Molecular Plant • Volume 4 • Number 6 • Pages 965–984 • November 2011 RESEARCH ARTICLE

Product Variability of the 'Cineole Cassette' Monoterpene Synthases of Related *Nicotiana* Species

Anke Fähnrich, Katrin Krause and **Birgit Piechulla**[1]

University of Rostock, Institute for Biological Sciences, Albert-Einstein-Str. 3, 18059 Rostock, Germany

ABSTRACT ***Nicotiana* species of the section *Alatae* characteristically emit the floral scent compounds of the 'cineole cassette' comprising 1,8-cineole, limonene, myrcene, α-pinene, β-pinene, sabinene, and α-terpineol. We successfully isolated genes of *Nicotiana alata* and *Nicotiana langsdorfii* that encoded enzymes, which produced the characteristic monoterpenes of this 'cineole cassette' with α-terpineol being most abundant in the volatile spectra. The amino acid sequences of both terpineol synthases were 99% identical. The enzymes cluster in a monophyletic branch together with the closely related cineole synthase of *Nicotiana suaveolens* and monoterpene synthase 1 of *Solanum lycopersicum*. The cyclization reactions (α-terpineol to 1,8-cineole) of the terpineol synthases of *N. alata* and *N. langsdorfii* were less efficient compared to the 'cineole cassette' monoterpene synthases of *Arabidopsis thaliana*, *N. suaveolens*, *Salvia fruticosa*, *Salvia officinalis*, and *Citrus unshiu*. The terpineol synthases of *N. alata* and *N. langsdorfii* were localized in pistils and in the adaxial and abaxial epidermis of the petals. The enzyme activities reached their maxima at the second day after anthesis when flowers were fully opened and the enzyme activity in *N. alata* was highest at the transition from day to night (diurnal rhythm).**

Key words: ***Nicotiana alata*; *Nicotiana langsdorfii*; cineole cassette; terpineol synthase; multiproduct enzyme; monoterpene synthase; 1,8-cineole, α-terpineol.**

INTRODUCTION

The terpene synthase gene family is presently subdivided into seven subfamilies, tps a–g (Bohlmann et al., 1998; Dudareva et al., 2003; Lee and Chappell, 2008). Ninety genes encoding monoterpene synthases are known; the enzymes were found in floral and vegetative tissues of angiosperms (branch b) and gymnosperms (branch d) (summarized in Degenhardt et al., 2009). Monoterpene synthases produce a wide variety of terpenoids. They require divalent metal ions as cofactors to operate an unusual electrophilic reaction mechanism. The tremendous range of possible variations of the carbocationic reactions of the substrate geranyl pyrophosphate (GPP) (cyclization, hydride shifts, rearrangements, termination steps) permits the production of essentially all feasible skeletal types, isomers, and derivatives (Williams et al., 1998; Trapp and Croteau, 2001). Some monoterpene synthases produce almost exclusively their name-giving compound, such as geraniol synthases (GES) and linalool synthases (LIS), and some myrcene synthases (MYR), ocimene synthases (OCI), and limonene synthases (LIM), while the majority of the up to now known isolated monoterpene synthases synthesize several products simultaneously (multiproduct enzymes) (Degenhardt et al., 2009). Table 1 summarizes and organizes all presently known plant monoterpene synthases according to their name-giving major product. Typical multiproduct enzymes are cineole synthases (CIN), terpinene synthases (TS), terpinolene synthases (TES), bornyl diphosphate synthases (BOR), carene synthases (CAR), and some myrcene synthases (MYR) and terpineol synthases (TER), which produce cyclic and acyclic compounds (Table 1 and Figure 1). Several multiproduct synthases produce the same compounds, such as limonene, β-myrcene, sabinene, α/β-pinene appear frequently in product spectra, while others such as α-terpineol and 1,8-cineole are only synthesized by CIN or TER enzymes. For better inspection, the mentioned compounds are colour-coded in Table 1.

α-Terpineol is proposed to be the precursor of 1,8-cineole. The bicyclic compound is most likely formed by monoterpene synthases that first form the intermediate α-terpineol from the

[1] To whom correspondence should be addressed. E-mail birgit.piechulla@uni-rostock.de, fax 0049 (0)381 4986132, tel. 0049 (0)381 4986130.

© The Author 2011. Published by the Molecular Plant Shanghai Editorial Office in association with Oxford University Press on behalf of CSPB and IPPE, SIBS, CAS.

doi: 10.1093/mp/ssr021, Advance Access publication 28 April 2011
Received 10 December 2010; accepted 10 February 2011

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

Table 1. Color-Coded Product Spectra of Monoterpene Synthases.

Monoterpene synthase	Products of recombinant enzyme	Reference
BOR (bornyl diphosphate)		
Salvia officinalis	Major: bornyl diphosphate (75%) Minor: α-pinene (3.4%), (+)-camphene (9.5%), (–)-camphene (0.5%), (+)-limonene (3.9%), (–)-limonene (3.9%), terpinolene (2.1%), β-myrcene (1.5%)	Wise et al., 1998
CAR (carene)		
Picea abies PaJF67	Major: carene (78%) Minor: terpinolene (11%), sabinene (5%), β-myrcene (3%), γ-terpinene (1%), α-pinene (0.9%), β-phellandrene (0.7%), α-terpinene (0.6%), limonene (0.4%)	Fäldt et al., 2003b
CAM (camphene)		
Abies grandis Ag6	Major: camphene (54%), α-pinene (32%) Minor: limonene (7%)	Bohlmann et al., 1999
CIN (cineole)		
Salvia officinalis	Major: 1,8-cineole (79%) Minor: β-myrcene (2.9%), sabinene (2.6%), limonene (1.5%), α-terpineol (1%), α-pinene (6.4%), β-pinene (6.8%)	Wise et al., 1998, Croteau et al., 1994
Salvia fruticosa	Major: 1,8-cineole (72%) Minor: β-myrcene (2.2%), sabinene (3.6%), limonene (⩽1%), α-terpineol (7%), α-pinene (4.6%), β-pinene (9%)	Kampranis et al., 2007
Arabidopsis thaliana At3g25820 At3g25830	Major: 1,8-cineole (52%) Minor: α-thujene (0.6%), α-pinene (1.9%), sabinene (14.5%), β-pinene (7.8%), β-myrcene (13,3%), limonene (4%), (*E*)-β-ocimene (2.7%), terpinolene (0.8%), α-terpineol (2.4%)	Chen et al., 2004
Citrus unshiu CitMTSL1	Major: 1,8-cineole (⩾98%)	Shimada et al., 2005
Nicotiana suaveolens CIN	Major: 1,8-cineole (62%) Minor: β-myrcene (13%), sabinene (9.4%), limonene (8.9%), α-terpineol (5.2%), α-pinene (1.2%)	Roeder et al., 2007
GES (geraniol)		
Ocimum basilicum	Major: geraniol (100%)	Iijima et al., 2004
Cinnamomum tenuipilum	Major: geraniol (100%)	Yang et al., 2005
Perilla citriodora *Perilla frutescens*	Major: geraniol (100%)	Ito and Honda, 2006
LIM (limonene)		
Mentha spicata *Mentha piperita*	Major: limonene Very minor: α- and β-pinene, β-myrcene	Colby et al., 1993, Rajaonarivony et al., 1992
Lavendula angustifolia LaLIMS	Major: limonene (37%) Minor: α-pinene (14%), α-phellandrene (1%), β-myrcene (8%), terpinolene (22%), camphene (16%)	Landmann et al., 2007
Schizonepeta tenuifolia	Major: γ-limonene	Maruyama et al., 2001
Carum carvi	Major: (+)-limonene (98.4%) Minor: (–)-limonene (1.6%)	Bouwmeester et al., 1998
Cannabis sativa	Major: 4(S)-limonene (97%)	Günnewich et al., 2007
Perilla frutescens	Major: (–)-limonene (97%)	Yuba et al., 1996
Citrus unshiu MTSE1, MTSE2	Major: d-limonene (⩾97%)	Shimada et al., 2004
Citrus limon LIMS1 (C62) and LIMS2 (M34)	Major: limonene (99%) Minor: α-pinene, β-myrcene (⩽1%)	Lücker et al., 2002
Abies grandis Ag10	Major: limonene (⩾70%) Minor: α-pinene, β-pinene, β-phellandrene	Bohlmann et al., 1997
Abies grandis Ag11	4(S)-limonene (35%), α-pinene (24%), sabinene (10%), β-pinene (11%)	Bohlmann et al., 1999
Agastache rugosa	Major: γ-limonene (100%)	Maruyama et al., 2002
Picea abies	Major: limonene (87%)	Martin et al., 2004

