Table 1 Floral biology of several species of the 5 *Nicotiana* sections *Alatae*, *Suaveolentes*, *Noctiflorae*, *Trigonophyllae* and *Petunioides*

					Scent emission [6, 7, this paper]			
	Flower morphology	Flower color	Flower opening [1,2,3]	Pollinator [1,4,5,6]	Number of compounds	Time of emission	Cineol cassette monoterpenes	Isolated gene [8,9,10]
Alatae								
N. alata		White	Around dusk	Hawkmoth	69	Nocturnal	7	TER
N. langsdorfii		Green	Early afternoon until senescence	Hummingbird	14	Nocturnal	7	TER
N. bonariensis		White	Around dusk	Moth	23	Nocturnal	6	CIN
N. forgetiana		Red	Late afternoon	Hummingbird	32	Nocturnal	7	CIN
N. longiflora		White	Around dusk	Hawkmoth	28	Nocturnal	6	CIN
N. mutabilis		White, pink, magenta	Late afternoon	Hummingbird	20	Nocturnal	7	Putative CIN
Suaveolentes								
N. suaveolens		White	Vespertine	Hawkmoth	41	Nocturnal	7	CIN
Noctiflorae								
N. noctiflora		White	Diurnal or vespertine	Unknown	6	Unknown	5	CIN
Trigonophyllae								
N. obtusifolia		White	Dusk	Unknown	5[a]	Unknown	only 1,8 cineol	–
Petunioides								
N. palmeri		White	Diurnal	Unknown	0	Unknown	ND	–
N. petunioides		White	Vespertine	Unknown	8[b]	Unknown	ND	–

ND not detectable, + detectable, – no synthase isolated, *TER* α-terpineol synthase, *CIN* 1,8-cineol synthase

[a] 1,8-cineole, lavendel lactone, lilac aldehyde, benzyl benzoate, demethoxy-4-vinylbenzene

[b] Germacrene D, gamma-cardienene, 2-phenylethanol, benzene aldehyde, hexenyl benzoate, trimethyl hexanone, methyl hexadecene

[1] Kaczorowski et al.(2005); [2] Knapp et al. (2004); [3] Anssour et al. (2009); [4] Ippolito et al. (2004); [5] Stehmann et al. (2002); [6] Raguso et al. (2006); [7] Raguso et al. (2003); [8] Fähnrich et al. (2011); [9] Fähnrich et al. (2012); [10] Roeder et al. (2007)

