

# Synthesis of ‘cineole cassette’ monoterpenes in *Nicotiana* section *Alatae*: gene isolation, expression, functional characterization and phylogenetic analysis

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**Abstract** The scent bouquets of flowers of *Nicotiana* species, particularly those of section *Alatae*, are rich in monoterpenes, including 1,8-cineole, limonene,  $\beta$ -myrcene,  $\alpha$ - and  $\beta$ -pinene, sabinene, and  $\alpha$ -terpineol. New terpene synthase genes were isolated from flowers of *Nicotiana bonariensis*, *N. forgetiana*, *N. longiflora*, and *N. mutabilis*. The recombinant enzymes synthesize simultaneously the characteristic ‘cineole cassette’ monoterpenes with 1,8-cineole as the dominant volatile product. Interestingly, amino acid sequence comparison and phylogenetic tree construction clustered the newly isolated cineole synthases (CIN) of section *Alatae* together with the catalytically similar CIN of *N. suaveolens* of section *Suaveolentes*, thus suggesting a common ancestor. These CIN genes of *N. bonariensis*, *N. forgetiana*, *N. longiflora*, and *N. mutabilis* are distinct from the terpeneol synthases (TERs) of the taxonomically related *N. alata* and *N. langsdorfii* (both *Alatae*), thus indicating gene diversification of monoterpene synthases in section *Alatae*. Furthermore, the presence of CINs in species of the American section *Alatae* supports the hypothesis that one parent of the Australian section *Suaveolentes* was a member of the present section *Alatae*. Amino acid sequences of the *Nicotiana* CINs and TERs were compared to identify relevant amino acids of the cyclization reaction from  $\alpha$ -terpineol to 1,8-cineole.

**Keywords** *Nicotiana* · *Alatae* · *N. bonariensis* · *N. forgetiana* · *N. longiflora* · *N. mutabilis* · Monoterpene synthase · ‘Cineole cassette’ · 1,8-Cineole ·  $\alpha$ -Terpineol

## Abbreviations

TPS Terpene synthase  
CIN Cineole synthase  
TER Terpeneol synthase

## Introduction

The genus *Nicotiana* belongs to the *Solanaceae* and comprises about 75 species classified in 3 subgenera and 13 sections (Goodspeed 1954; Knapp et al. 2004; Chase et al. 2003; Clarkson et al. 2004). About 75 % of the *Nicotiana* species occur naturally in America, and 25 % in Australia, while only one species has so far been found in Africa (Aoki and Ito 2000; Merxmüller and Buttler 1975). The species of section *Alatae* originated in the Americas, comprising to date 8 species (*N. alata*, *N. bonariensis*, *N. langsdorfii*, *N. rastroensis*, *N. longiflora*, *N. plumbaginifolia*, *N. forgetiana*) (Lim et al. 2006; Knapp et al. 2004) and the recently discovered *Nicotiana mutabilis* in southern Brazil (Stehmann et al. 2002). The flowers of the *Alatae* species vary in shape and color, e.g. *Nicotiana longiflora* and *N. bonariensis* bear white flowers, whereas *N. forgetiana* petals are red and those of *N. mutabilis* change from white to pink during flower aging (Goodspeed 1954; Macnish et al. 2010). The floral scent compositions are also quite variable in *Nicotiana* species of section *Alatae* (Table 1), despite the conserved and characteristic

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**Table 1** Emission of monoterpenes from *Nicotiana* species of section *Alatae*

Scent compounds	<i>N. alata</i>		<i>N. langsdorfii</i>		<i>N. longiflora</i>	<i>N. mutabilis</i>		<i>N. bonariensis</i>		<i>N. forgetiana</i>	
1,8-cineole	+++	+++	+++	+++	+	+++	+++	+++	+++	+++	+++
$\alpha$ -terpineol	++	+++	+–	+	–	+–	++	–	+++	+	+
$\alpha$ -pinene	+–	nd	+	nd	+	++	nd	+	nd	+	+–
$\beta$ -pinene	+	nd	++	nd	+	+	nd	+	nd	+	nd
sabinene	+	+	+++	+	+	+++	nd	+++	++	++	+
$\beta$ -myrcene	+	+	+++	++	+	++	++	++	++	++	++
limonene	+	+	+++	++	+	+++	++	++	+	+++	+
( <i>E</i> )- $\beta$ -ocimene	+–	nd	+–	nd	+	+–	+++	+	nd	+–	++
linalool	+++	+++	+	nd	+	+++	nd	+–	nd	–	nd

Red: ‘cineole cassette’ monoterpenes of species of section *Alatae*

Relative emission levels based on Raguso et al. (2003, 2006), and own data (blue). *ND* not detectable; +–: detectable in small amounts and traces <1 %; +: 1–5 %; ++: 5–10 %; +++: >10 %

emission of a set of seven monoterpenes, the so-called ‘cineole cassette’ monoterpenes (Fig. 1; Table 1, 1,8-cineole, limonene,  $\beta$ -myrcene,  $\alpha$ - and  $\beta$ -pinene, sabinene,  $\alpha$ -terpineol) (Raguso et al. 2003, 2006).

Terpenoids are the largest group of natural products, and at least 40,000 compounds are known to date (Bohlmann and Keeling 2008). This wealth of terpenes is synthesized by terpene synthases (TPS), which catalyze the reaction of acyclic prenyl diphosphates into various acyclic and cyclic compounds. Depending on the substrate, the terpene synthases are classified as monoterpene synthases (substrate geranyl pyrophosphate), sesquiterpene synthases (substrate farnesyl pyrophosphate), diterpene synthases (substrate geranyl geranyl pyrophosphate) and are subdivided into seven subfamilies, TPS A to H (Chen et al. 2011).

Up to now, ninety monoterpene synthases have been isolated. The enzymes were found in floral and vegetative tissues of angiosperms (TPS B) and gymnosperms (TPS D) (Degenhardt et al. 2009). They produce the wide variety of terpenoids (summarized in Fährnich et al. 2011). Some monoterpene synthases synthesize almost exclusively their name-giving compound, e.g. geraniol synthases (GES), linalool synthases (LIS), and limonene synthases (LIM), while the majority of the isolated monoterpene synthases are multiproduct enzymes. Typical multiproduct enzymes are cineole synthases (CIN), terpinene synthases (TS), terpinolene synthases (TES), bornyl diphosphate synthases (BOR), carene synthases (CAR), some myrcene synthases (MYR) and terpineol synthases (TER), which produce cyclic and acyclic compounds (Fig. 1). Several compounds, such as limonene,  $\beta$ -myrcene, sabinene,  $\alpha/\beta$ -pinene are synthesized by several different multiproduct enzymes, while volatiles such as  $\alpha$ -terpineol and 1,8-cineole were only synthesized by one type of monoterpene synthases (CIN or TER enzymes) (Fährnich et al. 2011).

The ecological or biological relevance and the reason why some monoterpenes (e.g. limonene) may result from more than one enzymatic source while others terpenes are

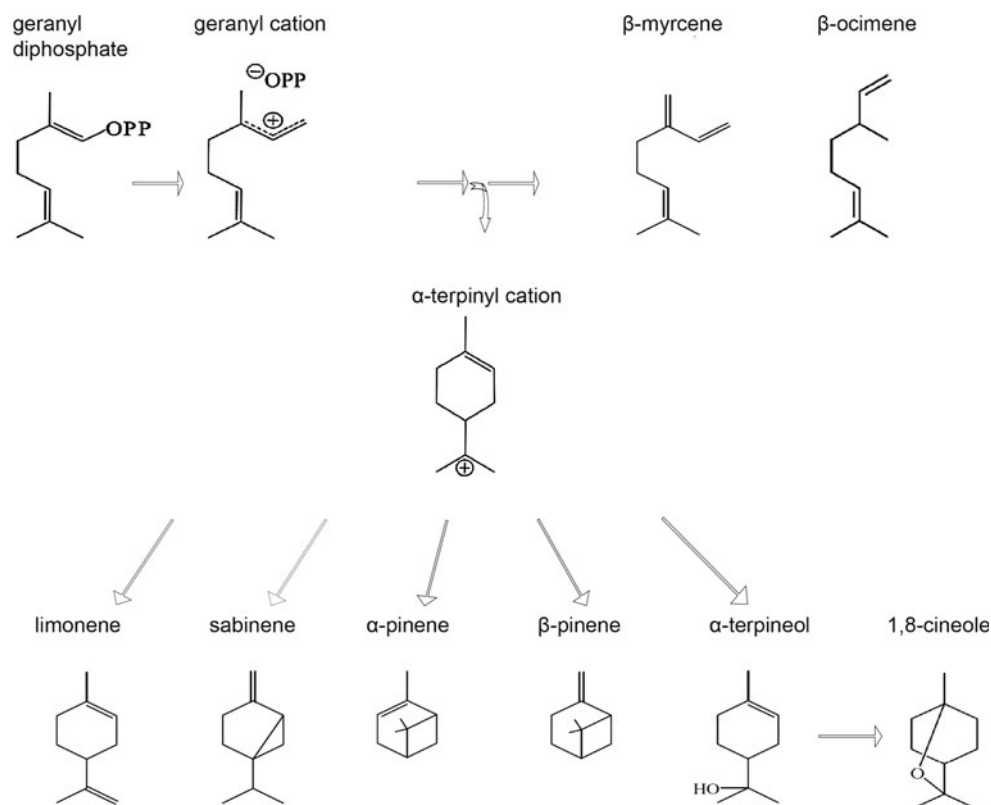
synthesized by just one enzyme are presently not known. This issue could be addressed after isolation and detailed studies of respective monoterpene synthase enzymes.

The characteristic ‘cineole cassette’ monoterpene emission in *Nicotiana* species of section *Alatae* was originally noticed by Raguso et al. (2003, 2006). Since 1,8-cineole was always the major product across the *Alatae* species, it was suggested that the *Nicotiana* CINs are similar to the CIN of *Salvia officinalis* (Kampranis et al. 2007). However, we observed that floral ‘cineole cassette’ emission profiles of *Nicotiana* species (Table 1) or the 1,8-cineole/ $\alpha$ -terpineol product ratios of in vitro assays are not identical (Fährnich et al. 2011), suggesting that the synthesis capacities of respective enzymes are distinct and cannot be unified. Additionally, it is presently not known which molecular features of the enzymes determine the 1,8-cineole/ $\alpha$ -terpineol ratio, which is an indicator of the efficacy of the cyclization reaction (Fig. 1). This demands clarifying studies of respective genes and enzymes.

Another interesting observation should be added to the list of open questions. A cineole synthase with 1,8-cineole as major product was previously isolated from *N. suaveolens* (Roeder et al. 2007) as well as two enzymes that produce  $\alpha$ -terpineol as major component within the ‘cineole cassette’ monoterpenes (*N. alata* and *N. langsdorfii*, Fährnich et al. 2011). It is interesting to note that species of the Australian clade *Suaveolentes* typically do not emit ‘cineole cassette’ monoterpenes. Therefore, the emission of the ‘cineole cassette’ monoterpenes from *N. suaveolens* flowers indicates an evolutionary interesting exception and suggests the existence of a common ancestral gene prior to gene duplication and diversification.