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

Table 1. Continued

Monoterpene synthase	Products of recombinant enzyme	Reference
Picea sitchensis	Major: limonene (100%)	Byun-McKay et al., 2006
LIS (linalool)		
Arabidopsis thaliana	Major: (S)-linalool (100%)	Chen et al., 2003
Artemisia annua QH1, QH5	Major: (R)-linalool (100%)	Jia et al., 1999
Antirrhinum majus AmNES	Major: (S)-linalool (100%)	Nagegowda et al., 2008
Fragaria ananassa FaNES1, FaNES2	Major: (S)-linalool (100%)	Aharoni et al., 2004
Lavendula angustifolia	Major: (R)-linalool (100%)	Landmann et al., 2007
Lycopersicon esculentum MTS1	Major: (R)-linalool (100%)	van Schie et al., 2007
Mentha citrata	Major: (R)-linalool (98%)	Crowell et al., 2002
Melaleuca alternifolia AY279379	Major: linalool (37.8%), *trans*-β-ocimene (20.4%), *cis*-ocimene (11.7%), β-myrcene (15.4%) Minor: limonene (2.5%), terpinolene (2.5%)	Shelton et al., 2004
Ocimun basilicum	Major: (R)-linalool (100%)	Iijima et al., 2004
Oryza sativa	Major: (S)-linalool (100%)	Yuan et al., 2008
Picea abies	Major: (–)-linalool (98%)	Martin et al., 2004
Perilla frutescens	Major: (–)-linalool (100%)	Masumoto et al., 2010
MYR (myrcene)		
Antirrhinum majus Ama0c15, Ama1e20	Major: β-myrcene (100%)	Dudareva et al., 2003
Arabidopsis thaliana AtTps10	Major: β-myrcene (56%) Minor: limonene (20%), ocimene, carene, tricyclene	Bohlmann et al., 2000
Humulus lupulus HlMTS2	Major:β-myrcene (100%)	Wang et al., 2008
Quercus ilex	Major: β-myrcene (98%) Minor: limonene, α/β pinene, sabinene	Fischbach et al., 2001
Abies grandis Ag2	Major: β-myrcene	Bohlmann et al., 1997
Arabidopsis thaliana At3g25810	Major: β-myrcene, (*E*)-β−ocimene, sabinene, α-pinene, limonene, β-pinene Minor: α-thujene, terpinolene, α-terpineol	Chen et al., 2003
Lycopersicon esculentum LeMTS2	β-myrcene (50%), sabinene, β-phellandrene	van Schie et al., 2007
Ocimum basilicum	Major: β-myrcene (100%)	Iijima et al., 2004
Oryza sativa Os08g07100	Major: β-myrcene (100%)	Yuan et al., 2008
Picea abies	Major: β-myrcene (100%)	Martin et al., 2004
Perilla frutescens PTS-5526	β-myrcene (53.8%), sabinene (20.9%), linalool (19.8%) (not verified!), limonene (5.5%)	Hosoi et al., 2004
OCI (ocimene)		
Antirrhinum majus Ama0a23	Major: ocimene (97%)	Dudareva et al., 2003
Arabidopsis thaliana AtTps03	Major: (*E*)-β−ocimene (94%) Minor: (*Z*)-β-ocimene (4%), β-myrcene (2%)	Fäldt et al., 2003a
Lotus japonicus LjEβOS	Major: (*E*)-β-ocimene (98%) Minor: (*Z*)-β-ocimene (2%)	Arimura et al., 2004
Medicago truncatula MtEBOS	Major: (*E*)-β-ocimene (98%)	Navia-Gine et al., 2009
Citrus unshiu CitMTS4	Major: (*E*)-β-ocimene (≥97%)	Shimada et al., 2005
Phaseolus lunatus	Major: (*E*)-β-ocimene (98%)	Arimura et al., 2008
PHL (phellandrene)		
Abies grandis Ag8	Major: β-phellandrene (52%), β-pinene (34%) Minor: α-pinene (8%), limonene (6%)	Bohlmann et al., 1999
PIN (pinene)		
Picea abies	Major: α-pinene (42%), β-pinene (58%)	Martin et al., 2004
Artemisia annua	Major: β-pinene (94%) Minor: α-pinene (6%)	Lu et al., 2002
Citrus unshiu MTS62	Major: β-pinene (82.4%) Minor: α-pinene (2.5%), sabinene (4.1%), limonene (11%), α-thujene (trace), γ-terpinene (trace)	Shimada et al., 2004

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

Table 1. Continued

Monoterpene synthase	Products of recombinant enzyme	Reference
Abies grandis Ag3	Major: β-pinene (58%), α-pinene (42%)	Bohlmann et al., 1999
Citrus limon βPINS (D85)	Major: β-pinene (81.4%) Minor: α-pinene (4%), sabinene (11%), limonene (3.5%), γ-terpinene (≤1)	Lücker et al., 2002
Fragaria vesca	Major: α-pinene (80%) Minor: β-myrcene, β-phellandrene	Aharoni et al., 2004
Picea sitchensis	β-pinene, α−pinene, ratio (10:35)	McKay et al., 2003
Pinus taeda Pt30	Major: (+)α-pinene (97%)	Phillips et al., 2003
Pinus taeda Pt1	Major: (−)α-pinene (79%) Minor: (–)β-pinene (4.2%), limonene, camphene	Phillips et al., 2003
Pseudotsuga menziesii	Major: α-pinene, camphene Minor: β-pinene, limonene, carene	Huber et al., 2005
SAB (sabinene)		
Salvia pomifera	Major: sabinene (100%)	Kampranis et al., 2007
Salvia officinalis	Major: sabinene (63%) Minor: γ-terpinene (21%), terpinolene (7%), limonene (6.5%), β-myrcene (2.5%)	Wise et al., 1998
TS (terpinene)		
Citrus unshiu MTSL3	Major: γ-terpinene (85.4%), α-pinene (9.1%), β-pinene (6.3%)	Shimada et al., 2004
Citrus unshiu MTSL61	Major: γ-terpinene (78.4%) Minor: α-thujene (2.8%), α-pinene (5.8%), β-pinene (3.7%), limonene (9.3%)	Shimada et al., 2004
Citrus limon γTS (B93)	Major: γ-terpinene (71.4%) Minor: α-thujene (2.5%), α-pinene (5.6%), sabinene (0.4%), β-pinene (4.7%), β-myrcene, α-terpinene (1.7%), p-cymene, limonene (9%), terpinolene (3.7%)	Lücker et al., 2002
TES (terpinolene)		
Abies grandis Ag9	Major: terpinolene (42%) Minor: α-pinene (18%), limonene (11%)	Bohlmann et al., 1999
Ocimum basilicum TES	Major: terpinolene Minor: β-pinene, limonene	Iijima et al., 2004
Pseudotsuga menziesii PmeTPS2	Major: terpinolene Minor: γ-terpinene, α-pinene, β-pinene, limonene, sabinene, α-terpinene, carene	Huber et al., 2005
TER (terpineol)		
Magnolia grandiflora Mg17	Major: α-terpineol (100%)	Lee and Chappell, 2008
Pinus taeda	Major: α-terpineol (57.3%) Minor: β-myrcene (2%), limonene (27.6%), terpinolene (8%), β-pinene (5%)	Phillips et al., 2003
Nicotiana alata	Major: α-terpineol (41%) Minor: 1,8-cineole (20%), β-myrcene (11%), limonene (15%), sabinene (13%)	Fähnrich et al., this publication
Nicotiana langsdorfii	Major: α-terpineol (36%) Minor: 1,8-cineole (30%), β-myrcene (8%), limonene (11%), sabinene (13%), α-pinene (2%)	Fähnrich et al., this publication
Santalum album	Major: α-terpineol, limonene	Jones et al., 2008
Vitis vinifera VvTPS1891, VvTPS4568	Major: α-terpineol (50,1%) Minor: 1,8-cineole (11%), β-myrcene (2.5%), sabinene (1.3%), limonene (2.8%), α-pinene (4.3%), β-pinene (8.5%)	Martin and Bohlmann, 2004
Zea mays STC-B73, TPS26_B73	Major: α-terpineol Minor: limonene, γ-terpinene, terpinolene	Lin et al., 2008

Colour code for monoterpnes: 1,8-cineole limonene β-myrcene α/β pinene sabinene terpineol.

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

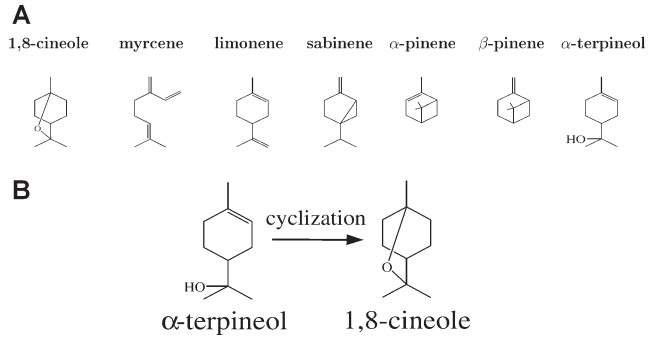

Figure 1. Chemical Structures of Monoterpenes.
(A) Chemical structures of seven monoterpenes of the 'cineole cassette'.
(B) Cyclization reaction of α-terpineol to 1,8-cineole.