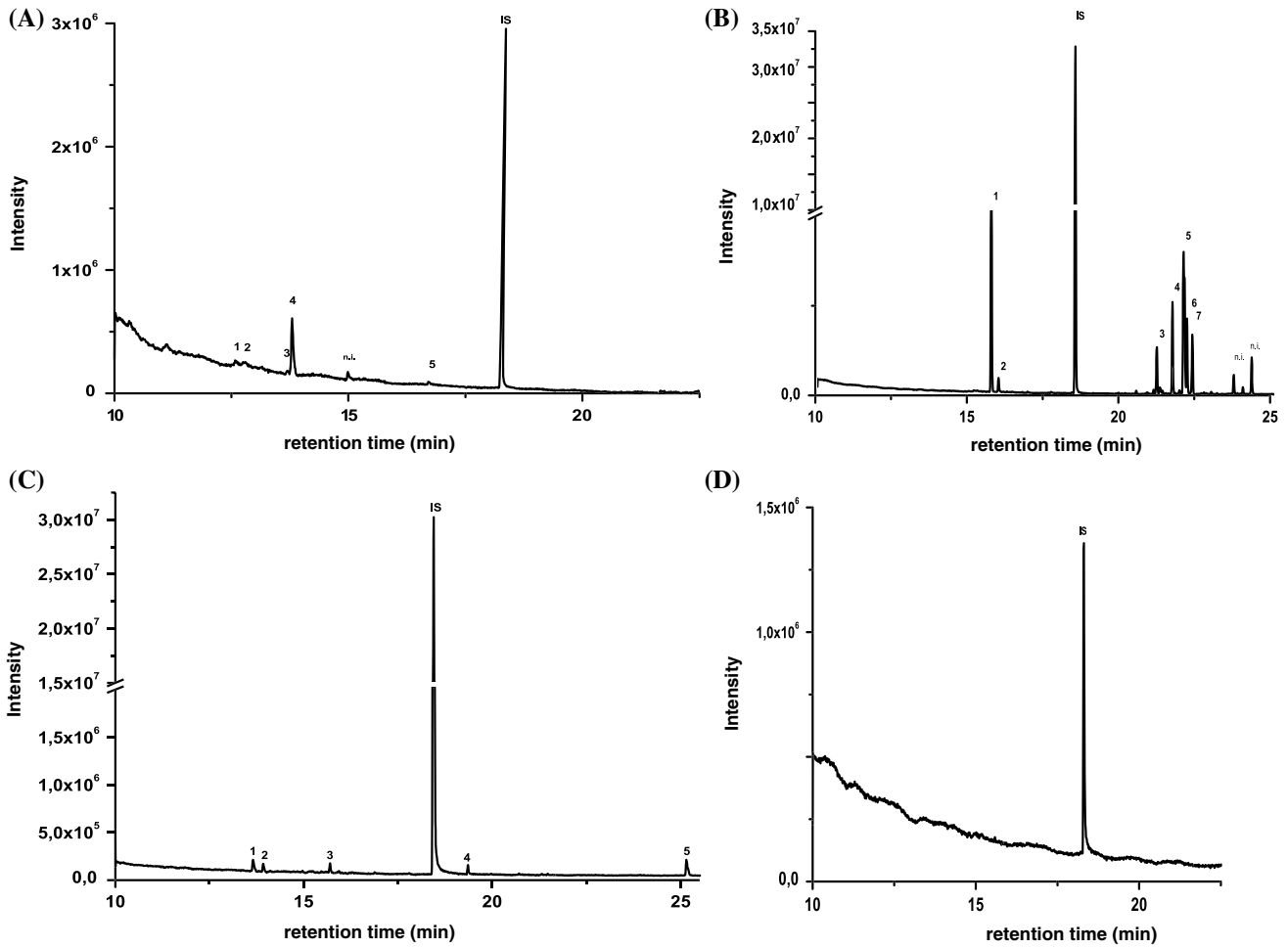

Fig. 2 Floral volatiles emitted from different *Nicotiana* species *Nicotiana* flowers (while attached to the plants) were transferred into a glass globe (one flower per globe). Headspace volatiles of 4 flowers were investigated for each species. The volatiles of the *Nicotiana* species were collected and amounts were calculated in ng per flower and per h (2 experimental replicates, each with 4 flowers). Statistical analyses were performed using Sigma Plot. Compounds were analyzed by GC/MS, identified by their retention index, by comparison of mass spectra of the library of the National Institute of Standards and Technology (NIST147) and by comparison with the authentic standards. **a** GC chromatogram of headspace volatiles of flowers of *N noctiflora* were collected between 6 and 8 p.m. (*1*) sabinene, (*2*) β-myrcene, (*3*) limonene, (*4*) 1,8-cineole, (*5*) α-terpineol, (*n.i.*) not identified compound, (*IS*) internal standard (5 ng nonyl acetate). **b** GC chromatogram of headspace volatiles of flowers of *N. petunoides* were collected for 24 h. (*1*) 2-phenylethylethanol, (*2*) isophorene, (*3*) germacrene D, (*4*) γ-cardinene (*5*) hexenyl benzoate, (*6*) benzene acetaldehyde, (*7*) methylhexadecene, (*n.i.*) not identified. (*IS*) internal standard (5 ng nonyl acetate). **c** GC chromatogram of headspace volatiles of flowers of *N. obtusifolia* were collected for 24 h (*1*) lavendel lactone, (*2*) cineole, (*3*) lilac aldehyde, (*4*) dimethoxy-4-vinylbenzene, (*5*) benzyl benzoate, (*IS*) internal standard (5 ng nonyl acetate). **d** GC chromatogram of headspace volatiles of flowers of *N. palmeri* were collected for 24 h. (*IS*) internal standard (5 ng nonyl acetate)

of respective CIN/TER monoterpene synthases can be regarded as a shared trait of *Nicotiana* section *Suaveolentes* and *Alatae.* Furthermore it was questioned, whether these genes/enzymes can be used as a phylogenetic tool to search for the ancestor of both sections. Consequently, we analyzed species of putative progenitors such as *N. noctiflorae* and *N. petunioides* of section *Noctiflorae,* and *N. obtusifolia* and *N. palmeri* of section *Trigonophyllae* to search for the emission of the 'cineole cassette' monoterpenes and the presence of respective CIN/TER monoterpene synthase genes.

Results

Floral volatile emission of *Nicotiana* species of section *Noctiflorae and Trigonophyllae*

The progenitor species of section *Alatae* and *Suaveolentes* were hypothesized to be members of the section *Petunioides* or section *Noctiflorae*, or they may also originate from the more distantly located N. section *Trigonophyllae* (Clarkson et al. 2004, Fig. 1). The headspace volatiles of