Considering the mentioned aspects, further studies have to be undertaken to fully understand the production, catalytic reaction, and evolution of the ‘cineole cassette’ monoterpene synthases from *Nicotiana* flowers. As a first step in reaching these goals we present here the isolation and functional characterization of monoterpene synthases

**Fig. 1** Chemical structures of monoterpenes. Schematic overview of the biosynthesis of acyclic and cyclic ‘cineole cassette’ monoterpenes and ocimene. The substrate geranyl pyrophosphate is converted into an acyclic cation and further to the cyclic precursor  $\alpha$ -terpinyl cation. 1,8-cineole is synthesized via  $\alpha$ -terpineol



from *N. bonariensis*, *N. forgetiana*, *N. longiflora*, and *N. mutabilis*.

## Materials and methods

### Plant growth

*Nicotiana bonariensis* (TW28) (Raguso et al. 2003), *Nicotiana forgetiana* (TW 50, Raguso et al. 2003), *Nicotiana longiflora* (TW78, Raguso et al. 2003), *Nicotiana mutabilis* (collected by Rainee Kaczorowski in Quebra Cabo, acc. RK200813120; it is not ‘Rastroensis’) were grown on Vermiculite (Deutsche Vermiculite Dämmstoffe GmbH, Sprockhövel, Germany) in growth chambers under long day conditions (illumination: 16 h, 6 am–10 pm,  $160 \mu\text{Em}^{-2} \text{s}^{-1}$ , mercury vapor lamp (Osram, München, Germany) 22 °C, 8 h darkness: 8 h, 10 pm–6 am, 21 °C). Plants were watered with Hoagland’s solution (Hoagland and Arnon 1938).

Volatile collection of flowers of *N. bonariensis*, *N. forgetiana*, *N. mutabilis*

The collection of volatiles from whole flowers was performed using the open loop system (Heath and Manukian 1994). One flower was kept in each glass globe, and

headspace volatiles of four globes were investigated individually for each species. Collection began on the day of anthesis of flowers, when they were placed into glass globes. A compressor (Schneider Werkstatt- und Maschinenfabrik, Bräunlingen, Germany) delivered a constant air flow of  $5 \text{ l min}^{-1}$ , which was divided between the four glass globes. Headspace volatiles of flowers of *N. bonariensis* were collected between 6 and 8 pm, and of *N. forgetiana* and *N. mutabilis* between 10 and 12 am. The volatile-enriched air was sucked through a 100 mg SuperQ column (Alltech Associates, Deerfield, IL, USA) using a vacuum pump with  $2.8 \text{ l min}^{-1}$  (KNF Neuberger, Freiburg, Germany) (Effmert et al. 2008). For quantification, nonyl acetate or cis-nerolidol ( $5 \text{ ng } \mu\text{l}^{-1}$ ) was used as internal standard and volatiles were eluted in fractions of 200 and 100  $\mu\text{L}$  dichloromethane and analyzed by GC/MS (Effmert et al. 2008).

### GC/MS analysis

The volatile compounds were analyzed with a Shimadzu QP5000 gas chromatograph connected to a mass spectrometer for identification (GC/MS). Separation was performed on a DB5-MS column ( $60 \text{ m} \times 0.25 \text{ mm} \times 0.25 \text{ mm}$ ; J+W Scientific Folsom, CA, USA) with helium as carrier gas (flow rate of  $1.4 \text{ ml min}^{-1}$ ) at a temperature gradient from 35 °C (2 min hold) to 275 °C (3.5 min hold)

using a ramp of  $10\text{ }^{\circ}\text{C min}^{-1}$ . Mass spectra were obtained by using the scan mode (total ion count,  $40\text{--}280\text{ m/z}^{-1}$ ). Compound identity was confirmed by (1) comparison of mass spectra and retention times with those of available authentic standards (terpenoid standards from Sigma-Aldrich, St. Louis, MS, USA; nonyl acetate from Roth, Karlsruhe, Germany), (2) comparison of obtained spectra with spectra in the library of the National Institute of Standards and Technology (NIST147), and (3) by comparison of the Kovats indices.

#### Crude protein extracts from petals

Petals were harvested every 3 h (starting at 9 am) 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  $\text{MgCl}_2$ , 1 mM  $\text{CaCl}_2$ , 25 mM  $\text{Na}_2\text{S}_2\text{O}_5$ , 2 mM DTT, 5 mM ascorbate, 2  $\mu\text{l}$  2-mercaptoethanol, 0.1 g PVPP (polyvinyl polypyrrolidone) and protease inhibitor cocktail tablets (Roche, Mannheim, Germany). The crude extracts were centrifuged for 30 min at  $13,000\text{ g}$  ( $4\text{ }^{\circ}\text{C}$ ). The supernatant was collected. After addition of 10 % glycerol, the crude extract was stored at  $-70\text{ }^{\circ}\text{C}$  until used for Western blots. Extracts for enzyme activity determination were always freshly prepared.

#### RNA extraction

RNA from *Nicotiana bonariensis* (TW28), *Nicotiana forgetiana* (TW50), *Nicotiana longiflora* (TW78), *Nicotiana mutabilis* (acc. RK2008131201) plants were isolated according to Chang et al. (1993). In brief, 0.5–1 g petals were ground in liquid nitrogen and incubated for 15 min with 400  $\mu\text{l}$  2-mercaptoethanol and 15 ml CTAB buffer (2 % CTAB (hexadecyltrimethyl ammonium bromide), 2 % PVP (polyvinylpyrrolidone K40), 100 mM Tris-HCl, 25 mM EDTA, 2 M NaCl add  $\text{H}_2\text{O}$  to 500 ml). Samples were extracted with 15 ml of chloroform: isoamyl alcohol (24:1) added twice and centrifuged for 20 min ( $10,000\text{ g}$ ). The samples were resuspended in 0.25 M LiCl buffer to allow precipitation overnight at  $8\text{ }^{\circ}\text{C}$ . RNA was centrifuged for 30 min at  $4\text{ }^{\circ}\text{C}$  and the pellet was resuspended in 700  $\mu\text{l}$  SSE buffer (1 M NaCl, 0.5 % SDS, 10 mM Tris-HCl, 1 mM EDTA) and again precipitated with ethanol (99.8 %) for 1 h at  $-70\text{ }^{\circ}\text{C}$ . The RNA pellet was gently washed twice with ethanol (70 %).

#### Isolation of monoterpene synthase genes

A homology-based RT-PCR strategy was used to clone genes of interest. Oligonucleotides of recently described cineole synthases were deduced (Fährnich et al. 2011). The RT reaction was performed with the SuperScript III<sup>TM</sup>

Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to manufacturer's recommendation. For cDNA synthesis, 2  $\mu\text{g}$  RNA, 25 pmol oligo dT (GAC TGG TCA ATC AGT TAC (T)<sub>16</sub>) primer, 10 mM dNTPs, 0.1 M DTT, 5  $\mu\text{l}$  5 $\times$  cDNA synthesis buffer were incubated at  $58\text{ }^{\circ}\text{C}$  for 1 h. The reaction was terminated by heating at  $85\text{ }^{\circ}\text{C}$  for 5 min. Subsequently the PCR reaction was performed by using the following components: 1  $\mu\text{g}$  first strand cDNA, 5  $\mu\text{l}$  10 $\times$  *Pfu* buffer, 1.25 units *Pfu* DNA polymerase (Invitrogen, Karlsruhe Germany), 25 pmol of each anti-sense/sense primer and sterile  $\text{H}_2\text{O}$  was added to a final volume of 50  $\mu\text{l}$ . To amplify the monoterpene synthase of *N. bonariensis*, *N. forgetiana*, *N. longiflora* and *N. mutabilis*, the primer combination CINS6 (5'AGA CGT TCG GGG AAT TAC CAA CCT 3'), which binds to the RR(X)<sub>8</sub>W motif, and R2 (5'GAC TGG TCA ATC AGT TAC3'), which binds to the adapter sequence of oligo dT was used. The PCR reactions were performed at standard conditions:  $98\text{ }^{\circ}\text{C}$  2 min ( $1\times$ ),  $98\text{ }^{\circ}\text{C}$  30 s,  $54\text{ }^{\circ}\text{C}$  30 s,  $68\text{ }^{\circ}\text{C}$  1 min  $\text{kb}^{-1}$  ( $30\times$ ) and 10 min  $72\text{ }^{\circ}\text{C}$ . Products were separated on an 1.2 % agarose gel and extracted with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). For sequencing, the full-length monoterpene synthase cDNAs were ligated into the cloning vector pJET1.2/blunt and subsequently the plasmid was transformed into *E. coli* TOP 10 cells (Invitrogen, Karlsruhe, Germany). After incubation overnight on Luria-Bertani (LB) agar containing  $100\text{ }\mu\text{g ml}^{-1}$  ampicillin, the transformants were analyzed by colony PCR using the vector primers T7 Promotor (5'TAA TAC GAC TCA CTA TAG GG3') and pJET1.2 reverse (5'AAG AAC ATC GAT TTT CCA TGG CAG3') by using Master mix (Qiagen, Hilden Germany) at standard conditions.

#### Gene expression analysis

For temporal expression analysis RT-PCR was applied using *N. forgetiana* and *N. bonariensis*. Total RNA was isolated every 3 h according to Chang et al. (1993). 2  $\mu\text{g}$  of DNase I (Sigma-Aldrich, St. Louis, MS, USA) digested RNA was used for cDNA synthesis performed with the SuperScript III<sup>TM</sup> Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to manufacturer's recommendation. Subsequently PCR reactions were performed by using the following components: 1  $\mu\text{g}$  first strand cDNA, 25 pmol of each antisense/sense primer, 14  $\mu\text{l}$  Master mix (Fermentas, Thermo Fisher Scientific Inc) and sterile  $\text{H}_2\text{O}$  was added to a final volume of 50  $\mu\text{l}$ . To amplify a CIN gene fragment the primer combination CINS2 and R2 was used and a 1.1 kbp fragment was obtained. The PCR reactions were performed at standard conditions:  $98\text{ }^{\circ}\text{C}$  2 min ( $1\times$ ),  $98\text{ }^{\circ}\text{C}$  30 s,  $50\text{ }^{\circ}\text{C}$  30 s,  $68\text{ }^{\circ}\text{C}$  1 min  $\text{kb}^{-1}$  (20/30/40 $\times$  cycles) and 10 min  $72\text{ }^{\circ}\text{C}$ . For quantification the CIN transcript was adjusted to the control (elongation

factor  $1\alpha = EF - 1\alpha$ , Dean et al. 2002). A 370 bp fragment of this housekeeping gene was obtained by PCR reaction using 1 µg cDNA, 14 µl Master mix (Fermentas, Thermo Fisher Scientific Inc) and 25 pmol of primer EF1 fow and EF1 rev. The PCR reactions were performed at standard conditions: 94 °C 90 s (1×), 94 °C 30 s, 50 °C 30 s, 72 °C 1 min  $kb^{-1}$  (20/30/40× cycles) and 10 min 72 °C. The amplifications were analyzed on an 1.2 % agarose gel. Fragments of the CIN gene of *N. forgetiana* and *N. bonariensis* were extracted with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced to verify the correct sequence.