α-terpinyl cation, which then undergoes an internal cyclization reaction between the hydroxyl group and the double bond (Croteau et al., 1994; Wise et al., 1998; Chen et al., 2004; Figure 1B). Due to the linked biosynthesis of α-terpineol and 1,8-cineole, it is expected that the enzymes catalyzing this reaction were related. Terpineol synthases (TER) were so far isolated from five plant species: *Magnolia grandiflora*, *Pinus taeda*, *Santalum album*, *Vitis vinifera*, and *Zea mays* (summarized in Table 1). The TER of *M. grandiflora* is a single product enzyme, while the enzymes of the other species produced additional compounds, such as limonene, sabinene, β-myrcene, (*E*)-β-ocimene, α-pinene, and β-pinene. It is interesting to note that both TERs from *V. vinifera* synthesize the epoxide monoterpenoid 1,8-cineole. Cineole synthases (CIN) were so far isolated from *Arabidopsis thaliana*, *Citrus unshiu*, *Nicotiana suaveolens*, *Salvia fruticosa*, and *Salvia officinalis* (summarized in Table 1). Their major product 1,8-cineole contributed between 52 and 98% to the synthesized product spectra, while β-myrcene, limonene, sabinene, α- and β-pinene, (*E*)-β-ocimene, and α-terpineol accomplished amounts between 1 and 15% in different species.

1,8-cineole, also known as eucalyptol, and α-terpineol are monoterpenes that appear widely distributed in the plant kingdom. They are present in leaves, roots, twigs (= shoots), and are emitted from flowers of 30 plant families (Knudsen et al., 2006). For example, respectable amounts of 1,8-cineole were emitted from certain *Nicotiana* flowers and inflorescences, while the monoterpenes limonene, sabinene, β-myrcene, (*E*)-β-ocimene, α-pinene, β-pinene, and α-terpineol were emanated at lower levels (Raguso et al., 2003, 2006). Since 1,8-cineole was the major compound, this set of monoterpenes was named 'cineole cassette' (Figure 1A) (Raguso et al., 2006). The simultaneous emission of 'cineole cassette' monoterpenes was found in *Nicotiana* species of section *Alatae* (*N. alata*, *N. bonariensis*, *N. langsdorfii*, *N. mutabilis*, *N. rastroensis*, *N. longiflora*, *N. plumpaginifolia*, *N. forgetiana*), but not in species of the sister taxa sections (N. section *Sylvestres* (*N. sylvestris*—emits only myrcene and ocimene), N. section *Rusticae* (*N. rustica*), N. section *Suaveolentes* (*N. africana*, *N. cavicola*, *N. ingulba*) (Raguso et al., 2003, 2006). Interestingly, the name-giving species *N. suaveolens* of the section *Suaveolentes* emits the complete set of 'cineole cassette' monoterpenes including 1,8-cineole and it therefore presents an evolutionarily interesting exception.

Various *Nicotiana* species emit different 'cineole cassette' monoterpene quantities and ratios; for example, the total emission of scent is sevenfold higher in *N. alata* compared to *N. suaveolens* and *N. langsdorfii*. Limonene, myrcene, and sabinene dominate beside 1,8-cineole, the scent of *N. langsdorfii* and *N. alata*, while the scent of *N. suaveolens* contains less myrcene but significant levels of *E*-β-ocimene (Raguso et al., 2003). While α-terpineol is present in the scent of *N. alata*, it was not obvious in the odor of *N. langsdorfii* and *N. suaveolens* and each species emitted a defined ratio of α-terpineol and 1,8-cineole. These qualitative and quantitative differences in the 'cineole cassette' monoterpenes emission profiles of *Nicotiana* species are most likely due to characteristic features of the underlying monoterpene synthases. Therefore, we wanted to elucidate similarities and differences between the catalytic capabilities of these enzymes. The isolated monoterpene synthases from *Nicotiana* species will be characterized and determination of the product spectra will help to explain the different scent emission profiles (e.g. 1,8-cineole/α-terpineol ratios). Sequence comparison as well as detailed analysis of the homology-based 3-D structures with particular focus on the active pocket will be used to highlight amino acids with importance for product formation. The previously isolated CIN from *N. suaveolens* flowers paved the way to isolate and study related genes in species of section *Alatae*, such as *N. alata* and *N. langsdorfii*.

RESULTS

Monoterpene Emission in Flowers of *Nicotiana alata* and *Nicotiana langsdorfii*

Flowers of *Nicotiana alata* and *Nicotiana langsdorfii* emitted most of the characteristic monoterpenes of the 'cineole cassette', the profile of headspace collections of *N. alata* comprized nine compounds (sabinene, β-myrcene, limonene, 1,8-cineole, linalool, α-terpineol, eugenol, isoeugenol, nerolidol), while only five compounds were identified in the *N. langsdorfii* spectra (sabinene, β-myrcene, limonene, 1,8-cineole, α-terpineol) (Figure 2). The total emission of 1,8-cineole (ng per flower per h) was approximately 17-fold higher in *N. alata* than *N. langsdorfii* (Figure 2C and 2D). *N. alata* released almost equal amounts of 1,8-cineole and α-terpineol (28 and 24%, respectively, ratio 1.2), while 74% of the emanated monoterpenes of *N. langsdorfii* was 1,8-cineole, and α-terpineol was emitted at very low levels (ratio 15) (Figure 2E and 2F), while myrcene, sabinene, and limonene can be regarded as side products (contributions: 1–2% in *N. alata* and 5–8% in *N. langsdorfii*). (5–8%).

Subsequently, the enzymatic capacity of monoterpenoid synthesis was determined in petals of both *Nicotiana* species. Petal protein raw extracts of flowers of different developmental

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

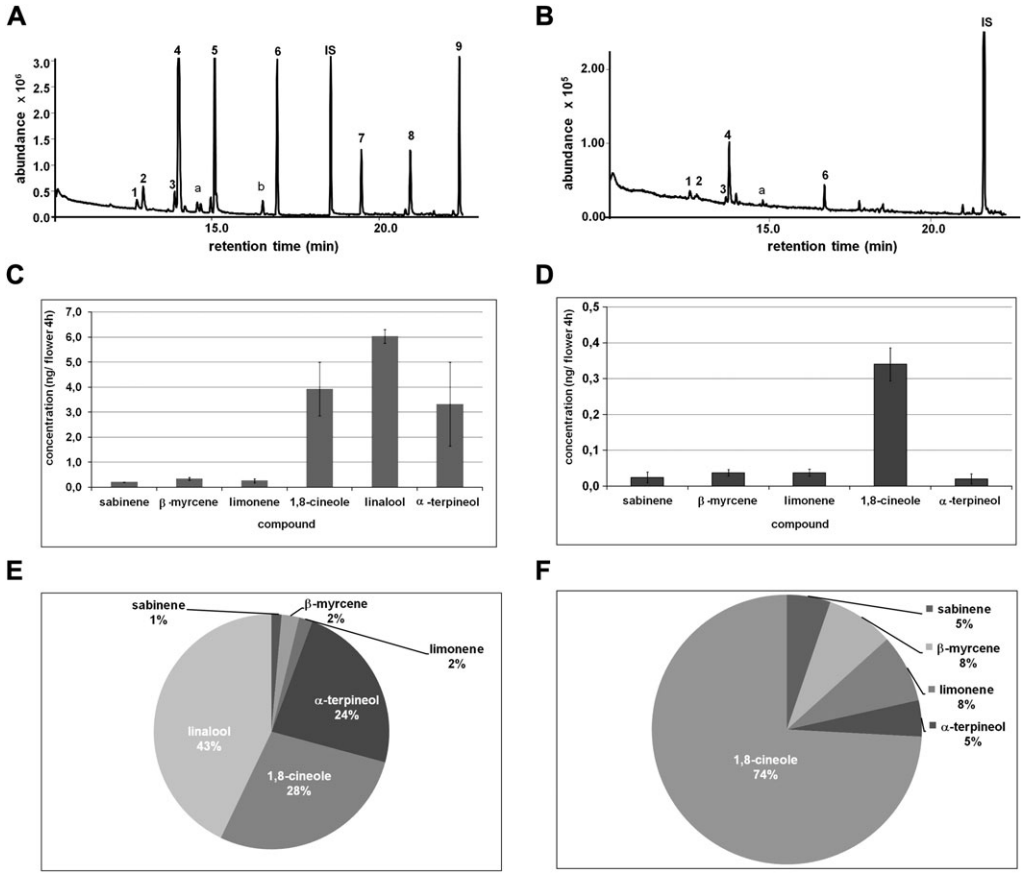

Figure 2. Floral Volatiles Emitted from *Nicotiana alata* and *Nicotiana langsdorfii*.