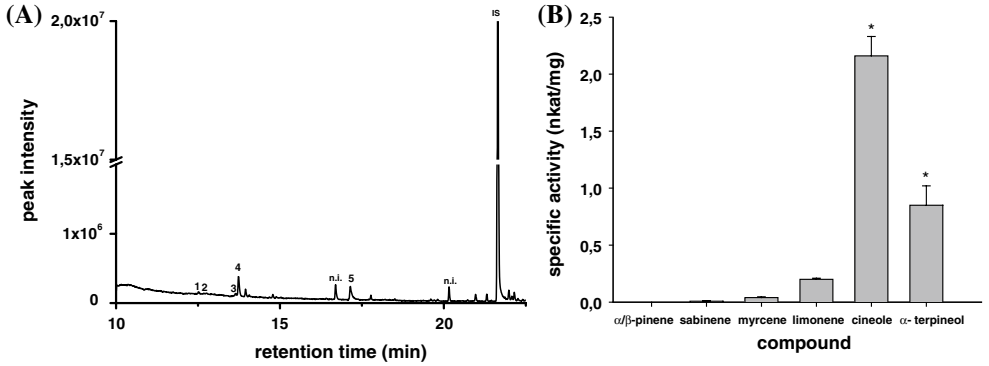

Fig. 3 Monoterpene synthase enzyme activity in petals of *N. noctiflora*. The precursor geranyldiphosphate was provided as substrate for enzyme activity assay with petal extracts of *N. noctiflora*. The petal raw extracts *of N. noctiflora* were used for an enzyme assay and the produced compounds in the hexane phase were analyzed by GC/MS. Product identification was based on comparison with authentic standard compounds, by comparison with the mass spectra of the NIST 147 database and their retention index (**a**) GC/MS-chromatogram of the synthesized products by petal raw extracts of *N. noctiflora*. (**b**) Specific activity was calculated for each 'cineole cassette' monoterpene (nkat/mg^{-1}). Statistical analyses were performed using ANOVA and *t* test calculated with SigmaPlot (*significance: <0,05). (*1*) sabinene, (*2*) myrcene, (*3*) limonene, (*4*) 1,8-cineole, (*5*) α-terpineol, (*IS*) internal standard (5 ng cis-nerolidol), n = 3. Statistics were performed using *t* test of Sigma Plot, *significance: $p < 0.05$

the flowers of species of section *Noctiflorae* (*N. noctiflora* and *N. petunioides*) and *Trigonophyllae* (*N. obtusifolia* and *N. palmeri*) were collected and analyzed via GC/MS. The floral bouquet of *N. noctiflora* consists of six detectable compounds, five of them belong to the 'cineole cassette': 1,8-cineole, α-terpineol, myrcene, sabinene and limonene (Fig. 2a; Table 1). Within this floral emission pattern cineole is the major monoterpene (120 ng flower^{-1} h^{-1}). The other compounds were released in lower amounts, (α-terpineol 30 ng flower^{-1} h^{-1}, sabinene 10 ng flower^{-1} h^{-1}, myrcene and limonene 4–5 ng flower^{-1} h^{-1}). To show that this emission of volatiles is based on the presence of an active enzyme, tissue extracts of petals of *N. noctiflora* flowers were prepared and incubated with the substrate geranyl pyrophosphate (GPP). The products were analyzed via GC/MS and enzyme activity was calculated for each product (Fig. 3a, b). Highest enzyme activity was reached for 1,8-cineole with approximately 2,160 pkat/mg protein. α-terpineol, limonene and myrcene were synthesized as minor compounds with an enzyme activity of approximately 100–700 pkat/mg protein. Antibodies against the CIN of *N. suaveolens* cross reacted with a potential terpene synthase of ca. 60 kD in petal extracts of *N. noctiflora* (Fig. S1). *N. petunioides* released nine volatile compounds and seven of them could be identified such as aromatic aldehydes, alcohols and esters (e.g. 2-phenylethylethanol, benzene acetaldehyde, hexenyl benzoate) and sesquiterpenes (germacrene D, γ-cardinene) (Fig. 2b; Table 1), however *N. petunioides* flowers did not emit monoterpenes. The headspace volatiles of species of section *Trigonophyllae* were also analyzed, *N. obtusifolia* flowers emitted five compounds, one monoterpene (1,8-cineole) and four aromatic compounds, lavendel lactone, lilac aldehyde, benzyl benzoate and dimethoxy-4-vinylbenzene (Fig. 2c; Table 1), while no volatiles were detected in the headspace of *N. palmeri* (Fig. 2d; Table 1). The volatile spectra of crude petal extracts of *N. petunioides, N. obtusifolia, N. palmeri* did not indicate monoterpene synthesis in petals (Fig. S2 A-C, respectively).

Out of the four *Nicotiana* species investigated here only *N. noctiflora* and *N. obtusifolia* flowers emitted monoterpenes of the 'cineole cassette' (Table 1).

Isolation and characterization of a cineole synthase of *Nicotiana noctiflora*

The emission of the 'cineole cassette' monoterpenes and the detection of an immunological cross reacting protein in petal tissue was taken as indication for the existence of a monoterpene synthase in *N. noctiflora* (Figs. 2a, 3, S1). Subsequently, a monoterpene synthase gene was isolated via RT-PCR. An open reading frame comprizing 1,572 nucleotides, encoding a mature protein of 524 aa (starting from the RR $(X)_8W$ motif), was isolated (Accession No. KF958292). The function of the new enzyme from *N. noctiflora* was determined by overexpressing the protein in *E. coli.* The supernatant was supplemented with GPP and the volatiles were analyzed by GC/MS (Fig. 4). The enzyme of *N. noctiflora* turned out to be a multi product enzyme synthesizing simultaneously the seven monoterpenes of the 'cineole cassette': α-pinene, β-pinene, sabinene, β-myrcene, limonene, 1,8-cineole and α-terpineol. The bicyclic epoxide 1,8-cineole was the major compound comprising ca. 50 % of the products. α-terpineol contributed approximately 25 %, while the other components of the 'cineole cassette' were minor products (each contributing