#### 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 monoterpene synthase. A his-tag was added to the N-terminus. To avoid formation of inclusion bodies in *E. coli* the truncated monoterpene synthases without target sequence were amplified (Bohlmann et al. 1998). RT-PCR reactions were performed with 1 µg first strand cDNA, 10 mM dNTPs, 25 pmol primer (each), 5 µl 10× *Pfu* buffer, 1.25 units *Pfu* DNA polymerase (Invitrogen, Karlsruhe, Germany) and sterile water added to a final volume of 50 µl. PCR products were purified according the manufacturer's recommendation (Qiagen, Hilden, Germany), ligated into the Champion™ pET SUMO vector (Invitrogen, Karlsruhe, Germany), and sequenced to assure the correctness of the sequence. The plasmid was transformed into *E. coli* HMS 174 (DE3) (Novagene, Darmstadt, Germany) which were then cultivated overnight at 37 °C in 5 ml LB medium supplemented with 50 µg  $ml^{-1}$  kanamycin. 1 ml of an overnight preculture was transferred into 50 ml LB medium containing 50 µg  $ml^{-1}$  kanamycin and 1 % glucose and growth was continued at 37 °C until an  $OD_{600}$  of 0.6 was reached. For functional expression the cultures were induced with 1 mM isopropyl- $\beta$ -D-thiogalactopyranoside (final concentration) and growth continued for 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  $\beta$ -mercaptoethanol). The cells were frozen in liquid nitrogen and immediately thawed at 42 °C. Freeze-thaw cycles were repeated three times and followed by incubation with 1 mg  $ml^{-1}$  lysozyme for 1 h on ice. After centrifugation at 4 °C for 30 min (8,000 g), the resulting supernatant was either transferred to a Ni-NTA column or directly used in an enzyme assay.

Overexpressed protein was purified by Ni-NTA affinity chromatography according to the manufacturer's recommendations (Qiagen, Hilden, Germany). In brief, the supernatant was incubated for 1 h with the column matrix and then washed with 50 mM  $NaH_2PO_4$ , 300 mM NaCl and 40–60 mM imidazole. The protein was eluted with 500 µl elution buffer (50 mM  $NaH_2PO_4$ , 300 mM NaCl, 80–200 mM imidazole). The protein concentration was analyzed using Bradford reagent (Bradford et al. 1976).

#### SDS-polyacrylamide gel electrophoresis and Western blot analysis

Samples (15 µg) of the crude protein extract or the purified recombinant enzyme (5 µg) were loaded onto polyacrylamide gels (12.5 %), electrophoretically separated (Mini-protean, Bio-Rad Laboratories, Hercules, CA, USA), and finally transferred to PVDF (polyvinylidene difluoride) membrane (Roth, Karlsruhe, Germany) by using a mini-tank blotting gel cassette (XCell II, Novex, San Diego, CA, USA). The membranes were placed in a Tris-buffered blocking solution overnight (TBS 20 mM) with 0.05 % ( $vv^{-1}$ ) Tween 20, 4 % ( $wv^{-1}$ ) skim milk, and 1 % ( $wv^{-1}$ ) BSA. After incubation with the specific antibody against the CIN from *N. suaveolens* (Roeder et al. 2007) (diluted 1:3,000 in blocking solution, Davids Biotechnology, Regensburg, Germany) for 1.5 h, repeated washes with TBS containing 0.05 % ( $vv^{-1}$ ) Tween 20 (TBS-Tween) were performed. The membrane was incubated with the secondary antibody (anti-rabbit alkaline phosphatase-conjugate, diluted 1:20,000 in TBS-Tween (Sigma-Aldrich, St. Louis, MS, USA) and washed again with TBS-Tween and TBS. The membrane was equilibrated in detection buffer (100 mM Tris-HCl, 150 mM NaCl, 50 mM  $MgCl_2$ ) and incubated with CDP-Star (Roche, Mannheim, Germany; 0.25 µM in detection buffer) in darkness and analyzed in a luminescence image analyser (LAS-1000, Fujifilm, Japan). Its luminescence after 30 min was quantified with Fujifilm Image Gauge software. After measuring the luminescence, the proteins were stained with NBT/BCIP (50 mg  $ml^{-1}$  in dimethylformamide, 2:1; Roche, Mannheim, Germany).

#### Enzyme assay

The Ni-NTA affinity chromatography purified enzyme (2 µg) or the purified supernatant was used for the enzyme assay, which was performed as described by Fähnrich et al. 2011. The assay buffer consisted of 250 mM Hepes/KOH buffer (pH 8) containing 10 % glycerol, 100 mM  $MgCl_2$  and 0.25 mM  $MnCl_2$ . The putative terpene synthase was incubated with an assay buffer, 147 µM GPP (Echelon Biosciences) and 5 mM DTT (final volume of 200 µl). Crude protein extracts (100 µl) were incubated with the



enzyme assay buffer, 7  $\mu\text{M}$  GPP and 5 mM DTT. The assay samples were overlaid with 200  $\mu\text{l}$  hexane and incubated for 3 h at 32 °C. To quantify the products of the TPS, 5 ng internal standard were added (5 ng  $\mu\text{l}^{-1}$  cis-nerolidol or nonyl acetate, Roth, Karlsruhe, Germany). The products were extracted by vortexing 2 min followed by a centrifugation for 2 min at 2,000 g. Aliquots of the hexane phase were analyzed by GC/MS.

#### Sequence analysis and phylogenetic tree construction

The genes were sequenced by GATC Biotech AG (Konstanz, Germany). Homologous monoterpene synthases were identified with the BLAST search tool of NCBI (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Altschul et al. 1990). The complete sequences were aligned with the ClustalW program of EMBL (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>). Phylogenetic analysis was performed by BioEdit version 7.0.5. The phylogenetic tree was constructed by using the neighbor joining algorithm, maximum parsimony algorithm and MEGA Software v 4.0 (Tamura et al. 2007). To display the phylogenetic tree, the TreeExplorer 2.12 was used. 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, *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. Newly isolated gene sequences were submitted to the EMBL data databank, accession numbers are: *Nicotiana forgetiana* CIN JX028206, *Nicotiana bonariensis* CIN JX028207, *Nicotiana longiflora* CIN JX040448, *Nicotiana mutabilis* putative CIN JX040449.

#### 3D modeling

To build the homology-based 3D structure of the *N. alata* TER, *N. forgetiana* CIN, *N. longiflora* CIN and *N. bonariensis* CIN, the limonene synthase (LIM) of *Mentha spicata* (Hyatt et al. 2007) was chosen because of the high sequence similarity of 96 % (recommendation  $\geq 30$  %). Using ClustalW or BioEdit version 7.0.5 as alignment tools, the cDNA sequence of *N. alata* TER was adjusted to the *M. spicata* LIM sequence. The program SPDBV3.7. (Swiss Institute of Bioinformatics) was used to perform and visualize the 3D structure. The pdb-file was loaded using the program Pymol (version 0.98, Delano scientific LLC) to get a better 3D view of the protein model.