(A) Headspace volatiles of flowers of *N. alata* were collected between 6 and 10 pm. Compounds were analyzed by GC–MS, identified by their retention index, by comparision of mass spectra of the library of the National Institute of Standards and Technology (NIST147), and by comparision with the authentic standards: (1) sabinene, (2) β-myrcene, (3) limonene, (4) 1,8-cineole, (5) linalool, (6) α-terpineol, (7) eugenol, (8) isoeugenol, (9) nerolidol, (a) linalool-oxide (tentatively identified), (b) not identified. (IS) internal standard (5 ng nonyl acetate).

(B) Headspace volatiles of flowers of *N. langsdorfii* were collected between 10 am and 2 pm. Compounds were analyzed by GC–MS, identified by their retention index, by comparision of mass spectra of the NIST147 library, and by comparision with the authentic standards: (1) sabinene, (2) β-myrcene, (3) limonene, (4) 1,8-cineole, (6) α-terpineol, (a) linalool-oxide (tentatively identified). (IS) internal standard (5 ng nerolidol).

(C) Quantification of emitted monoterpenes (sabinene, β-myrcene, limonene, 1,8-cineole linalool, α-terpineol) of flowers from *N. alata*. $n = 3$.

(D) Quantification of monoterpene emission (sabinene, β-myrcene, limonene, 1.8-cineole, α-terpineol) of flowers from *N. langsdorfii*, $n = 3$.

(E) Relative contribution (%) of the emitted monoterpenes of flowers from *N. alata*.

(F) Relative contribution (%) of the emitted monoterpenes of flowers from *N. langsdorfii*.

stage of both species were prepared, incubated with geranyl pyrophosphate (GPP), and the product 1,8-cineole was used to calculate the enzyme activities (Figure 3). During a period of 9 d, *N. alata* buds developed to senescent flowers (Figure 3A). The monoterpene enzyme activities reached highest levels on the second day after flower opening (approximately 800 pkat 1,8-cineole mg^{-1} protein) (Figure 3B). Thereafter, the enzyme activities decreased gradually to ~200 pkat mg^{-1} at day 5 and remained at this level until day 7. The same profiles were observed for β-myrcene and α-terpineol (data not shown). The duration of flower opening was shorter in *N. langsdorfii*; it lasts only for 3 d (Figure 3D). The monoterpene synthase enzyme activities reached maximum levels of ~500 pkat 1,8-cineole mg^{-1} at day 2 and decreased thereafter (Figure 3E). In order to study the emanation of 1,8-cineole during the day and night, the volatile emission was determined using 6-h intervals at different time points during the day and night. *N. alata* enzyme activities oscillated throughout the day, reached highest levels just before the transition from light to darkness and low levels at the end of the dark phase (Figure 3C), while oscillations could not be detected in *N. langsdorfii* (Figure 3F).

Localization of Monoterpene Synthases in *Nicotiana* Flowers

To determine which floral organs were involved in the synthesis and emission of the monoterpenes of the 'cineole cassette',

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

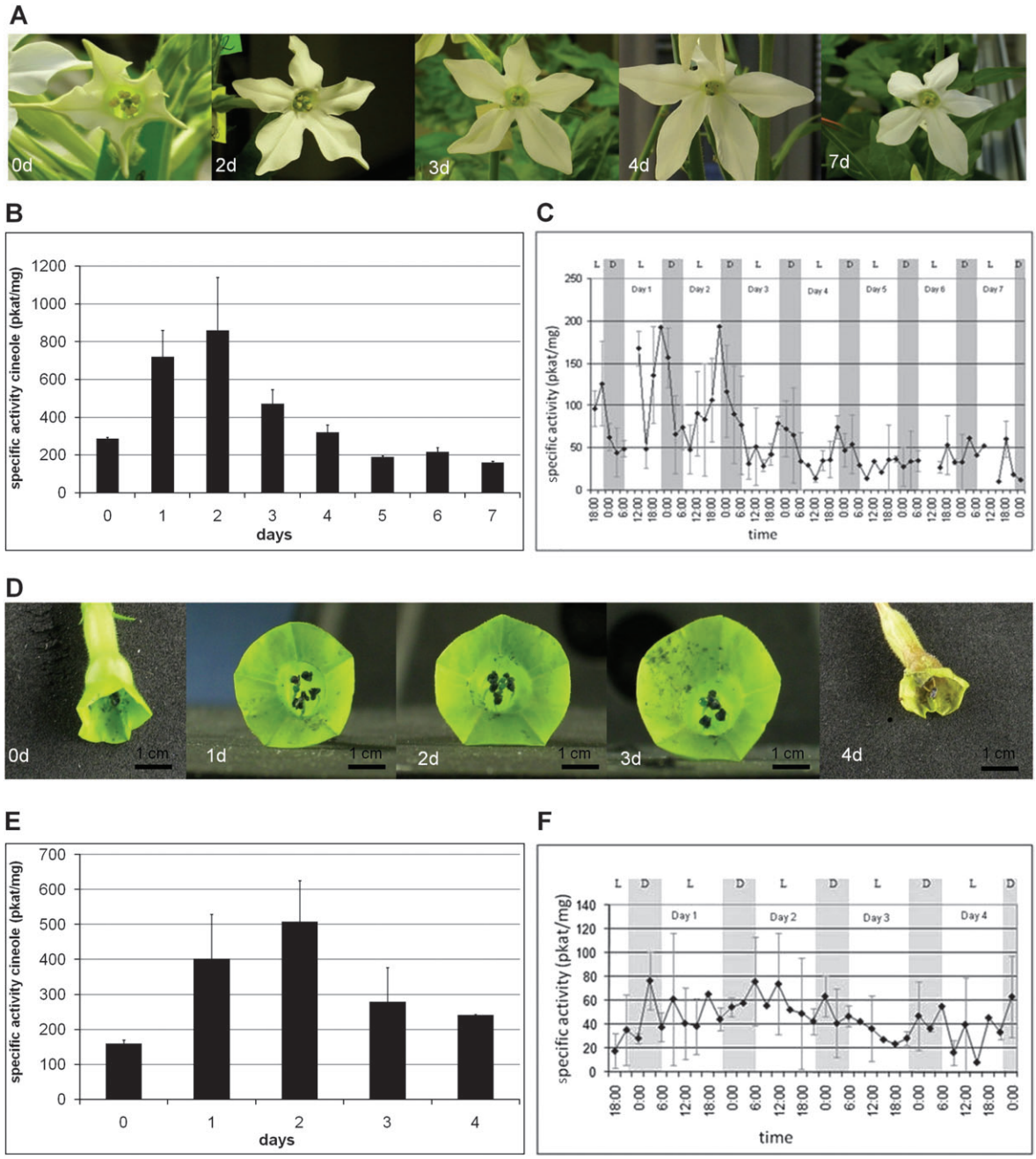

Figure 3. Monoterpene Synthase Activities during Flower Development.

(A) The development of the *Nicotiana alata* flower is depicted. Day of anthesis (day 0), senescence (day 8/9).

(B) Specific activities of a monoterpene synthase in petal extracts of *N. alata* flowers throughout flower development (0–7 d). The specific activities were calculated: 1,8-cineole pkat mg^{-1} total protein. Harvest started at the day of anthesis (n = 8).

(C) Determination of the specific enzyme activities in *N. alata* flowers in 6-h intervals (6 am, noon, 6 pm, and midnight). Harvest started at the day of anthesis (two independent experiments).

(D) The development of the *Nicotiana langsdorfii* flower is depicted. Day of anthesis (day 0), senescence (day 4).

(E) Specific activities of a monoterpene synthase in petal extracts of *N. langsdorfii* flowers throughout flower development (0–4 d). The specific activities were calculated: 1,8-cineole pkat mg^{-1} total protein. Harvest started at the day of anthesis (n = 8).

(F) Determination of the specific enzyme activities in *N. langsdorfii* flowers at 6-h intervals (6 am, noon, 6 pm, and midnight). Harvest started at the day of anthesis (two independent experiments).

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

proteins of various floral tissues and organs were extracted and analyzed via Western blot analysis (Figure 4A). Protein preparations of *N. alata* petals, sepals, pistil, stigma, and style were exposed to cineole synthase-specific antibodies (anti-CIN from *N. suaveolens*), and homologous enzymes were detected in the petals and pistils, but not in the other tissues.

Thin sections of the petal lobes of *N. alata* and *N. langsdorfii* were prepared in order to localize the cells or cell layers harboring the enzyme. Petals were incubated with the primary polyclonal antibody against the *N. suaveolens* CIN followed by a goat anti-rabbit antibody labeled with Alexa Fluor 488. In both species, the CIN was localized in the adaxial and abaxial epidermis cells and was almost not detectable in mesophyll cells (Figure 4B and 4D). The thin sections of petals were also incubated with pre-immune serum (= control) and showed autofluorescence (Figure 4C and 4E).