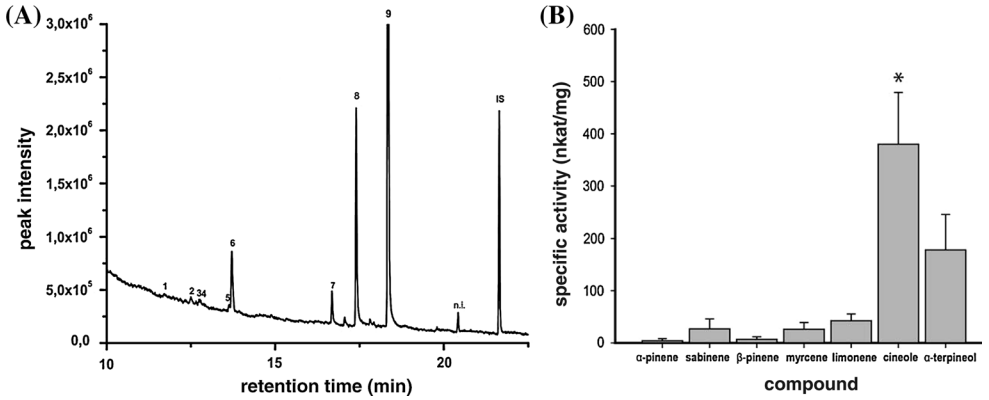

Fig. 4 GC chromatogram of overexpressed cineole synthase of *Nicotiana noctiflora* The gene was overexpressed in E. *coli*. The monoterpene synthase in the supernatant was used in an enzyme assay, GPP was provided as substrate. Volatiles synthesized were analyzed by GC/MS. Product identification was based on comparison with authentic standard compounds and with mass spectra of the NIST147 library. **a** GC chromatogram of the supernatant of recombinant cineole synthase of *N. noctiflora*. (*1*) α-pinene, (*2*) sabinene, (*3*) β-pinene, (*4*) β-myrcene, (*5*) limonene, (*6*) 1,8-cineole, (*7*) α-terpineol, (*8*) nerol, (*9*) indole, (*IS*) internal standard (5 ng cis-nerolidol), n.i.: not identified. **b** Specific activity of recombinant cineole synthases from *N. noctiflora* were calculated for each of the seven ‚cineole cassette' monoterpenes. n = 3, statistics were performed using *t* test of Sigma Plot, *significance: $p < 0.05$

<10 %). Subsequently, this monoterpene synthase of *N. noctiflora* was named cineole synthase (CIN).

Phylogenetic analysis

The new cineole synthase gene of *Nicotiana noctiflora* together with known CIN and TER genes of the *Nicotiana* section *Alatae* were aligned (Fig. 5). Interestingly, the cineole synthase of *N. noctiflora* was comprized of 524 amino acids, similar as CINs from previously isolated *Nicotiana* species, while the two TER enzymes of *N. alata* and *N. langsdorfii* shared a deletion of two amino acids. Beside this, the amino acid comparison revealed high amino acid identities. The natural amino acid variation between sequences was between 3 and 6 amino acids. The *N. noctiflora* enzyme harbors five amino acid alterations, two of them are at positions which were not changed in the previously isolated enzymes (serin at position 198, histidin at position 265). The comparison of all up to now isolated CIN and TER enzymes exhibited four sequence positions (aa 167, aa 202, aa 352, aa 471/472) were amino acid alterations appeared more frequently, while the other amino acid divergences were scattered over the sequence.

The sequences of all *Nicotiana* species were used to construct a phylogenetic tree using the neighbor joining method (Fig. 6). The TER/CIN genes of the Solanaceae family form a monophyletic clade (BP 93 %). The genus *Nicotiana* and the genus *Solanum* were separated clades with bootstraps of 100 and 99 %, respectively. Within the genus *Nicotiana* five branches were observed, (1) the CIN of *N. suaveolens* was separated from *Alatae* and *Noctiflorae* species, (2) the CIN genes of *N. forgetiana, N. longiflora* and *N. mutabilis* cluster together and (3) the TER genes of *N. alata* and *N. langsdorfii* form a branch. It is interesting to note that the CINs of *N. bonariensis* and *N. noctiflora* were separated from the other three branches and it came as a surprise that the CIN of *N. noctiflora* (section *Noctiflorae*) was embedded in the sequences of section *Alatae*. Based on these monoterpene synthase sequence alignments and the floral emission of the 'cineole cassette' monoterpenes *N. noctiflora* is apparently more closely related to *Alatae* species than to *Suaveolentes*.

Discussion

The question about evolution and relationships of species within the genus *Nicotiana* had been attracted several scientific groups in the past, starting with Goodspeed (1954), and more recently Aoki and Ito (2000), Chase et al. (2003), Knapp et al. (2004), Clarkson et al. (2004). However, the relationship of N. sections *Alatae, Noctiflorae* and *Suaveolentes* were not solved to satisfaction. Here we tried to use the characteristic floral scent composition of 'cineole cassette' monoterpenes of *Alatae* species as a phylogenetic marker because all so far investigated species of section *Alatae* emit the 'cineole cassette' monoterpenes. The sister taxa *Suaveolentes* (with the exception of *N. suaveolens*) does not release these typical compounds (Raguso et al. 2003, 2006). One hypothesis indicates that the Australien section *Suaveolentes* originated from an ancient hybridization event between a member of *Alatae* and a member of *Petunioides* or *Noctiflorae* (Goodspeed 1954; Aoki and Ito 2000) and apparently most species of *Suaveolentes* silenced or lost the '*Alatae*'-typical capability