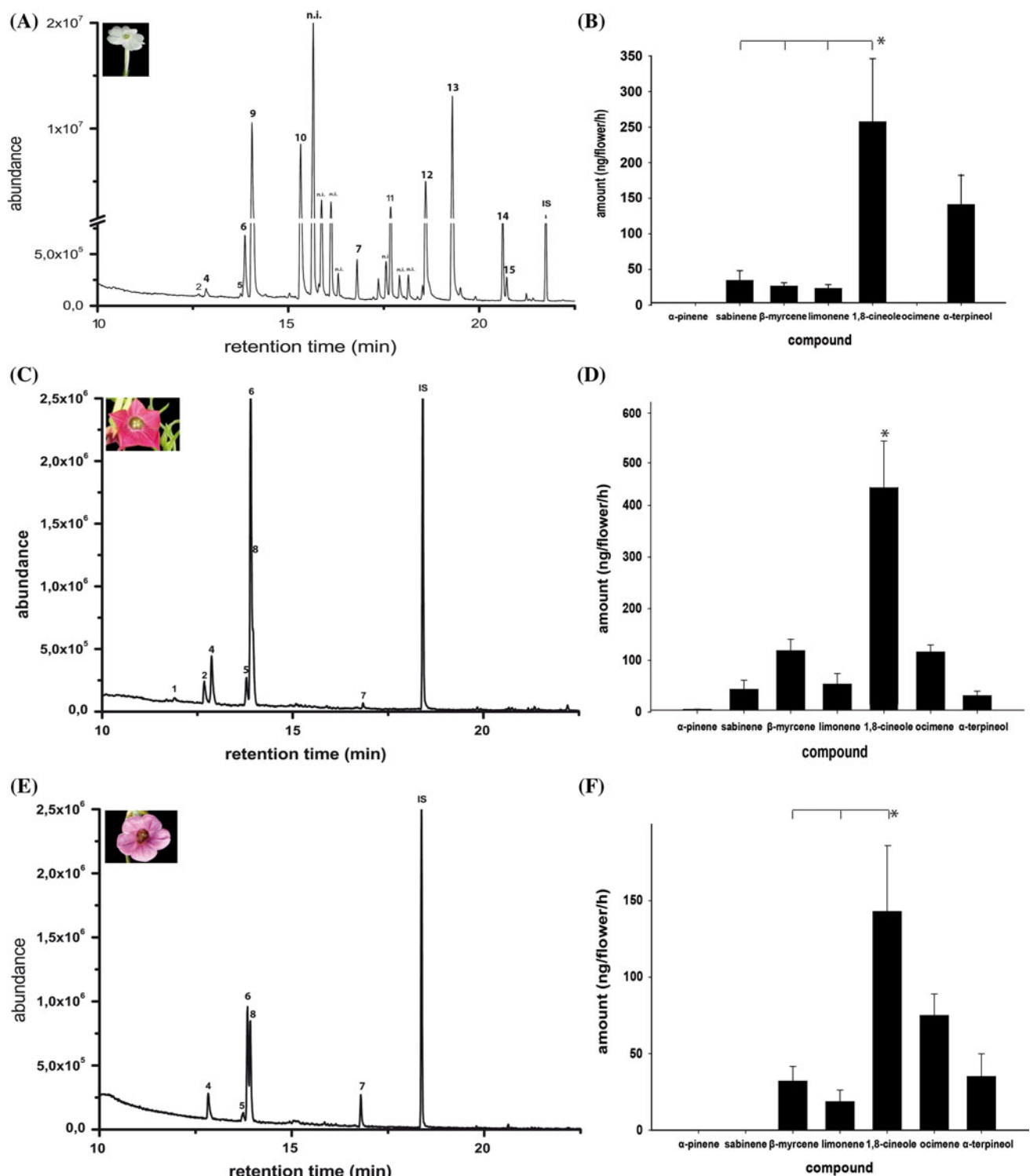
## Results

#### Floral emission of ‘cineole cassette’ monoterpenes of *Nicotiana* species of section *Alatae*

*Nicotiana* species of section *Alatae* release monoterpenes from their flowers (Raguso et al. 2003, 2006; Table 1). Here the emission of floral monoterpenes of *N. bonariensis*, *N. forgetiana*, *N. mutabilis* and *N. longiflora* was examined in detail. A complex volatile emission pattern comprising 12 identified compounds (sabinene,  $\beta$ -myrcene, limonene, 1,8-cineole, phenylacetaldehyde, phenylethanol,  $\alpha$ -terpineol, 2-phenylethylacetate, cinnamylalcohol, eugenol, phenylacetate, isoeugenol) and an unidentified compound was obtained for *N. bonariensis* (Fig. 2a). 1,8-cineole and  $\alpha$ -terpineol were the major compounds of the ‘cineole cassette’ with a 1,8-cineole/ $\alpha$ -terpineol ratio of 2:1 (Fig. 2b). The emission profiles of *N. forgetiana* were composed of seven compounds ( $\alpha$ -pinene, sabinene,  $\beta$ -myrcene, limonene, 1,8-cineole, (*E*)- $\beta$ -ocimene and  $\alpha$ -terpineol) with 1,8-cineole dominating the scent bouquet (Fig. 2c). A high 1,8-cineole/ $\alpha$ -terpineol ratio of 12:1 was determined (Fig. 2d). The scent of *N. mutabilis* was composed of  $\beta$ -myrcene, limonene, 1,8-cineole, (*E*)- $\beta$ -ocimene and  $\alpha$ -terpineol, with a 1,8-cineole/ $\alpha$ -terpineol ratio of 4:1 (Fig. 2e, f). Since the scent emissions of the ‘cineole cassette’ monoterpenes of *N. longiflora* flowers were very low (Raguso et al. 2003), we were unable to measure volatiles in the headspace. According to Raguso’s measurements, limonene is the major compound followed by 1,8-cineole and  $\alpha$ -pinene, whereas  $\alpha$ -terpineol was not detected (Table 1).

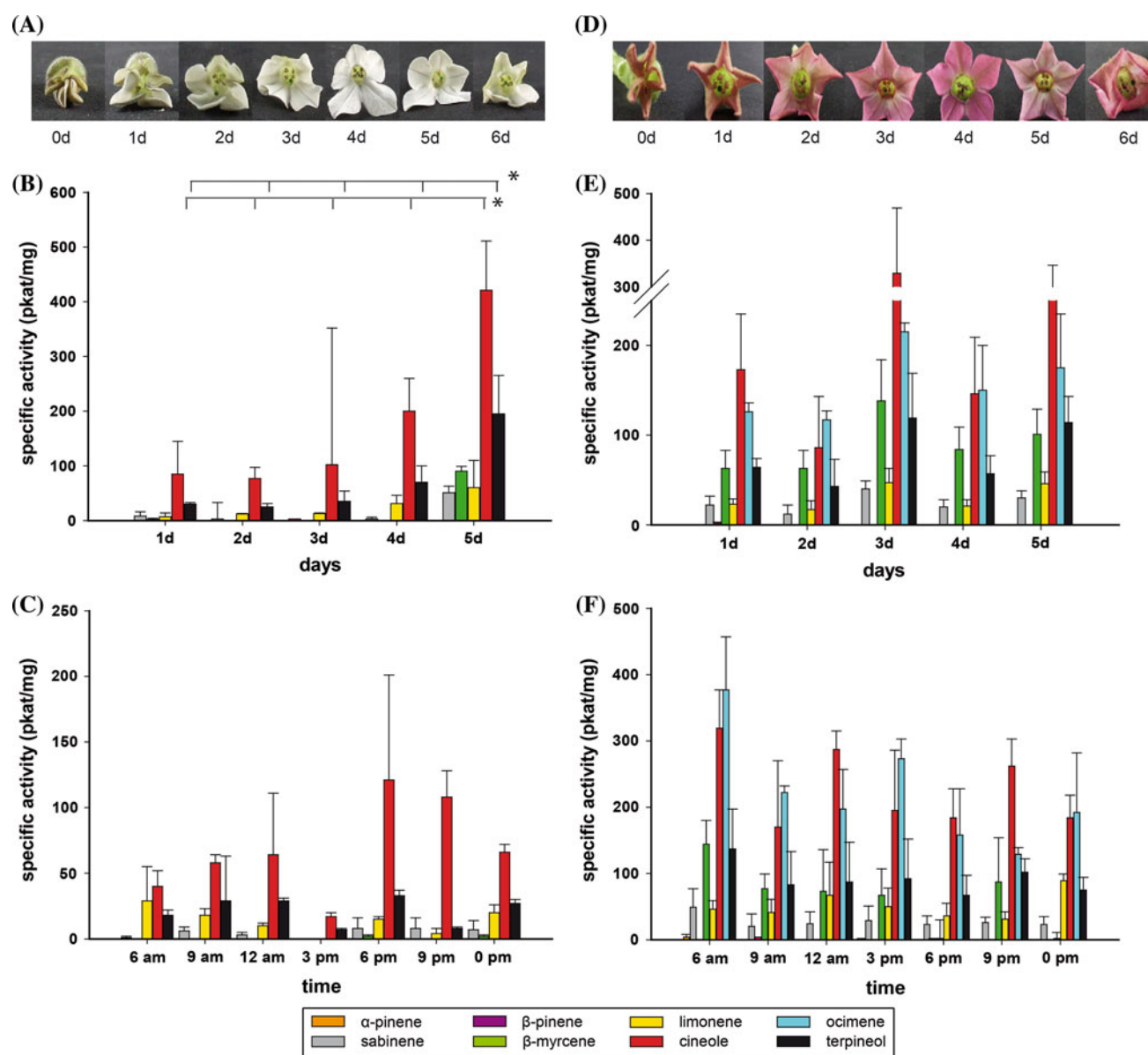
#### Synthesis of monoterpenes in *Nicotiana* flowers

As a next step we determined terpene synthase activities in petal raw extracts. After addition of the substrate GPP to petal protein extracts, five and eight monoterpenes were synthesized by *N. bonariensis* and *N. forgetiana*, respectively (Fig. 3). Almost identical product spectra were obtained when neryl pyrophosphate (NPP) was applied as substrate (Fig. S3). In the white flowers of *N. bonariensis* (Fig. 3a), the enzyme activities increased continuously from day 1 to day 5, reaching a specific activity of 0.42 nkat 1,8-cineole/mg protein at day 5 (Fig. 3b). The same patterns were observed for the other monoterpenes of the ‘cineole cassette’ (maximum activities at day 5 were: 0.2 nkat  $\alpha$ -terpineol/mg protein, 0.09 nkat  $\beta$ -myrcene/mg protein, 0.05 nkat sabinene/mg protein, 0.06 nkat limonene/mg protein; total activity 0.8 nkat/mg protein) (Fig. 3b). The cineole and terpeneol synthase activities were significantly higher at day 5 compared to day 1–4. Enzyme activity levels peaked with 0.33 nkat 1,8-cineole/mg protein at day 3 in the red



**Fig. 2** Floral volatiles emitted from different *Nicotiana* species. **a** Headspace volatiles of flowers of *N. bonariensis* were collected between 6 and 8 pm. **c** Headspace volatiles of flowers of *N. forgetiana* were collected between 10 and 12 am. **e** Headspace volatiles of flowers of *N. mutabilis* were collected between 10 and 12 am. Each glass globe contained one flower, headspace volatiles of 4 flowers were investigated for each species. 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. (1)  $\alpha$ -pinene, (2)-sabinene, (3)  $\beta$ -pinene, (4) myrcene, (5) limonene, (6) cineole, (7) terpineol, (8) E- $\beta$  ocimene, (9) phenylacetaldehyde, (10) phenylethanol, (11) phenylethylacetate, (12) cinnamylalcohol, (13) eugenol, (14) phenylacetate, (15) isoeugenol. (IS) internal standard (5 ng cis-nerolidol or nonyl acetate). **b, d, f** Quantification of emitted monoterpenes from flowers of *N. bonariensis*, *N. forgetiana*, *N. mutabilis*, respectively. The volatiles were collected for 2 h, amounts were calculated in ng per flower and per h,  $n = 3$ . Statistical analyses were performed using ANOVA and *t* test calculated with Sigma Plot (\*significance  $p < 0.05$ )



**Fig. 3** Monoterpene synthase activities during flower development and during the day. The precursor geranyl pyrophosphate (GPP) was provided as substrate to the newly isolated monoterpene synthases. **a, d** The development of the flowers of *Nicotiana bonariensis* and *Nicotiana forgetiana* respectively is depicted. Day of anthesis (day 0), senescence (day 6). **b** Monoterpene synthase activities were determined in petal extracts of *N. bonariensis* flowers throughout flower development (day 1–day 5). The specific enzyme activities (pkat/mg total protein) were calculated for sabinene (gray),  $\beta$ -myrcene (green), limonene (yellow) 1,8-cineole (red) and  $\alpha$ -terpineol (black),  $n = 3$ . **c** Determination of the specific enzyme activities for sabinene (gray),  $\beta$ -myrcene (green), limonene (yellow) 1,8-cineole (red) and  $\alpha$ -terpineol (black) in *N. bonariensis* flower petals at different time points during the day (6 am, 9 am, noon, 3 pm, 6 pm, 9 pm and midnight),  $n = 3$ . Statistical analyses were performed using ANOVA and  $t$  test calculated with Sigma Plot (\*significance:  $p < 0.05$ )

6 pm, 9 pm and midnight),  $n = 3$ . **e** Monoterpene synthase activities were determined in petal extracts of *N. forgetiana* flowers throughout flower development (day 1–day 5). The specific enzyme activities (pkat/mg total protein) were calculated for  $\alpha$ -pinene (orange), sabinene (gray),  $\beta$ -pinene (violet),  $\beta$ -myrcene (green), limonene (yellow) 1,8-cineole (red), (*E*)- $\beta$ -ocimene (cyan), and  $\alpha$ -terpineol (black),  $n = 3$ . **f** Determination of the specific enzyme activities for  $\alpha$ -pinene (orange), sabinene (gray),  $\beta$ -pinene (violet),  $\beta$ -myrcene (green), limonene (yellow) 1,8-cineole (red), (*E*)- $\beta$ -ocimene (cyan), and  $\alpha$ -terpineol (black) in *N. forgetiana* flower petals at different time points during the day (6 am, 9 am, noon, 3 pm, 6 pm, 9 pm and midnight),  $n = 3$ . Statistical analyses were performed using ANOVA and  $t$  test calculated with Sigma Plot (\*significance:  $p < 0.05$ )

flowers of *N. forgetiana* (Fig. 3d, e). The enzyme activities regarding other monoterpene products were lower (0.12 nkat  $\alpha$ -terpineol/mg protein, 0.14 nkat  $\beta$ -myrcene/mg protein, 0.04 nkat sabinene/mg protein, 0.04 nkat

limonene/mg protein, total activity 0.6 nkat/mg protein at day 3).