Isolation and Structural Characterization of Monoterpene Synthases of *N. alata* and *N. langsdorfii*

The presence of monoterpene synthases in *N. alata* and *N. langsdorfii* was predicted to occur in flowers. The isolation of the genes and characterization of the overexpressed recombinant enzymes will provide information about the production capabilities of these monoterpene synthases. Via RT–PCR, two sequences were cloned; both exhibited high sequence identities with the CIN of *N. suaveolens* (Figure 5A). The cloned coding sequences of *N. alata* and *N. langsdorfii* comprised 1 644 and 1 566 nucleotides and encoded mature proteins of 548 and 522 amino acids, respectively. The N-terminus of the *N. langsdorfii* protein started with the $RR(X)_8W$-motif, while the *N. alata* sequence extended 26 amino acids to the N-terminus. The sequences of both enzymes were 95% identical at the nucleotide and 99% at the amino acid level (Figure 5A). Phylogenetic analysis showed that the new sequences of *N. alata* and *N. langsdorfii* cluster together with the cineole synthase from *N. suaveolens* and a monoterpene synthase 1 (MTS1) of *Solanum lycopersicum* (Figure 5B). The enzymes of the species of section *Alatae* were more similar to each other than to the CIN of *N. suaveolens* or to other cineole or terpineol synthases from other plant species. Therefore, the enzymes classify to their taxonomic relationship (> Solanaceae) rather than to their product spectra, although similarties to the compound profiles of other TER and CIN enzymes were obvious (Table 1).

The sequences of both enzymes of *Nicotiana* species of section *Alatae* in comparison to *N. suaveolens* lacked two or six amino acids at three defined positions (Figure 5A, highlighted in gray). Furthermore, 38 amino acids were altered between the enzymes of *N. alata* and *N. langsdorfii* versus *N. suaveolens* (highlighted in gray). These variations were evenly distributed thoughout the entire protein sequences. Only two amino acid variations occur between *N. alata* and *N. langsdorfii* sequences (arrow in Figure 5A, indicated by* in Figure 6A). These two amino acids were closely located to the NALV- and DDXXD-motifs. 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 directs the formation of different product amounts and influences probably the enzyme specificity of *Salvia* monoterpene synthases (Kampranis et al., 2007). Since monoterpene synthases are functionally active in plastids, they harbor a transit peptide of 50–60 amino acids upstream of the $RR(X)_8W$-motif (RR-motif). So far, only 26 amino acids of the transit peptide were cloned for the enzyme of *N. alata* containing several serine and threonine residues (T^3, T^{21}, S^6, S^{11}, S^{14}, S^{15}, S^{16}). 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) and was conserved in the N-terminal region of the three *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 approximately 35–40 amino acids upstream of the DDXXD-motif was present in the monoterpene synthase sequences of *N. alata* and *N. langsdorfii*. The function of this motif was 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).

To get a better idea about the 3-D structure of the newly isolated genes, homology-based protein models constructed on the 3-D crystal structure of the limonene synthase from *Mentha spicata* (Hyatt et al., 2007) were performed (Figure 6). The structures of the enzymes were dominated by 25 α-helices. The sequences of *N. alata* and *N. langsdorfii* differ only by two amino acids (indicated in Figure 6A); consequentely, both 3-D structures were virtually identical. To allow a detailed view, 19 amino acids of the active pocket were enlarged (Figure 6B). The amino acids of the DDXXD motif (amino acids 300–304, indicated with a black dot in Figure 5A) as well the Arg263 of the RXR-motif (black dot in Figure 5A) formed the lower part of the active pocket, while the sequence of the amino acids 436–446 (Leu, Ala, Asn, Asp, Leu, Gly, Thr, Ser, Ser, Asp/Glu, Glu, black dot in Figure 5A) shaped the upper part of the active pocket. Two atoms of the cofactor Mg^{2+} (violet) stabilized the active site by coordinating with the aspartate-rich region. The substrate GPP (green and red) was docked into the model of the *N. alata* monoterpene synthase and the pyrophosphate (red) formed hydrogen bonds to the acidic amino acids of the DDXXD-motif as well as to Asn438. The Asn293 of the NALV-motif, Trp272, and Ile 395 (black dot in Figure 5A) also participate in the formation of the active pocket and are involved in the conversion of the substrate (Kampranis et al., 2007). Since the configuration of the active pockets of the enzymes of *N. alata* and *N. langsdorfii* are identical, identical reaction mechanisms and product spectra of both enzymes were expected.

Functional Analysis of Monoterpene Synthases from *N. alata* and *N. langsdorfii*

A putative function of the newly isolated enzymes from *N. alata* and *N. langsdorfii* was predicted based on the amino acid sequence similarity with the cineole synthase of *N.*

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

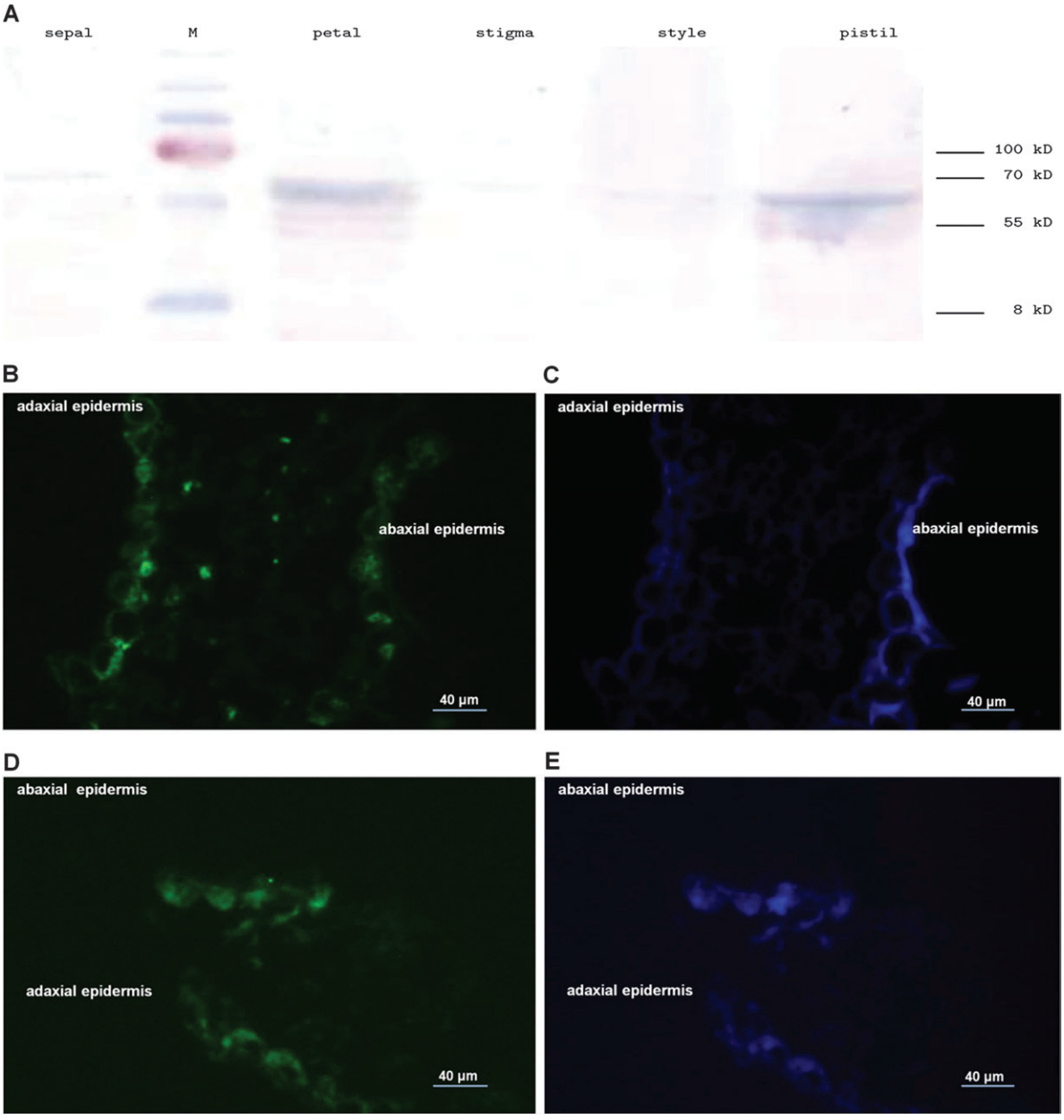

Figure 4. Organ-Specific Expression and Cellular Localization of the Monoterpene Synthase in Flowers of *Nicotiana alata* and *Nicotiana langsdorfii*.

(A) Detection of the monoterpene synthase in different organs and tissues of *N. alata* (harvest time point at 9 pm). 5 µg of total protein extracts of different floral organs were separated on a SDS polyacrylamide gel (12.5%) and Western blots were incubated with specific antibodies against the cineole synthase of *N. suaveolens* (see Methods).

(B) Thin sections of *N. alata* petals (2 d past anthesis, 9 pm) were incubated with the antibodies against the *N. suaveolens* CIN. The monoterpene synthase of *N. alata* was visualized by incubation with a second antibody labeled with Alexa Fluor 488.