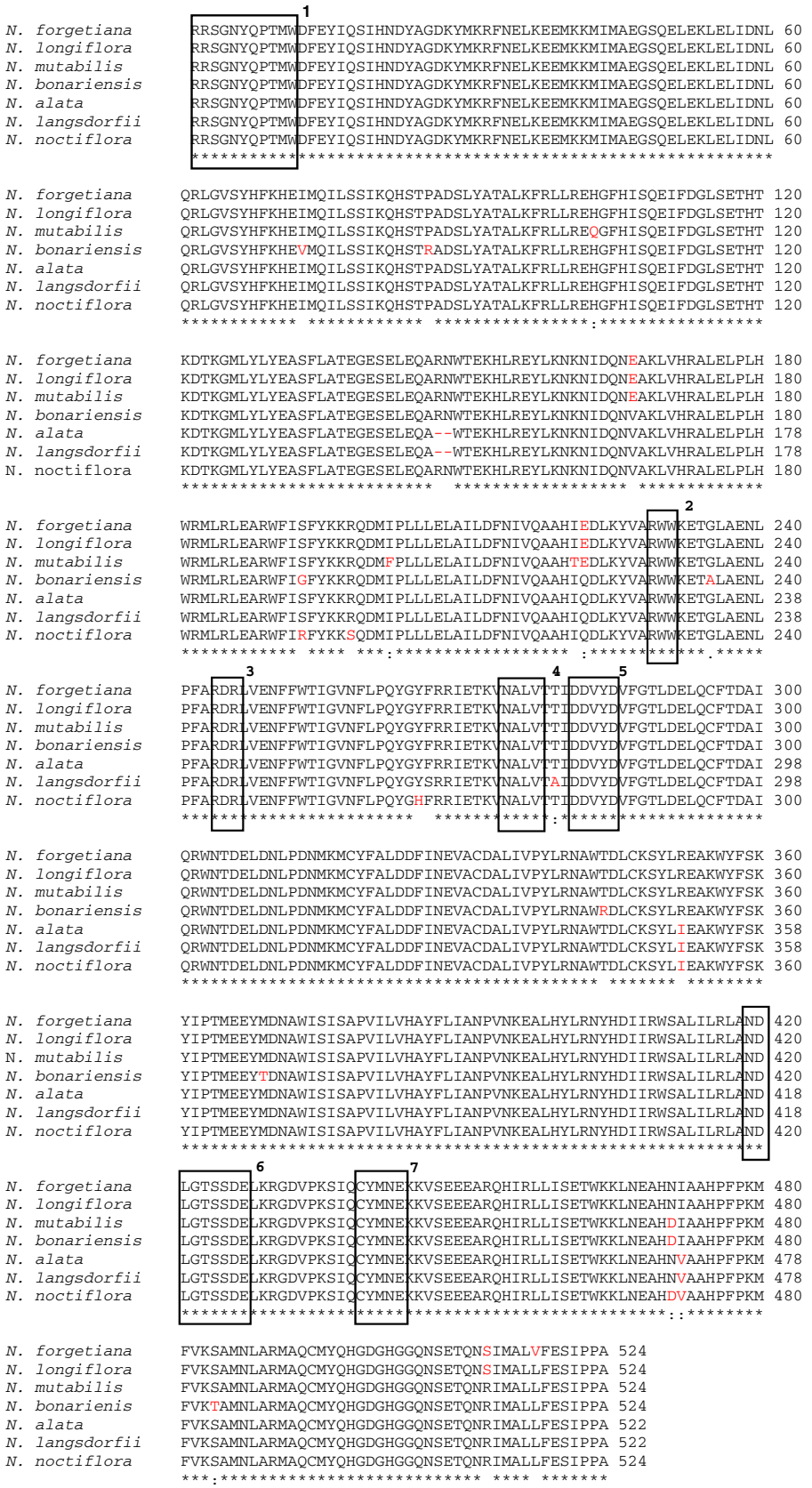

Fig. 5 Sequence alignment of cineole and terpineol terpene synthases. Amino acid sequences of monoterpene synthases of seven *Nicotiana* species were aligned using the Clustal W-algorithm. Conserved sequence motifs are indicated (*1*) $RR(X)_8W$ motif, (*2*) RWW motif, (*3*) RXR motif, (*4*) NALV motif, (*5*) DDXXD motif, (*6*) NSE/DTE motif, (*7*) CYMNE motif. Amino acid alterations between the species are in red