The activities of the terpene synthases in petal raw extracts not only vary during flower development but also



throughout the day. Exemplified analysis of the pattern of the major product 1,8-cineole revealed higher monoterpene synthase activities at the beginning of the night phase (6 and 9 pm) compared to lower levels during the day in *N. bonariensis* flowers (Fig. 3c), while *N. forgetiana* did not exhibit enzyme activity alterations throughout the day (Fig. 3f). Maximum enzyme activities of 0.12 nkat 1,8-cineole/mg protein were reached in petal extracts of *N. bonariensis* at 6 pm, while the enzyme activities in flowers of *N. forgetiana* leveled around 0.23 nkat 1,8-cineole/mg protein. The specific activity of *N. mutabilis* petals was investigated only once at day 3 post-anthesis at 9 am, and the enzyme activity was highest for 1,8-cineole (0.167 nkat/mg protein) while activities with other monoterpenes were very low (sabinene 0.01 nkat/mg protein,  $\beta$ -myrcene 0.005 pkat/mg protein,  $\beta$ -pinene 0.02 pkat/mg protein, limonene 0.01 pkat/mg protein,  $\alpha$ -terpineol 0.057 pkat/mg protein).

1,8-cineole is synthesized from  $\alpha$ -terpineol by one reaction step (Fig. 1). The 1,8-cineole/ $\alpha$ -terpineol ratio therefore expresses the performance capability of the cyclization reaction. In *N. bonariensis* and *N. forgetiana*, the 1,8-cineole/ $\alpha$ -terpineol ratios varied (only) between 2:1 and 3:1 during flower development and at different time points of the day (Table S1). These constant compound ratios, also supported by correlation coefficient of  $R = 0.9$  (Table S2), were evaluated as an indication of precisely regulated catalysis rather than randomized product formation.

#### Expression of TPS in *N. forgetiana*

The presence of monoterpene synthases in flower extracts of the investigated *Nicotiana* species was further underpinned by determination of the cineole synthase specific mRNAs. The mRNAs were isolated from *N. forgetiana* flowers at different time points during the day and RT-PCR reactions were performed (Fig. S1). The PCR products of the expected size of circa 1200 nucleotides were obtained with specific cineole synthase primers. The mRNA levels were high between 2, 6 and 8 pm in *N. forgetiana* prior to the elevated enzyme activities.

Taken together, these examinations support the presence of cineole monoterpene synthases in petals of these *Nicotiana* species of section *Alatae*.

#### Gene isolation and sequence analysis of 'cineole cassette' monoterpene synthases from *Nicotiana* species of section *Alatae*

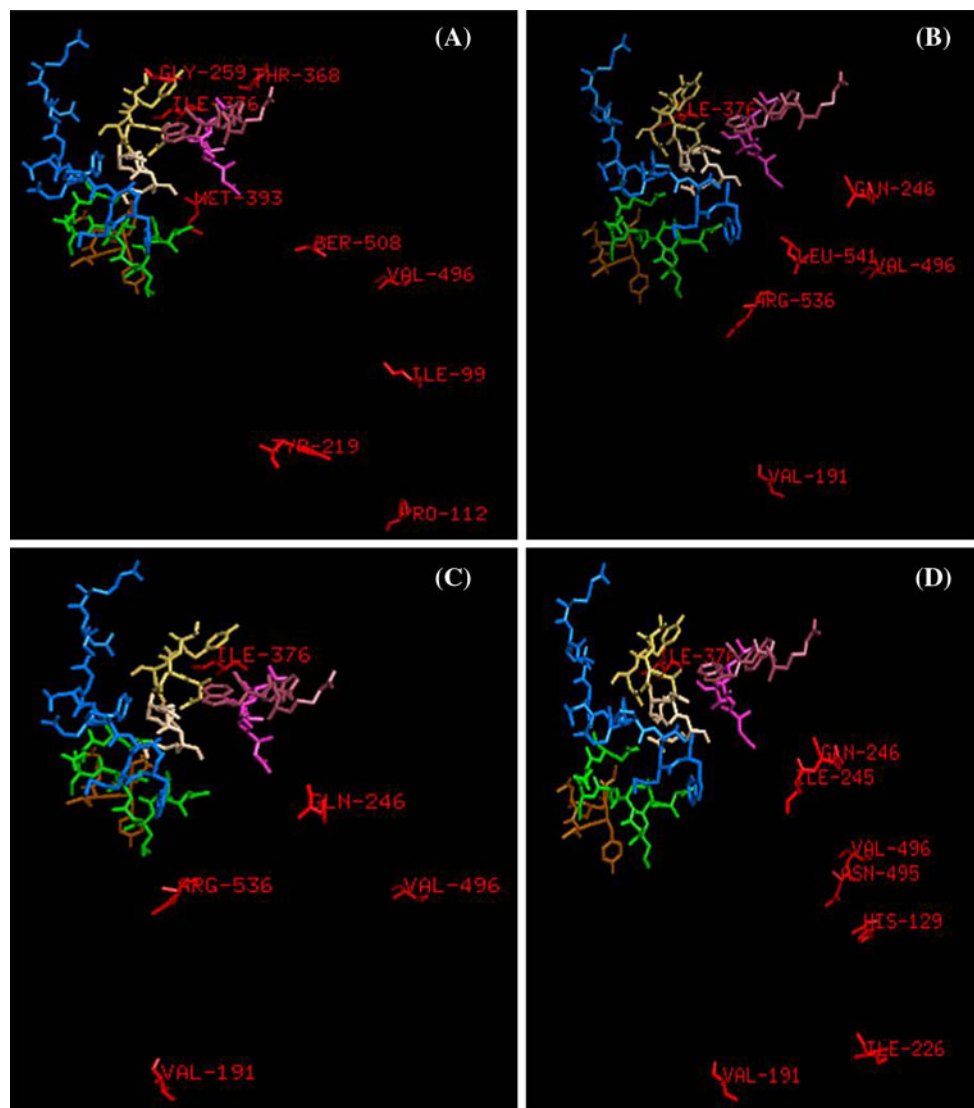
Via RT-PCR we successfully isolated putative terpene synthases from petals of *N. bonariensis*, *N. forgetiana*, *N. longiflora* and *N. mutabilis*. The amino acid sequences

were aligned with the previously isolated terpineol synthases of *N. alata* and *N. langsdorfii* and cineole synthase of *N. suaveolens* (Fig. 4) with which they share high sequence identity (89–99 %, Table S3). The newly cloned coding sequences comprised 1,572 bp and encode mature proteins of 524 amino acids. The N-terminus of all four enzymes contained the typical RR(X)<sub>8</sub>W-motif (1), and other motifs (2–7) characteristic of monoterpene synthases were also present (Fig. 4). The DDXXD-motif is known to be involved in divalent metal ion binding (Starks et al. 1997; Bohlmann et al. 1998), while the NALV sequence directed the formation of different product amounts and probably influences the enzyme specificity of *Salvia* monoterpene synthases (Kampranis et al. 2007). Since monoterpene synthases are functionally active in plastids, they should harbor a transit peptide upstream of the RR(X)<sub>8</sub>W-motif (RR-motif). The transit sequences were not cloned for the four new genes. The RR-motif is involved in binding the substrate GPP and isomerization of the initial carbocation to the linalyl diphosphate intermediate by recapture of the diphosphate (Williams et al. 1998). This motif was conserved in the N-terminal region of all *Nicotiana* monoterpene synthases. The RWW- and CYMNE-motifs were also found in these terpene synthase sequences, but their functions remain unknown (Roeder et al. 2007). Another highly conserved RXR-motif was localized circa 35–40 amino acids upstream of the DDXXD-motif. The function of this motif is related to the complexation of the diphosphate after ionization of the substrate preventing nucleophilic attack on any of the carbocationic intermediates (Starks et al. 1997).

Amino acid alterations within the new and known *Nicotiana* monoterpene synthases are highlighted in gray (Fig. 4). Not a single amino acid alteration was observed in the sequence motifs or within the active pocket (amino acids indicated with black dots in Fig. 4). In total, eleven amino acid positions were altered in one of the four newly isolated genes compared to the TER of *N. alata*. To get a better idea of the location of these altered amino acids, we have shown them in a homology-based protein model constructed on the 3-D crystal structure of the limonene synthase from *Mentha spicata* (Hyatt et al. 2007) (Fig. 5). Four of the eleven altered amino acids of *N. bonariensis* (Fig. 5a; Table S4A) were located close to conserved motifs: gly259 and ile 376 were found near the DDXXD motif, thr368 was near RWW and NALV motifs, met393 was close to the DTE motif. One of the eight altered amino acids of *N. forgetiana* (Fig. 5b; Table S4B) was closely located to conserved motifs DDXXD and RWR. One (ile 376) of the seven altered amino acids of *N. longiflora* (Fig. 5c; Table S4C) was located close to conserved motifs DDXXD and RWW. One (ile 376) of the ten altered amino acids of *N. mutabilis* (Fig. 5d; Table

**Fig. 4** Sequence of *Nicotiana* monoterpene synthases. Alignment of the newly isolated monoterpene synthase genes from *N. bonariensis*, *N. forgetiana*, *N. longiflora*, *N. mutabilis* with the terpineol synthase (TER) sequences of *N. alata*, *N. langsdorfii* and the cineole synthase (CIN) sequence of *N. suaveolens* (BLAST Search, Altschul et al. 1990, alignment with Clustal W). Conserved sequence motifs are indicated: (1) RR(X)<sub>8</sub>W-motif, (2) RWW-motif, (3) RXR-motif, (4) NALV-motif, (5) DDXXD-motif, (6) CYMNE-motif. Indicated in gray: Sequence differences between TER/CIN sequences. Amino acids involved in formation of the active pocket are marked with filled dots: a = arg263, b = trp272, c = asn293, d = asp300-asp304, e = ile395, f = leu436-glu446. Lines above the sequence compilation ( $\alpha 1$ – $\alpha 22$ ) indicate amino acids involved in the formation of helices