(C) See (B), incubation with pre-immune serum (control).

(D) Thin sections of *N. langsdorfii* petals (2 d past anthesis, noon) were obtained and incubated with the antibodies against cineole synthase of *Nicotiana suaveolens*. The monoterpene synthase of *N. langsdorfii* was visualized by incubation with a second antibody labeled with Alexa Fluor 488.

(E) See (D), incubated with pre-immune serum (control).

suaveolens (Figure 5A). To ultimately determine the product spectra of these new enzymes, the recombinant genes were overexpressed in *E. coli* and purified via his-tag affinity chromatography (Supplemental Figure 1). The enzymes were cloned using PCR techniques specifically designed to only introduce the $RR(X)_8W$ 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.,

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

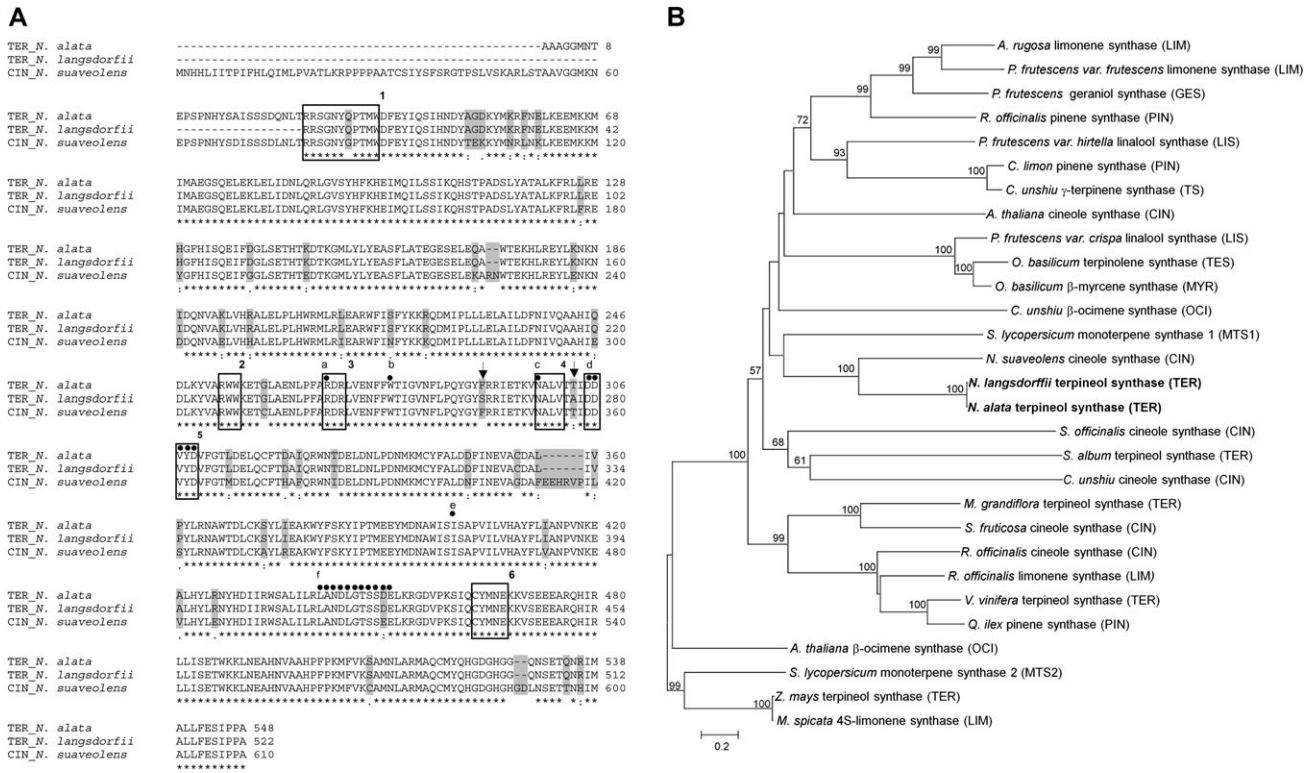

Figure 5. Monoterpene Synthase Sequence Alignment and Phylogeny.

(A) Alignment of the terpineol synthase (TER) amino acid 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) $RR(X)_8W$-motif, (2) RWW-motif, (3) RXR-motif, (4) NALV-motif, (5) DDXXD-motif, (6) CYMNE-motif. Indicated in gray: sequence differences between TERs of *N. alata* and *N. langsdorfii*, and CIN of *N. suaveolens*. Sequence differences between TERs of *N. alata* and *N. langsdorfii* (arrows). Amino acids involved in formation of the active pocket shown in Figure 6: a, Arg 263; b, Trp272; c, Asn293; d, Asp300–Asp304; e, Ile395; f, Leu436–Glu446.
(B) Phylogenetic relationship of terpineol synthases of *Nicotiana alata* and *Nicotiana langsdorfii* to other monoterpene synthases. Unrooted neighbor-joining phylogenetic tree based on amino acid sequence similarities. The tree was created with MEGA 4.0 and displayed by using TreeView. Gaps (Clustal W) and the target sequence upstream of the $RR(X)_8W$ motif of the alignment were removed. Plant species used for the tree construction (for accession numbers, see Methods).

2000). The purified enzymes were tested with the substrate GPP and the volatiles were analyzed by gas chromatography. The enzyme of *N. alata* produced five monoterpenes of the 'cineole cassette', sabinene, β-myrcene, limonene, 1,8-cineole, and α-terpineol (Figure 7A) and the enzyme of *N. langsdorfii* synthesized the same compounds and additionally α-pinene (Figure 7B). The major compound of both enzymes was α-terpineol (41 and 36%, *N. alata* and *N. langsdorfii*, respectively), suggesting the name terpineol synthase (TER) (Figure 7C and 7D). The other characteristic monoterpenes of the 'cineole cassette', sabinene, β-myrcene, limonene, and 1,8-cineole, contributed between 8 and 30%, while α-pinene was produced only at very low levels or was not detectable. As expected from the high sequence similarity, both terpineol synthases of *Nicotiana* species of section *Alatae* synthesized very similar volatile spectra. Whether the small qualitative and quantitative differences of the product profiles resulted from the two amino acid divergences between the *N. alata* and *N. langsdorfii* TER sequences needs to be investigated in the future.

Knowing the *in vitro* production capabilities of the TERs of *N. alata* and *N. langsdorfii*, it was interesting to correlate them to the floral emission patterns. In this comparative study, we included the data available for the closely related cineole synthase of *N. suaveolens* (Raguso et al., 2006; Roeder et al., 2007). The floral 'cineole cassette' monoterpene emissions of *N. alata* and *N. langsdorfii* species (Figure 8A and 8B, respectively) showed low concordance with the profiles of the isolated enzymes (Figure 8D and 8E, respectively), while both profiles were similar in the case of *N. suaveolens* (Figure 8C and 8F). Regarding the major compounds 1,8-cineole and α-terpineol, 1,8-cineole was the major compound emitted from *N. langsdorfii* and *N. suaveolens* flowers, while 1,8-cineole and α-terpineol were emitted at almost equal levels from *N. alata*. Controversily, the *in vitro* enzyme profiles were different from the emission profiles and varied for the *Alatae*

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

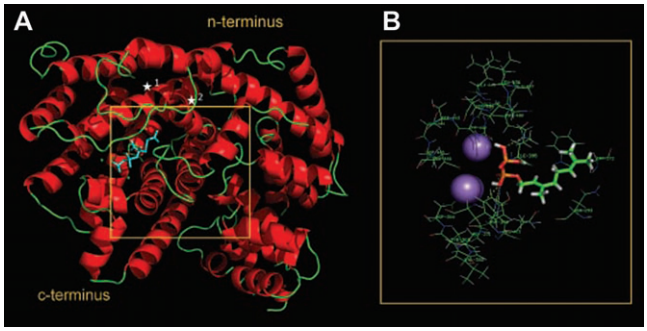

Figure 6. 3-D Model of *N. alata* Terpineol Synthase Active Pocket.
(A) 3-D structure was modeled using swiss pdb viewer. The crystal structure of the limonene synthase of *Mentha spicata* was the master sequence for modeling the 3-D structure of the *N. alata* enzyme (gaps in the alignment were deleted). Enlargement of the putative active site of the TER of *N. alata* and the two amino acids altered between *N. alata* and *N. langsdorfii* are indicated (star 1: thr303, star 2: phe290).
(B) The amino acids of the active pocket are shown. The substrate geranylpyrophosphate (green–red, respectively) and two Mg^{2+}-ions (violet) were docked into the active site using the program GOLD (Jones et al., 1997).