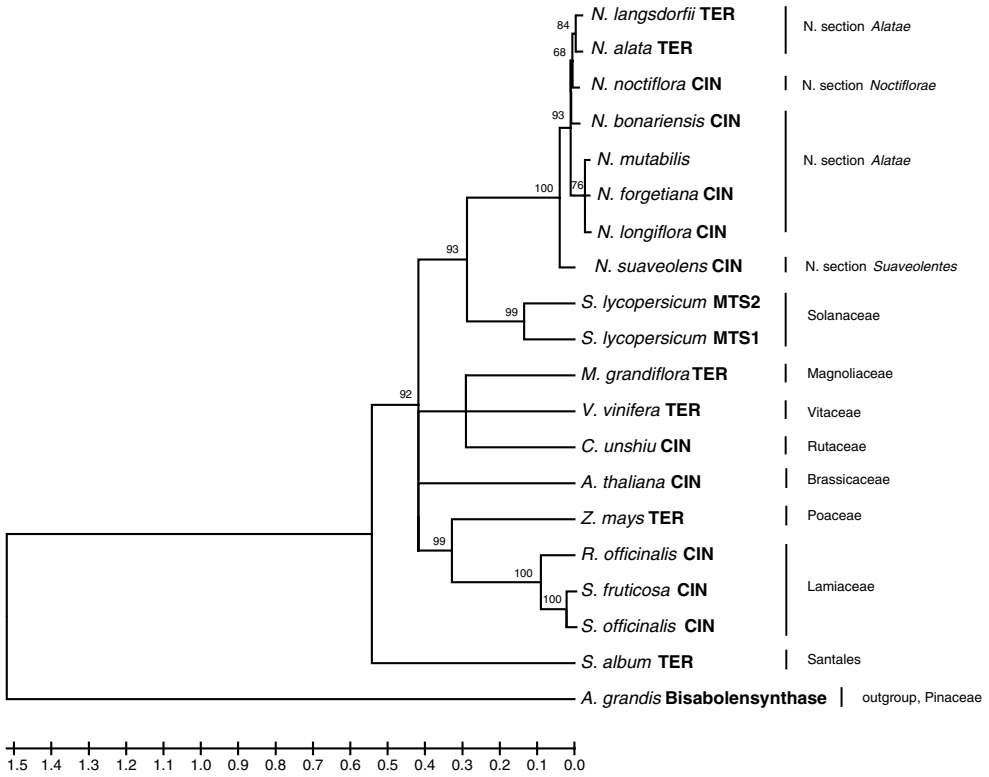

Fig. 6 Phylogeny of plant cineole and terpineol terpene synthases within the genus *Nicotiana.* Phylogenetic relationship of isolated cineole synthase genes (CIN) and terpineol synthase genes from *Nicotiana* section *Alatae*, *Suaveolentes* and *Noctiflora*e with related monoterpene synthases of other plant species. Rooted neighbor joining phylogenetic (100 % bootstrap) tree construction based on amino acid sequence similarities. The bisabolene synthase (a sesquiterpene synthase) was used as outgroup. The tree was created with MEGA 4.0 and displayed using TreeView. Gaps (Clustal W) and the target sequence upstream of the $RR(X)_8W$ motif of the alignment was removed. Plant species used for the tree construction (accession numbers see "Materials and methods")

of 'cineole cassette' monoterpene emission, while this 'alatoid' feature was conserved in *N. suaveolens*. Alternatively, it is also possible that the other parent of section *Suaveolentes*, *Noctiflorae* or *Petunioides*, contributed to the 'cineole cassette' monoterpene trait. Therefore, we analyzed the emission profiles of two species of section *Noctiflorae* (*N. noctiflora, N. petunioides*) and, additionally, two species of the distantly related section *Trigonophyllae* (*N. palmeri, N. obtusifolia*) to obtain information about 'cineole cassette' monoterpene emission in these sections/species. Only the eponymous species *N. noctiflora* emanated the set of respective monoterpenes. This result suggested a close relationship of *N. noctiflora* to section *Alatae*, which was further supported by the sequence comparison of the isolated CIN (Fig. 5). Based on these results it can be concluded that the phenotype of 'cineol cassette' monoterpene emission was most likely present in both parents of *Suaveolentes.*

Furthermore, it is interesting to note that the phylogenetic analysis of sequence comparisons embedded *N. noctiflora* into the section *Alatae* (Fig. 6). This observation was underpinned by the characteristic 'cineole cassette' monoterpene product profile of the recombinant enzyme. This finding contrasts previous results of marker gene comparison of Aoki and Ito (2000), Chase et al. (2003), and Clarkson et al. (2004), which placed *N. noctiflora* distantly to section *Alatae.* Furthermore, this analysis highlighted another interesting aspect: the new gene/enzyme from *N. noctiflora* clearly synthesizing more 1,8-cineole than α-terpineol (CIN), clustered closely to the TER genes of e.g. *N. alata* rather than to CIN genes of e.g. *N. forgetiana*. This result also would support an 'alatoid' ancestry. These contradictory as well as unexpected results ask for additional phylogenetic investigations.

Species of *Petunioides* were also hypothesized to be one parent of *Suaveolentes* (Goodspeed 1954). This was supported by the results of Kessler and Baldwin (2006) who showed that in the headspace of *N. attenuata*, a member of section *Petunioides*, two compounds of the 'cineole cassette', 1,8-cineole and limonene, were detected. This result suggested that a CIN might be present in *N. attenuata*, however, the gene and/or enzyme was not isolated up to now and therefore the biochemical and functional properties remain unknown. Furthermore, we were able to show a strong cross-reaction of antibodies of CIN of *N. suaveolens* with a