<i>N. alata</i>	-----MNT 3
<i>N. langsdorfii</i>	-----
<i>N. bonariensis</i>	-----
<i>N. forgetiana</i>	-----
<i>N. longiflora</i>	-----
<i>N. mutabilis</i>	-----
<i>N. suaveolens</i>	MNHHLITPIFHLQIMLPVATLKRPPPAATCSYFSRGTPLSVSKARLSTAAGVMGKN 60
<i>N. alata</i>	EPSPNHYSIAISSDQNLTRSGNYQPTMDFFEYIQSIHNDYAGDKYMKRFNELKEEMKKM 63
<i>N. langsdorfii</i>	-----RRSGNYQPTMDFFEYIQSIHNDYAGDKYMKRFNELKEEMKKM 42
<i>N. bonariensis</i>	-----RRSGNYQPTMDFFEYIQSIHNDYAGDKYMKRFNELKEEMKKM 42
<i>N. forgetiana</i>	-----RRSGNYQPTMDFFEYIQSIHNDYAGDKYMKRFNELKEEMKKM 42
<i>N. longiflora</i>	-----RRSGNYQPTMDFFEYIQSIHNDYAGDKYMKRFNELKEEMKKM 42
<i>N. mutabilis</i>	-----RRSGNYQPTMDFFEYIQSIHNDYAGDKYMKRFNELKEEMKKM 42
<i>N. suaveolens</i>	EPSPNHYSIDISSDLNLTARRSGNYGPTMDFFEYIQSIHNDYTEKKYMNRLNKLKEEMKKM 120
<i>N. alata</i>	IMAEGSQELEKLELDNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLRE 123
<i>N. langsdorfii</i>	IMAEGSQELEKLELDNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLRE 102
<i>N. bonariensis</i>	IMAEGSQELEKLELDNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLRE 102
<i>N. forgetiana</i>	IMAEGSQELEKLELDNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLRE 102
<i>N. longiflora</i>	IMAEGSQELEKLELDNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLRE 102
<i>N. mutabilis</i>	IMAEGSQELEKLELDNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLRE 102
<i>N. suaveolens</i>	IMAEGSQELEKLELDNLQRLGVSYHFKHEIMQILSSIKQHSTPADSLYATALKFRLRE 180
<i>N. alata</i>	HGFHISQEIFDGLSETHTKDTKGMPLYEASFLATEGESELEQA--WTEKHRLREYLNKN 181
<i>N. langsdorfii</i>	HGFHISQEIFDGLSETHTKDTKGMPLYEASFLATEGESELEQA--WTEKHRLREYLNKN 160
<i>N. bonariensis</i>	HGFHISQEIFDGLSETHTKDTKGMPLYEASFLATEGESELEQA--WTEKHRLREYLNKN 162
<i>N. forgetiana</i>	HGFHISQEIFDGLSETHTKDTKGMPLYEASFLATEGESELEQA--WTEKHRLREYLNKN 162
<i>N. longiflora</i>	HGFHISQEIFDGLSETHTKDTKGMPLYEASFLATEGESELEQA--WTEKHRLREYLNKN 162
<i>N. mutabilis</i>	HGFHISQEIFDGLSETHTKDTKGMPLYEASFLATEGESELEQA--WTEKHRLREYLNKN 162
<i>N. suaveolens</i>	HGFHISQEIFDGLSETHTKDTKGMPLYEASFLATEGESELEQA--WTEKHRLREYLNKN 240
<i>N. alata</i>	IDQNVAKLVHRALEPLHWRMLREARWFSFYKKRQDMIPLLLELAIDFNIVQAAHIQ 241
<i>N. langsdorfii</i>	IDQNVAKLVHRALEPLHWRMLREARWFSFYKKRQDMIPLLLELAIDFNIVQAAHIQ 220
<i>N. bonariensis</i>	IDQNVAKLVHRALEPLHWRMLREARWFSFYKKRQDMIPLLLELAIDFNIVQAAHIQ 222
<i>N. forgetiana</i>	IDQNEAKLVHRALEPLHWRMLREARWFSFYKKRQDMIPLLLELAIDFNIVQAAHIQ 222
<i>N. longiflora</i>	IDQNEAKLVHRALEPLHWRMLREARWFSFYKKRQDMIPLLLELAIDFNIVQAAHIQ 222
<i>N. mutabilis</i>	IDQNEAKLVHRALEPLHWRMLREARWFSFYKKRQDMIPLLLELAIDFNIVQAAHIQ 222
<i>N. suaveolens</i>	IDQNEAKLVHRALEPLHWRMLREARWFSFYKKRQDMIPLLLELAIDFNIVQAAHIQ 300
<i>N. alata</i>	DLKYVARWKKETGLAENLPFARDRLVENFFWTIGVNFPLQYGYFRRIETKVNALVTITDD 301
<i>N. langsdorfii</i>	DLKYVARWKKETGLAENLPFARDRLVENFFWTIGVNFPLQYGYFRRIETKVNALVTITDD 280
<i>N. bonariensis</i>	DLKYVARWKKETGLAENLPFARDRLVENFFWTIGVNFPLQYGYFRRIETKVNALVTITDD 282
<i>N. forgetiana</i>	DLKYVARWKKETGLAENLPFARDRLVENFFWTIGVNFPLQYGYFRRIETKVNALVTITDD 282
<i>N. longiflora</i>	DLKYVARWKKETGLAENLPFARDRLVENFFWTIGVNFPLQYGYFRRIETKVNALVTITDD 282
<i>N. mutabilis</i>	DLKYVARWKKETGLAENLPFARDRLVENFFWTIGVNFPLQYGYFRRIETKVNALVTITDD 282
<i>N. suaveolens</i>	DLKYVARWKKETGLAENLPFARDRLVENFFWTIGVNFPLQYGYFRRIETKVNALVTITDD 360
<i>N. alata</i>	VYDFVGTLDLQCFDTAQRWNTDELNDLPNNMKMCFALDDFINEVACDAL-----IV 355
<i>N. langsdorfii</i>	VYDFVGTLDLQCFDTAQRWNTDELNDLPNNMKMCFALDDFINEVACDAL-----IV 334
<i>N. bonariensis</i>	VYDFVGTLDLQCFDTAQRWNTDELNDLPNNMKMCFALDDFINEVACDAL-----IV 336
<i>N. forgetiana</i>	VYDFVGTLDLQCFDTAQRWNTDELNDLPNNMKMCFALDDFINEVACDAL-----IV 336
<i>N. longiflora</i>	VYDFVGTLDLQCFDTAQRWNTDELNDLPNNMKMCFALDDFINEVACDAL-----IV 336
<i>N. mutabilis</i>	VYDFVGTLDLQCFDTAQRWNTDELNDLPNNMKMCFALDDFINEVACDAL-----IV 336
<i>N. suaveolens</i>	VYDFVGTLDLQCFDTAQRWNTDELNDLPNNMKMCFALDDFINEVACDAL-----IV 420
<i>N. alata</i>	PYLRNAWTDLCKSYLLEAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLIANPVNKE 415
<i>N. langsdorfii</i>	PYLRNAWTDLCKSYLLEAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLIANPVNKE 394
<i>N. bonariensis</i>	PYLRNAWTDLCKSYLLEAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLIANPVNKE 396
<i>N. forgetiana</i>	PYLRNAWTDLCKSYLLEAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLIANPVNKE 396
<i>N. longiflora</i>	PYLRNAWTDLCKSYLLEAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLIANPVNKE 396
<i>N. mutabilis</i>	PYLRNAWTDLCKSYLLEAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLIANPVNKE 396
<i>N. suaveolens</i>	PYLRNAWTDLCKSYLLEAKWYFSKYIPTMEEYMDNAWISISAPVILVHAYFLIANPVNKE 480
<i>N. alata</i>	ALHYLRNYHDIIRWSALILRLANDLGTSSDELKRGDVPKSIQCYMNEKKVSEEEARQHIR 475
<i>N. langsdorfii</i>	ALHYLRNYHDIIRWSALILRLANDLGTSSDELKRGDVPKSIQCYMNEKKVSEEEARQHIR 454
<i>N. bonariensis</i>	ALHYLRNYHDIIRWSALILRLANDLGTSSDELKRGDVPKSIQCYMNEKKVSEEEARQHIR 456
<i>N. forgetiana</i>	ALHYLRNYHDIIRWSALILRLANDLGTSSDELKRGDVPKSIQCYMNEKKVSEEEARQHIR 456
<i>N. longiflora</i>	ALHYLRNYHDIIRWSALILRLANDLGTSSDELKRGDVPKSIQCYMNEKKVSEEEARQHIR 456
<i>N. mutabilis</i>	ALHYLRNYHDIIRWSALILRLANDLGTSSDELKRGDVPKSIQCYMNEKKVSEEEARQHIR 456
<i>N. suaveolens</i>	ALHYLRNYHDIIRWSALILRLANDLGTSSDELKRGDVPKSIQCYMNEKKVSEEEARQHIR 540
<i>N. alata</i>	LLISETWKKLNEAHNVAHPFKMFVKSAMNLARMAQCMYQHGDDGGHGG--QNSQTNRIM 513
<i>N. langsdorfii</i>	LLISETWKKLNEAHNVAHPFKMFVKSAMNLARMAQCMYQHGDDGGHGG--QNSQTNRIM 512
<i>N. bonariensis</i>	LLISETWKKLNEAHNVAHPFKMFVKSAMNLARMAQCMYQHGDDGGHGG--QNSQTNRIM 514
<i>N. forgetiana</i>	LLISETWKKLNEAHNVAHPFKMFVKSAMNLARMAQCMYQHGDDGGHGG--QNSQTNRIM 514
<i>N. longiflora</i>	LLISETWKKLNEAHNVAHPFKMFVKSAMNLARMAQCMYQHGDDGGHGG--QNSQTNRIM 514
<i>N. mutabilis</i>	LLISETWKKLNEAHNVAHPFKMFVKSAMNLARMAQCMYQHGDDGGHGG--QNSQTNRIM 514
<i>N. suaveolens</i>	LLISETWKKLNEAHNVAHPFKMFVKSAMNLARMAQCMYQHGDDGGHGG--QNSQTNRIM 600
<i>N. alata</i>	ALLFESIPPA 543
<i>N. langsdorfii</i>	ALLFESIPPA 522
<i>N. bonariensis</i>	ALLFESIPPA 524
<i>N. forgetiana</i>	ALLFESIPPA 524
<i>N. longiflora</i>	ALLFESIPPA 524
<i>N. mutabilis</i>	ALLFESIPPA 524
<i>N. suaveolens</i>	ALLFESIPPA 610



**Fig. 5** Localization of altered amino acids of newly isolated cineole synthases compared to *Nicotiana alata*. The 3D structure of *N. alata* was modelled using Swiss Pdb Viewer (Fähnrich et al. 2011). The crystal structure of the limonene synthase of *Mentha spicata* was a master sequence (gaps in the alignment were hidden). All amino acids were eliminated except those of the motifs (blue: RR(X)<sub>8</sub>W, beige: RWR, green: DTE, violet: RWW, pink: NALV, yellow: DDXXD, orange: CYMNE) and the amino acids that are different to the *N. alata* sequence. Presented is the perspective of the 3D structure where