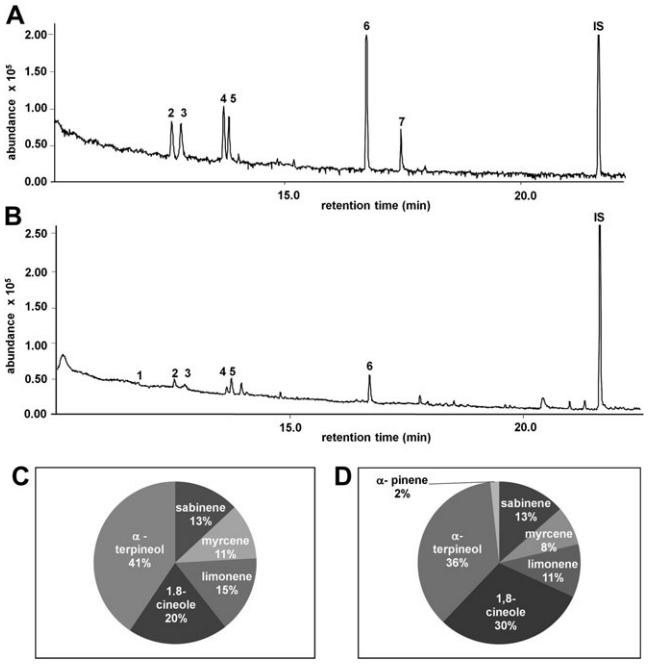

Figure 7. GC Chromatograms of the Terpineol Synthases of *Nicotiana alata* and *Nicotiana langsdorfii.* The recombinantly overexpressed his-tagged enzymes were purified via Ni-NTA affinity chromatography (Supplemental Figure 1). Enzyme assays were performed as described in Methods and volatile products 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 terpineol synthase of *N. alata*.
(B) GC chromatogram of the terpineol synthase of *N. langsdorfii.* (1) α-pinene, (2) sabinene, (3) β-myrcene, (4) limonene, (5) 1,8-cineole, (6) α-terpineol, (7) nerol, (IS) internal standard (5 ng *cis*-nerolidol). The spectra with non-induced *E. coli* culture (see Supplemental Figure 2) (= control).
(C) Relative contribution (%) of the 'cineole cassette' monoterpenes of the terpineol synthase of *N. alata* (n = 3).
(D) Relative contribution (%) of the 'cineole cassette' monoterpenes of the terpineol synthase of *N. langsdorfii* (n = 3).

species. The cineole/terpineol ratio was ~0.5 for *N. alata*, ~1 for the *N. langsdorfii* enzyme, and ~8 in the case of *N. suaveolens* cineole synthase (Table 2). Regarding the side products, the contribution of sabinene, β-myrcene, and limonene was <4% for each compound in the floral bouquets of *N. alata* and *N. langsdorfii*, while both recombinant enzymes released each volatile at levels between 8 and 15% *in vitro*.

Since a single cyclization reaction converts α-terpineol into 1,8-cineole (Figure 1B), the ratio of the product/substrate expresses the cyclization capacity of such enzymes. Consequently, the ratio was calculated for all known TERs and CINs (Table 2). Table 2 ordered terpineol and cineole synthases according to their product/substrate ratios. The enzymes from *Magnolia grandiflora*, *Santalum album*, *Zea mays*, and *Pinus taeda* synthesize α-terpineol and no 1,8-cineole. The enzymes of *Vitis vinifera* and *N. alata* synthesize more α-terpineol than 1,8-cineole, while 1,8-cineole was the dominant product of the cineole synthases of *N. suaveolens*, *Salvia fruticosa*, *A. thaliana*, *Salvia officinalis*, and *Citrus unshiu.*

DISCUSSION

Catalytic Reactions of TERs and CINs

Many *Nicotiana* species emit floral scents with characteristic compound compositions. Species of section *Alatae*, for example, emanate a defined set of monoterpenes, which are not released by species of the sister taxa N. section *Sylvestres*, N. section *Rusticae*, N. section *Suaveolentes* (Raguso et al., 2006). Here, we isolated monoterpene synthases of *Nicotiana alata* and *Nicotiana langsdorfii* of section *Alatae*, which synthesized the compounds 1,8-cineole, limonene, β-myrcene, α-pinene, sabinene, and α-terpineol simultaneously. Since the latter compound was the major product, the isolated enzymes were subsequently referred to terpineol synthases (TER). The monoterpenes produced by these newly isolated enzymes belong to the so called 'cineole cassette' (Raguso et al., 2006). While the minor products limonene, sabinene, pinene, and β-myrcene are common side products of many plant multiproduct enzymes (Table 1), the biosynthesis of the compounds 1,8-cineole and α-terpineol was established during the evolution of CINs and TERs (Table 1). α-Terpineol is the precursor of the bicyclic 1,8-cineole. In an additional reaction, the hydroxyl group reacts with the double bond to form the second cycle (Figure 1B; Chen et al., 2004; Degenhardt et al., 2009). The simultaneous appearance of both compounds can result from a non-efficient addition reaction of α-terpineol to 1,8-cineole or a premature termination reaction of the terpineol synthases

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

(Peters and Croteau, 2003; Iijima et al., 2004). While the conversion of the intermediate to the product of the enzymes of the *Alatae* species was limited, the enzyme of *Nicotiana suaveolens* produced significantly more 1,8-cineole than α-terpineol (Roeder et al., 2007). The cyclization reaction was even more efficient by the cineole synthases of *Arabidopsis thaliana*, *Salvia fruticosa*, *Salvia officinalis*, and *Citrus unshiu* (Table 2). The latter is the monoterpene synthase with highest efficacy regarding 1,8-cineole production. Controversily, enzymes that released α-terpineol but no 1,8-cineole are monoterpene synthases that most likely lack the ability to perform the cyclization reaction. Such enzymes were isolated from gymnosperms, monocots, and basal angiosperms, which represent the basal part of the plant kingdom. These TER enzymes may therefore be considered as precursor monoterpene synthases, which created and adapted the cyclization reaction during evolution and ultimately enzymes evolved that preferentially synthesize 1,8-cineole. The optimization of the cyclization reaction progressed differentially in different plant species and does not correlate with the taxonomic relationship of the plants (Bohlmann et al., 1998). The very similar 1,8-cineole/α-terpineol ratios (= cyclization efficiency) and volatile profiles of both species of section *Alatae* indicated a close relationship and late divergence during evolution of *N. alata* and *N. langsdorfii*, while the cineole synthase of the related species of the sister taxa *Suaveolentes* is more divergent and established a much better cyclization reaction during evolution. Interestingly, the *N. suaveolens* CIN varies in 38 amino acids from the *N. alata* and *N. langsdorfii* TERs and, consequently, it will be a future goal to determine which amino acid alteration(s) was/were important to establish an efficient cyclization reaction in these monoterpene synthases. Alternatively, the enzymes of the *Nicotiana* species can be compared with an enzyme that produces 100% cineole to see which amino acids play important roles.

Table 2. Cineole/Terpineol Ratios of Monoterpene Synthases of Different Plants.

	1,8-cineole/α-terpineol ratio
TER *Magnolia grandiflora*	α-terpineol, no 1,8-cineole
TER *Santalum album*	α-terpineol, no 1,8-cineole
TER *Zea mays*	α-terpineol, no 1,8-cineole
TER *Pinus taeda*	57% α-terpineol, no 1,8-cineole
TER *Vitis vinifera*	0.23
TER *Nicotiana alata*	0.5
TER *Nicotiana langsdorfii*	0.9
CIN *Nicotiana suaveolens*	7.8
CIN *Salvia fruticosa*	10.2
CIN *Arabidopsis thaliana*	21.7
CIN *Salvia officinalis*	79
CIN *Citrus unshiu*	1,8-cineole, no α-terpineol