protein of ca. 60 kD of petal extracts of *N. acuminata* which is another species of this section (Brosemann and Piechulla unpublished). Both results indicated the presence of CIN enzymes in section *Petunioides*. It would be of great interest to isolate respective genes and to study their enzymatic functionality. Similarly, the exclusive emission of 1,8-cineole of *N. obtusifolia* also suggests the presence of a cineole synthase in this distantly related species (Table 1). Together, the emission profiles of *N. attenuata* (1,8-cineole and limonene) and of *N. obtusifolia* (1,8-cineole) allow to hypothesize the following scenario: (1) the genes in both species originated from a typical 'cineole cassette' multi product monoterpene synthase and lost the ability to synthesize the complete set of monoterpenes, or (2) the gene of *N. attenuata* originated from a single product enzyme and gained the function of limonene synthesis. To the best of our present knowledge only the CIN of *Citrus unshiu* was described as a single product enzyme (Shimada et al. 2005) and no plant species is presently known that emits 1,8-cineole and limonene but not the other components of the 'cineole cassette' (summarized in Table 1 in Fähnrich et al. 2011). It is also interesting to note that in *Nicotiana* species of section *Alatae* (*N. alata, N. langsdorfii, N. bonariensis, N. forgetiana, N. longiflora, N. mutabilis*) always 1,8-cineole and α-terpineol contributed most to the monoterpene emission spectrum (Fähnrich et al. 2011, 2012), while α-terpineol is not present in the floral VOC spectrum of *N. attenuata* (Kessler and Baldwin 2006), *N. acuminata* and *N. obtusifolia*.

Another issue needs to be discussed in the context of evolution of floral scent and underlying biosynthetic pathways and their usage as reliable phylogenetic markers. Many results support the assumption that a high degree of selection pressure is on floral volatiles due to the importance of attracting pollinators (summarized by Raguso et al. 2006). Therefore the volatile blends might be altered frequently in the course of evolution depending on the presence of specific pollinator species, and therefore might not necessarily be a good indication for ancestry (Barkman et al. 1997). Furthermore, scent variation is widespread in section *Alatae*, and may reflect edaphic specialization, introgression, local pollinator shifts, genetic drift or artificial selection in cultivation (Raguso et al. 2006). Since the evolutionary pressure not only lies on the genes encoding the enzymes but also on genes of the transcriptional, translational machinery or regulatory system multiple levels have to be considered influencing scent emission. Even if CIN and TER gene/enzymes may not considered strong markers to study ancestry, it still might be an useful approach to isolate and study genes and enzymes of related *Nicotiana* species in the future to unravel the evolution of the reaction mechanisms and enzymatic catalysis of CIN and TER genes which also may help to substantiate the phylogeny and classification of this genus via biochemical parameters.

Materials and methods

Plant growth

Nicotiana noctiflora, *Nicotiana petunoides, Nicotiana palmeri* and *Nicotinana obtusifolia* plants were grown on Vermiculite (Deutsche Vermiculite Dämmstoffe GmbH, Sprockhövel, Germany) in growth chambers under long day conditions (16 h illumination at 160 μE m^{-2} s^{-1} and 22 °C, 8 h darkness at 21 °C). Plants were watered with Hoaglands solution (Hoagland and Aronon 1938).

Volatile collection of flowers from *Nicotiana species*

The collection of volatiles from whole flowers was performed by using the open loop system as described by Heath and Manukian (1994). The collection started on the day of anthesis and four flowers were placed into glas globes for analysis. A compressor (Schneider Werkstatt- und Maschinenfabrik, Bräunlingen, Germany) delivered a constant air flow of 5 l min^{-1}, which was divided between the four glass globes. The volatile-enriched air was sucked through a SuperQ-column (Alltech Associates, Deerfield, Illinois, USA) using a vacuum pump with 2.8 l min^{-1} (KNF Neuberger, Freiberg, Germany) (Effmert et al. 2008). For quantification nonyl acetate or cis nerolidol (5 ng μl^{-1}) was used as internal standard and volatiles were eluted with 300 μl dichloromethane and analyzed by GC–MS.

GC/MS analysis

The volatile compounds were analyzed with a Shimadzu QP5000 gas chromatograph coupled to a mass spectrometer for identification (GC/MS). Separation was performed on a DB5-MS column (60 m × 0.25 mm × 0.25 mm; J+W Scientific Folsom, CA, USA) with helium as carrier gas (flow rate of 1.4 ml min^{-1}) at a temperature gradient from 35 °C (2 min hold) to 275 °C (3.5 min hold with a ramp of 10 °C/min). Mass spectra were obtained by using the scan modus (total ion count, 40–280 m/z). Compound identity was confirmed by (1) comparison of mass spectra and retention times with those of available standards, and (2) by comparison of the obtained spectra with spectra in the library of the National Institute of Standards and Technology (NIST 147).

Crude protein extracts from petals

Petals were harvested and placed in an ice-cold mortar. Samples of 0.2 g of the petals were extracted with 1 ml buffer containing 0.1 M sodium phosphate, 0.25 mM saccharose, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 25 mM $Na_2S_20_5$, 2 mM DTT, 5 mM ascorbate, 2 μl mercaptoethanol, 0.1 g

PVPP (poly vinyl polypyrolidone) and protease inhibitor cocktail tablets (Roche Mannheim, Germany). The crude extracts were prepared as described by Fähnrich et al. (2011), (2012).

RNA extractions

RNA of the different *Nicotiana* species was isolated according to Chang et al. (1993) and Fähnrich et al. (2011, 2012).