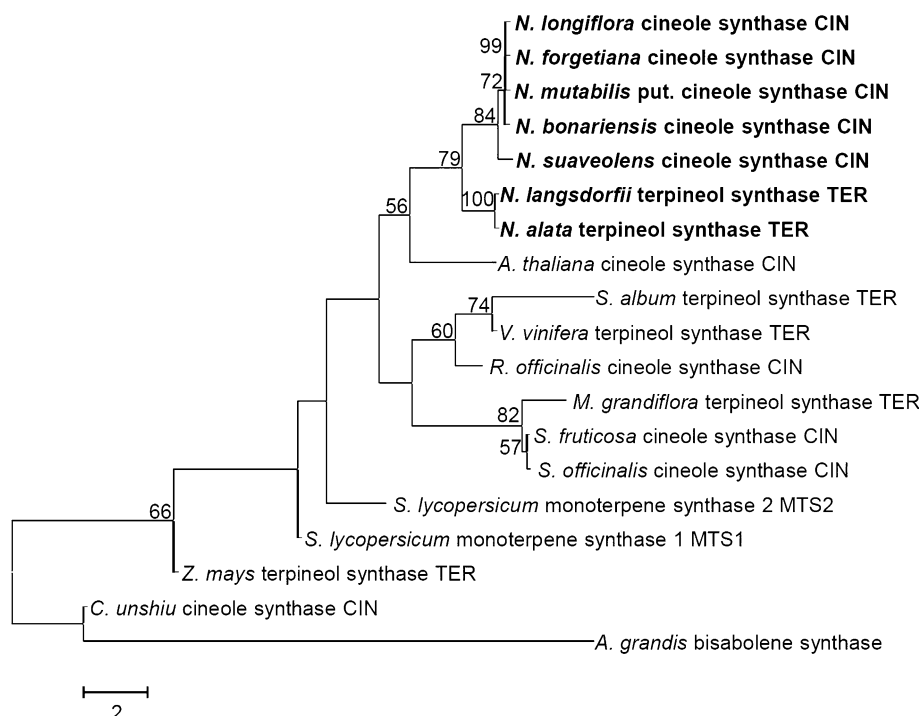
the distances of the altered amino acids are short to the motifs. **a** Altered amino acids of CIN of *N. bonariensis* compared to TER *N. alata*: ile99, pro112, tyr 219, gly259, thr368, ile376, met393, val496, ser508. **b** Altered amino acids of CIN of *N. forgetiana* compared to TER *N. alata*: val191, gln246, ile376, val496, arg536, leu541. **c** Altered amino acids of CIN of *N. longiflora* compared to TER *N. alata*: val191, gln246, ile376, val496, arg536. **d** Altered amino acids of CIN of *N. mutabilis* compared to TER *N. alata*: his129, val191, ile226, ile245, gln246, ile376, asn495, val496

S4D) was located close to conserved motifs DDXXD and RWR. This analysis highlights the altered amino acids at position 259 and 376, because they are in close vicinity to the catalytically important motif DDXXD and may influence the chemical reaction of the enzyme. It is interesting to note that at position 376 the TER genes of *N. alata* and *N. langsdorfii* display the non-polar amino acid isoleucine, while all isolated CIN genes carry the basic amino acid arginine (Fig. 4; Table S4).

The newly isolated terpene synthase genes together with the known CIN and TER genes of other plant species were used to construct a phylogenetic tree (Fig. 6, neighbor joining method). This analysis clustered the genes of *N. bonariensis*, *N. forgetiana*, *N. longiflora* and *N. mutabilis* to the closely related CINs and TERs of other *Nicotiana* species. Interestingly, the newly isolated sequences of the species of section *Alatae* are more closely related to the CIN of *N. suaveolens*, which belongs to the Australian



**Fig. 6** Plant cineole and terpineol terpene synthases. Phylogenetic relationship of newly isolated cineole synthase genes (CIN) from *N. bonariensis*, *N. forgetiana*, *N. longiflora*, *N. mutabilis* 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)<sub>8</sub>W motif of the alignment was removed. Plant species used for the tree construction (accession numbers see “Materials and methods”)



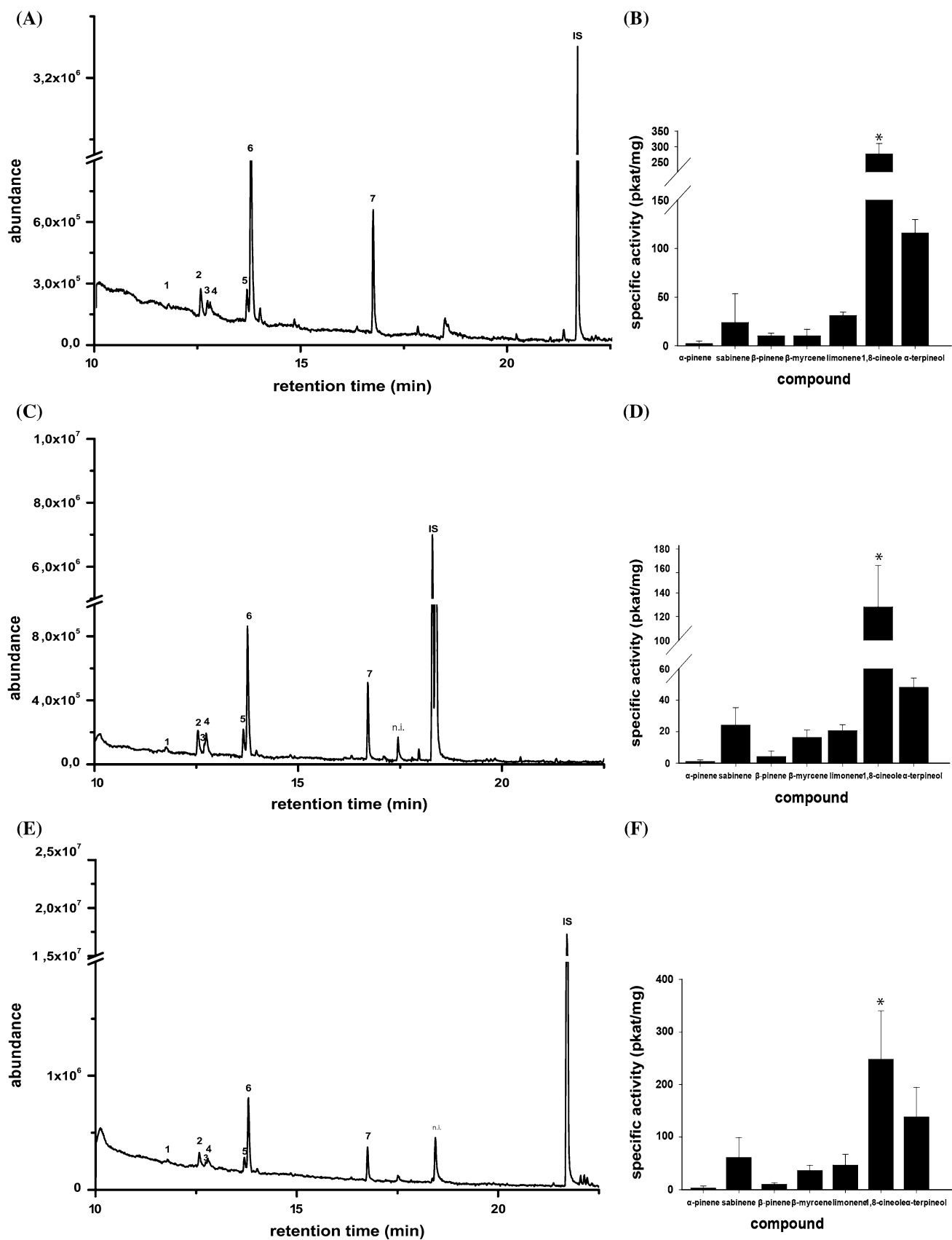
section *Suaveolentes*, than to the TERs of *N. alata* and *N. langsdorfii* of the American section *Alatae*. The same result was obtained when the parsimony algorithm was applied, which supports the conclusion that apparently the catalytic capabilities rather than the taxonomical relationships were decisive for the clustering.

#### Functional characterization of monoterpene synthases from *Nicotiana* species of section *Alatae*

To ultimately determine their biological functions, the newly isolated enzymes were overexpressed in *E. coli* and purified via his-tag affinity chromatography (Fig. S2, e.g. *N. forgetiana*). The enzymes were cloned using PCR-techniques specifically designed to only introduce the RR(X)<sub>8</sub>W truncated version of the enzyme into *E. coli* in order to get the highest expression and to prevent inclusion body formation (Williams et al. 1998; Peters et al. 2000). The purified enzymes were tested with the substrates GPP and NPP, and the volatiles were analyzed and identified by GC/MS (Figs. 7a, c, e, S4). The recombinant enzymes of *N. bonariensis*, *N. forgetiana* and *N. longiflora* reached enzyme activities in the same range (0.12–0.27 nkat 1,8-cineole/mg protein) and produced the seven compounds of the ‘cineole cassette’ (Figs. 7b, d, f, S4). While  $\alpha$ - and  $\beta$ -pinene, sabinene,  $\beta$ -myrcene and limonene were minor products, contributing between 1 and 10 % to the synthesized blend, 1,8-cineole and  $\alpha$ -terpineol were the major compounds of the volatile profiles. All three enzymes

synthesized Two- to threefold more 1,8-cineole than  $\alpha$ -terpineol and were therefore named cineole synthases (CIN). These similar ratios were supported by high correlation coefficients (Table S2). The quantitative product profiles were almost identical regarding the products 1,8-cineole,  $\alpha$ -terpineol, sabinene, limonene, and  $\alpha$ - and  $\beta$ -pinene. The contribution of  $\beta$ -myrcene was nearly identical for both enzymes of *N. forgetiana* and *N. longiflora*, whereas  $\beta$ -myrcene synthesis by the *N. bonariensis* enzyme was lower. Furthermore, this high similarity of the product profiles (of *N. forgetiana* and *N. longiflora*) correlates with the close correspondence of these enzymes at the level of amino acid identities (99 %). However, both enzymes have a distinct protein sequence compared to the CIN of *N. bonariensis* and a different product pattern.

**Fig. 7** GC chromatograms of overexpressed cineole synthases of *Nicotiana* species. The recombinantly overexpressed his-tagged enzymes were purified via Ni-NTA affinity chromatography (Fig. S2). Volatiles synthesized in enzyme assays 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 cineole synthase of *N. bonariensis*. **c** GC chromatogram of the cineole synthase of *N. forgetiana*. **e** GC chromatogram of the cineole synthase of *N. longiflora*. (1)  $\alpha$ -pinene, (2) sabinene, (3)  $\beta$ -pinene, (4)  $\beta$ -myrcene, (5) limonene, (6) 1,8-cineole, (7)  $\alpha$ -terpineol. (IS) internal standard (5 ng cis-nerolidol), n.i.: not identified. **b, d, f** Specific activities of recombinant cineole synthases from *N. bonariensis*, *N. forgetiana*, *N. longiflora* were calculated for each of the seven ‘cineole cassette’ monoterpenes. GPP was provided as substrate.  $n = 3$  (\*significance:  $p < 0.05$ )





## Discussion

Many species of the genus *Nicotiana* developed scented flowers, of which several emit the characteristic monoterpenes of the ‘cineole cassette’. This set of monoterpenes has primarily been found in species of section *Alatae* (Raguso et al. 2003, 2006; 1,8-cineole, limonene,  $\beta$ -myrcene,  $\alpha$ -pinene,  $\beta$ -pinene, sabinene, and  $\alpha$ -terpineol). However, the contributions of the individual monoterpenes to the emission profiles vary in the different species (Table 1). These variations might result from intrinsic features of the enzymes catalyzing the synthesis of these compounds or might be due to different physiological and cellular conditions in the petals. To investigate the first issue in more detail we isolated and characterized ‘cineole cassette’ monoterpene synthases from *N. bonariensis*, *N. forgetiana*, *N. longiflora*, and *N. mutabilis*. It turned out that the recombinant enzymes exhibit very similar properties with respect to product profiles, efficiencies of the cyclization reaction, maximum enzyme activities, and amino acid sequences.

### Emission profiles versus synthesis profiles

Floral scents are usually traits to communicate with the environment, for example, by attracting pollinators, or protecting against herbivores and pathogens via direct or indirect defense mechanisms (Pichersky and Gershenzon 2002; Raguso et al. 2003). Different *Nicotiana* species exhibit various flower colors and flower morphologies to interact with nocturnal hawkmoths as well as diurnal hummingbirds and bees (Kaczorowski et al. 2005; Knudsen et al. 2004; Raguso et al. 2003; Stehmann et al. 2002). The flowers of *N. alata* and *N. bonariensis* open around dusk and *N. alata* is pollinated by the hawkmoths *Agrius cingulatus* and *Eumorphia labruscae* (Sphingidae) and *N. bonariensis* is pollinated by small perching moths (Kaczorowski et al. 2005). Therefore it comes as no surprise that species-specific daily emission patterns as well as enzyme activities were observed for *N. bonariensis* and *N. alata* (Figs. 2, 3; Fährnrich et al. 2011), which correlated with the pollination of night-active organisms (Kaczorowski et al. 2005). For *N. forgetiana*, hummingbirds and small hawkmoths have been documented as pollinators before dusk (Ippolito et al. 2004), which is in accordance with the continuous emission profile (Fig. 3).

The scent profiles of *Nicotiana* flowers described by Raguso et al. (2003, 2006) were verified here, with particular focus on the presence of the ‘cineole cassette’ monoterpenes for *N. bonariensis*, *N. forgetiana*, and *N. mutabilis*. While these three species emitted relatively high levels of volatiles, in the headspace of *N. longiflora* only small amounts or none ( $\alpha$ -terpineol) were detected (Raguso

et al. 2003). Despite these very low emission levels of *N. longiflora* flowers, we were able to isolate a terpene synthase from this and the other three *Nicotiana* species. The synthesis profiles of these four overexpressed enzymes turned out to be very similar, they produced predominantly 1,8-cineole followed by  $\alpha$ -terpineol, and the side products occurred in significantly smaller amounts. These recombinant enzymes always synthesized in vitro twice as much 1,8-cineole as  $\alpha$ -terpineol (Tables S1, S2). An exception was *N. forgetiana*, which emitted 12-fold 1,8-cineole compared to  $\alpha$ -terpineol. The latter is in discrepancy to the in vitro capacity of the enzyme, but presently we have no sound explanation for this.

In the GC/MS profiles of the cineole synthases of *N. bonariensis*, *N. forgetiana* and *N. longiflora* neither *(E)*- $\beta$ -ocimene nor linalool was detected (Fig. 7). Since *(E)*- $\beta$ -ocimene was present in the floral scents of *N. forgetiana*, *N. longiflora* and *N. mutabilis* and linalool in *N. longiflora* and *N. mutabilis*, it is very likely that beside the CIN additional genes were expressed in petals. *(E)*- $\beta$ -ocimene does not belong to the monoterpenes of the ‘cineole cassette’ as described by Raguso et al. (2006). Our observation in this investigation as well as the profiles of the previously isolated terpene synthases of *N. alata* and *N. langsdorfii* support the notion that *(E)*- $\beta$ -ocimene is not a member of the ‘cineole cassette’ monoterpenes (Fährnrich et al. 2011). *(E)*- $\beta$ -Ocimene has also not been demonstrated as a product of other CIN or TER multiproduct enzymes of various other plant species (summarized in Fährnrich et al. 2011). The only known exception is the CIN of *N. suaveolens* (Roeder et al. 2007). In conclusion, for the synthesis of *(E)*- $\beta$ -ocimene additional enzymes with the capability to synthesize *(E)*- $\beta$ -ocimene must exist in respective *Nicotiana* species (Table 1; Fährnrich et al. 2011).

### Molecular analysis of the cineole synthases of *Nicotiana* species

In each case investigated so far, the emission of ‘cineole cassette’ monoterpenes was due to the presence of multiproduct enzymes. 1,8-cineole was the major compound of the enzymes isolated from *N. bonariensis*, *N. forgetiana* and *N. longiflora*, while  $\alpha$ -terpineol was the major compound of the enzymes isolated from the related species *N. alata* and *N. langsdorfii* (Fährnrich et al. 2011). The bicyclic 1,8-cineole is synthesized by a reaction of the hydroxyl group of  $\alpha$ -terpineol with the double bond to form a second cycle (Fig. 1). When both compounds appear simultaneously in the product profile, it is reasoned that either the addition reaction is not efficient or a premature termination reaction occurs (Peters and Croteau 2003; Iijima et al. 2004). The conversion of the intermediate to the final product was apparently particularly limited in the TER

genes of *N. alata* and *N. langsdorfii*, while the cyclization reaction was more pronounced by enzymes of *N. bonariensis*, *N. forgetiana*, *N. longiflora*, and *N. suaveolens* (Roeder et al. 2007). The ratio 1,8-cineole/ $\alpha$ -terpineol (expressing the efficacy of the cyclization reaction) of the newly isolated genes was 2:1 and ranged between those of the TERs of *N. alata* and *N. langsdorfii* (0.5:1 and 0.9:1, respectively) and the CIN of *N. suaveolens* (7.8:1) (Fähnrich et al. 2011).

It remains to be explained why the CIN enzymes more efficiently perform the cyclization reaction than the TER enzymes. During the search for key amino acids important for the catalytic reaction mechanisms, amino acids of the active pocket have often been considered, but recently it was shown that also amino acids distant to the active pocket influence the product spectrum (Hyatt and Croteau 2005; Greenhagen et al. 2006; Yoshikuni et al. 2006). Both alternative possibilities might also explain the different efficacies in the *Nicotiana* species. Therefore, the amino acid variations of TERs/CINs of *Nicotiana* were localized in the amino acid sequences (Fig. 4) and it turned out that neither amino acids in conserved motifs nor of the active pocket were altered in respective *Nicotiana* species (Fig. 4). Kampranis et al. (2007) indicated amino acids at positions 338 and 327 in region 1 of monoterpene synthases of *Salvia fruticosa* and *Salvia pomifera*, respectively, and at 436 (*S. fruticosa*) in region 2 to be relevant for the 1,8-cineole and  $\alpha$ -terpineol production. It was also concluded that serine 436 prevents 1,8-cineole production and therefore limits the cyclization reaction. However, the amino acids shown to be important in *Salvia* species were conserved in the enzymes of all isolated CINs and TERs of *Nicotiana* species (*N. alata*: asn292 and ala293 in the NALV motif, ser396) and therefore could not be decisive for the efficiency of the cyclization reaction in the *Nicotiana* species. We therefore searched for amino acids that were closely positioned to those conserved motifs or the active pocket that may influence catalysis (Fig. 5). The amino acid isoleucine at position 376 in the two TER enzymes was altered to arginine in all CIN enzymes. It is possible that this change from a non-polar to a basic amino acid had an impact on the catalytically active motif DDXXD of *Nicotiana* enzymes. At the equivalent position (#412) *S. fruticosa*'s isoleucine resulted in a 1,8-cineole/ $\alpha$ -terpineol ratio of 10:1, while *S. pomifera* harbors a positively charged lysine, which favors  $\alpha$ -terpineol emission (ratio 0.3:1). These apparent controversial results indicate once more that amino acids relevant in one species may not play the same role in another species, even if the enzymes are related. Future research in the *Nicotiana* enzymes will focus on the amino acids (ile and arg at position 376) to evaluate their impact on the  $\alpha$ -terpineol to 1,8-cineole cyclization reaction.

Phylogenetic and evolutionary analysis of 'cineole cassette' monoterpene synthases from related *Nicotiana* species

The genus *Nicotiana* is the fifth largest group within the *Solanaceae* and comprises 75 species. Goodspeed (1954) provided detailed information on the taxonomy, cytology, biogeography, and the floral and vegetative morphologies. The data indicated that *N. sec. Suaveolentes* is monophyletic due to the tree generated from internal transcribed spacers (Chase et al. 2003) and RFLP analysis of the plastid *matK* gene (Aoki and Ito 2000; Clarkson et al. 2004). Based on ancient chromosome hybridizations and the present chromosome number (amphidiploidy) Goodspeed (1954) hypothesized that one parent of the Australian section *Suaveolentes* is a member of the present section *Alatae* and the other parent either a member of the section *Acuminatae* (= *Petunioides*) or *N. sect. Noctiflorae*. We isolated and functionally analyzed related CINs from *Nicotiana* species of section *Alatae* and compared them with TER enzymes of *N. alata* and *N. langsdorfii* of the same section as well as CIN of *N. suaveolens* of section *Suaveolentes*. The three isolated enzymes of *N. bonariensis*, *N. forgetiana* and *N. longiflora* (section *Alatae*) were clearly cineole synthases (Fig. 7) and clustered together with the CIN of *N. suaveolens* (Fig. 6), supporting the hypothesis that one parent of the Australian *Suaveolentes* descended from the American *Alatae* (Goodspeed 1954). Furthermore, our results are in agreement with the phylogenetic trees that position *Alatae* next to *Suaveolentes* (Chase et al. 2003) or where *Noctiflorae*, *Repandae*, and *Sylvestres* are located between the sections *Alatae* and *Suaveolentes* (Clarkson et al. 2004). To understand the evolution of the TER/CIN genes of *Nicotiana* species in more detail, a future goal is to find and characterize a progenitor TER/CIN enzyme.

A novel finding of this research is that the newly isolated CINs of *Alatae* do not cluster with the taxonomically more closely related TERs of *Alatae* but with the catalytically similar CIN of *N. suaveolens*, which apparently indicates an amino acid sequence-based phylogeny rather than the taxonomy-based phylogeny. This is unusual for enzymes of the specialized metabolism (e.g. terpene synthases) but is common for enzymes of plant primary metabolism (Martin et al. 1993). A plausible explanation may apply: since *N. suaveolens* is an amphidiploid species with one assumed parent from *Alatae* tribe it can be speculated that the CIN is inherited from this parent. In this scenario, the isolated CINs of *N. bonariensis*, *N. forgetiana*, *N. longiflora* kept the original cyclization capability, while the TERs of *N. alatae* and *N. langsdorfii* diverged during evolution to establish a less efficient cyclization reaction.

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