Isolation of the cineole synthase genes and phylogenetic tree construction

A homology-based RT-PCR strategy was used to clone the respective gene. Oligonucleotides of recently described cineole synthases and terpineol synthases of the section *Alatae* were deduced (Fähnrich et al. 2011, 2012). The RT reaction was performed with the ThermoScript™ Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to manufacturer`s recommendation and as described by Fähnrich et al. (2011, 2012). To amplify the CIN of *N. noctiflora* the primer combination CINS6 ($RR(X)_8W$ motif, (5′AGA CGT TCG GGG AAA A3′) and R2 (5′GAC TGG TCA ATC AGT TAC 3′). The PCR reactions were performed at standard conditions (98 °C 1 min (1x), 98 °C 30 s, 54 °C 40 s, 68 °C 1 min/kbp (30x) and 10 min at 72 °C). For sequencing, we ligated the CIN gene into the vector pJet1.2 blunt (Fermentas, Hilden, Germany) and subsequently transformed the plasmid into *E. coli* TOP 10 cells (Invitrogen, Karlsruhe, Germany). The genes were sequenced using the ABI 3730xl sequencer (Roche/454 GS FLX) by GATC Biotech AG, Konstanz, Germany). Homologous monoterpene synthases were found using BLAST search tool at NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) (Altschul et al. 1990). The complete sequences were aligned with the ClustalW programm at EMBL (http://www.ebi.ac.uk/Tools/clustalw2/index.html). Phylogenetic analysis was performed by BioEdit v.7.0.5. and the phylogenetic tree was constructed by using the neighbour joining algorithm and MEGA Software v 4.0 (Tamura et al. 2007). Plant species and accession numbers were used for the tree construction: *Arabidopsis thaliana* CIN AY691947, *Citrus unshiu* CIN BAD91045, *Magnolia grandiflora* TER ACC66282, *Nicotiana alata* TER JQ346173 *Nicotiana langsdorfii* TER JN989317, *Nicotiana suaveolens* CIN EF175166, *Nicotiana bonariensis* CIN JX028207, *Nicotiana forgetiana* CIN JX028206, *Nicotiana longiflora* CIN JX 040448, *Nicotiana mutabilis* JX040449 *Rosmarinus officinalis* CIN DQ839411, *Salvia fruticosa* CIN ABH07677, *Salvia officinalis* CIN AAC26016, *Santalum album* TER ACF 24767, *Solanum lycopersicum* MTS2 AY840092, *Solanum lycopersicum* MTS1 AY840091, *Vitis vinifera* TER AAS79351, *Zea mays* TER AAL59230, *Abies grandis* bisabolene synthase AF006194. The newly isolated gene sequence of *N. noctiflora* was submitted to the NCBI database and has the Accession Number KF958292.

Heterologous protein expression

The protein was overexpressed by using the Expression Champion™ pET SUMO Protein kit (Invitrogen, Karlsruhe, Germany). The forward primer Sumo (5′AGA CGT TCG GGG AAT TAC CAA CCT3′) and a reverse primer Sumo (5′TCA GGC TGG AGG AAT AGA TTC AAA GAC3′) without stop codon were applied to amplify a truncated CIN. The RT-PCR reactions were performed according the standard protocols (Qiagen, Hilden, Germany) and than ligated into the Champion™ pET SUMO vector. *E.coli* HMS 174 (DE3) (Novagene, Darmstadt, Germany) was transformed. The bacteria were cultivated in 5 ml LB medium supplemented with 50 µg/ml kanamycin overnight at 37 °C. 1 ml of an overnight preculture was inoculated into 50 ml LB medium containing 50 µg/ml kanamycin and 1 % glucose and the culture was grown at 37 °C to an OD600 of 0.6. For functional expression, the cultures were induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside (final concentration) and growth continued for an additional 48 h at 13 °C in a rotary shaker. The cells were then harvested by centrifugation at 4 °C for 30 min (8,000*g*) and resuspended in 2 ml lysis buffer (50 mM Tris–HCl, pH 7.5, 10 % glycerol, 10 mM β-mercaptoethanol). The cells were frozen in liquid nitrogen and immediately thawed at 42 °C. The freeze–thaw cycle was repeated three times and followed by incubation with 1 mg/ml lysozyme for 1 h on ice. After centrifugation at 4 °C for 30 min (8,000*g*), the resulting supernatant was used for enzyme assays.

Enzyme assay and headspace volatile collection

The enriched supernatant was used for the enzyme assay. The overexpressed putative synthase was incubated with 200 µl assay buffer according to Fähnrich et al. (2011, 2012), at a temperature of 32 °C for 3 h.

Crude protein extracts (100 µl) were incubated with the enzyme assay buffer, 7 µM GPP and 5 mM DTT. The assay samples were overlaid with 200 µl hexane and incubated for 3 h at 32 °C. To quantify the products of the TPS, 1 µl internal standard was applied (cis-nerolidol 5 ng/µl). The products were extracted by vortexing 2 min and followed by a centrifugation for 2 min at 2,000*g*. Aliquots of the hexane phase were analyzed by GC/MS. Negative control experiments were performed routinely some examples are presented in Fig. S3.

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