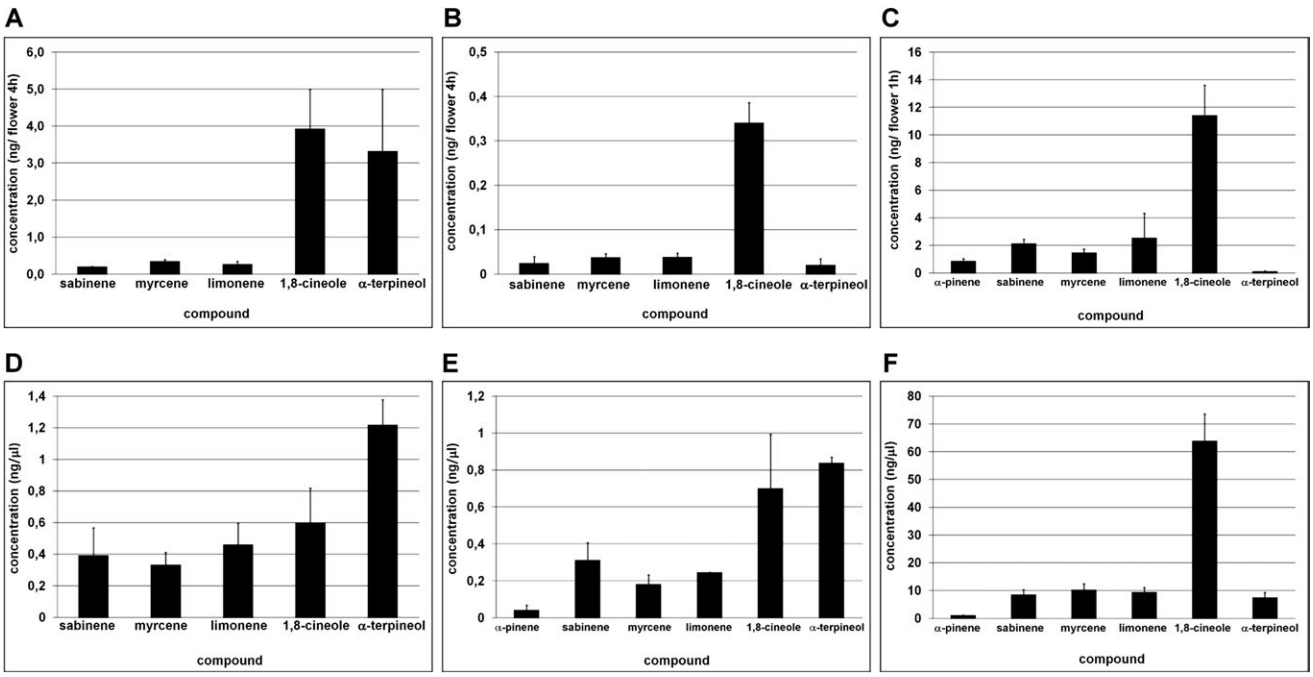

Figure 8. Comparison of Volatile Profiles of Floral Emission and Recombinant Monoterpene Synthase.

The 'cineole cassette' monoterpenes emitted from flowers of **(A)** *N. alata*, **(B)** *N. langsdorfii*, and **(C)** *N. suaveolens* were quantified (n = 3). The 'cineole cassette' monoterpenes synthesized from the recombinant TER and CIN enzymes of *N. alata* **(D)**, *N. langsdorfii* **(E)**, and *N. suaveolens* **(F)** were quantified using 5 ng μl^{-1} *cis*-nerolidol as internal standard (n = 3).

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

Several approaches were undertaken to determine amino acids that have important impact on the catalytic reaction mechanism determining the product formation. Domain swapping experiments of terpene synthases from, for example, *S. officinalis* and *Citrus limon* indicated the C-terminal region to influence the product specificity (Back and Chapell, 1996; El Tamer et al., 2003). Site-directed mutagenesis approaches allowed the identification of key amino acids of the active site as well as distant from it, which altered the product spectrum of several terpene synthases, such as *Abies grandis* pinene synthase (Hyatt and Croteau, 2005), *N. tabacum* 5-epi-aristolochene synthase, and *Hyoscyamus muticus* premnaspirodiene synthase (Greenhagen et al., 2006) and the *A. grandis* γ-humulene synthase (Yoshikuni et al., 2006). In the past, often, the capabilities of monoterpene synthases of phylogenetically unrelated plant species were compared with each other, but Kampranis et al. (2007) used a different strategy and studied similar enzymes in the related species *Salvia fruticosa*, *Salvia pomifera*, and *S. officinalis*. Amino acid sequences in two regions were different in *S. fruticosa* and *S. pomifera*, which attracted attention and were consequently mutagenized. Two mutations altered the 1,8-cineole and α-terpineol production significantly. Asn at position 338 of *S.f.* CINS1 enabled the enzyme to perform hydroxylation reactions to synthesize α-terpineol and 1,8-cineole at almost identical quantities. The mutated enzyme Ile338 changed the enzyme into a sabinene synthase and prevented the production of 1,8-cineole and α-terpineol. Conversely, alterations of the *S. pomifera* sabinene synthase at positions Ile327Asn and Thr328Ala favoured a threefold higher production of α-terpineol over 1,8-cineole. An additional mutation of Ser436Gly shifted the enzyme to the production of α-terpineol and prevented the cyclization reaction to 1,8-cineole. All three amino acids (*S.p.* Asn327, Ala328, Ser436) that were shown to be important for the terpineol and 1,8-cineole formation in *Salvia* species were conserved in the enzymes of the three *Nicotiana* species (Asn and Ala in the NALV motif and Ser at position 396 in *N. alata*, Figure 5A). Nevertheless, the *N. suaveolens* CIN produced a different cineole/terpineol ratio (approximately eightfold) than the enzymes of *N. alata* and *N. langsdorfii* (0.5–1-fold). Therefore, the amino acids emphasized in *Salvia* may not be decisive for the efficiency of the cyclization reaction in *N. alata*, *N. langsdorfii*, and *N. suaveolens*. The 3-D structures constructed on homology-based modeling did not identify amino acids or regions that may be relevant for the formation of the cineole/terpineol ratio. The two amino acids that differ between the *N. alata* and *N. langsdorfii* sequences are not located inside the active pocket, but rather in close vicinity to it. It is possible that these second-tier residues influence the active site surface and architecture to alter the cineole/terpineol ratio (Greenhagen et al., 2006). Ultimately, only 3-D structures based on protein crystallization including the substrate GPP might point out subtle differences that favor or prevent the cyclization reaction. Furthermore, detailed analysis of amino acids of enzymes of other species of section *Alatae* and section *Suaveolentes* and closely related species in section *Noctiflorae*, *Repandae*, *Sylvestres*, and *Petunioides* will further help to unravel the amino acid alterations that created differences in volatile qualities and quantities and directed the divergent evolution of the multiproduct cineole/terpineol synthases.

Function and Biological Relevance of TERs and CINs

The emission of the volatiles α-terpineol and 1,8-cineole appears widespread in the plant kingdom (Knudsen et al., 2006). 1,8-cineole has long been known to be active against plant pests; for example, it is toxic and a deterrent against certain insects and mammalian herbivores (Tripathi et al., 2001; Wiggins et al., 2003) and has antimicrobial activity (Hammer et al., 2003; Pina-Vaz et al., 2004). 1,8-Cineole also exhibits allelopathic activity, reducing germination and growth of a variety of plant species (Romagni et al., 2000; Singh et al., 2002). Beside these important roles of monoterpenes, up to now, only eight genes were cloned that synthesize α-terpineol and 1,8-cineole simultaneously (Tables 1 and 2). CIN/TER genes were isolated from *Pinus taeda*, *Vitis vinifera*, *Arabidopsis thaliana*, *N. alata*, *N. langsdorfii*, *N. suaveolens*, *S. fruticosa*, and *S. officinalis*. These genes were expressed in different plant organs. The mRNA from the *A. thaliana* CIN appeared in roots, while respective genes were expressed in flowers of *C. unshiu*, *V. vinifera*, *N. alata*, *N. langsdorfii*, and *N. suaveolens* and in leaves of *M. grandiflora* and *S. officinalis*. While the presence of cineole or terpineol monoterpene synthase was expected in petals, the appearance of a very strong signal in pistil tissue was a surprise. The role of this terpene synthase in pistils and its contribution to the floral scent bouquet are presently not understood. The different organ and tissue localizations of the respective mRNAs and enzymes suggest that the biological roles of the emitted volatiles might be quite different.

A correlation between scent emission of *Nicotiana* flowers and the activities of hawkmoths was observed, suggesting a function in pollination (pollination syndrome) (Raguso et al., 2003, 2006). The 'cineole cassette' TERs of *N. alata* and *N. langsdorfii* localized in the epidermis of the petals, which was also the case for the CIN of *N. suaveolens* (Roeder et al., 2007). Since the enzymes were present in the adaxial as well as abaxial epidermis, they facilitate the distribution of the monterpenes into all spherical directions. The detection of the enzyme in the pistils of *N. alata*, but not in the style or stigma, suggests its expression in the ovary. That ovaries can be the location of cineole synthases was documented for the enzyme of *C. unshiu* (Shimada et al., 2005). In contrast, in *N. suaveolens*, high expression of the cineole synthase occurred in stigma and styles (Roeder et al., 2007). The expression patterns of the TERs and CINs in pistil tissue correlated well with a high cineole emission from pistils of *N. suaveolens* (Effmert and Piechulla, unpublished results). In other plant species, other floral organs were employed in scent emissions or emit different volatile compounds/compositions (summarized in Effmert et al., 2005); for example, *Clarkia breweri* petals emit primarily S-linalool, while

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

linalool oxide is released from pistils (Pichersky et al., 1994). Further investigations are necessary to clarify whether 1,8-cineole emission from pistils is important for pollinator attraction, or necessary for defending pathogens, or functions as a communication signal. The latter two biological functions may apply for the TER and CIN genes that were expressed in leaves or roots. For the root-specific cineole synthase, it was shown that strongest expression was detected in the outer cell layers, the cortex, and the epidermis of mature roots, from which the lipophilic compounds synthesized by the enzyme could readily be released (Chen et al., 2004). In fact, it was shown that 1,8-cineole was a volatile compound emitted from hairy-root cultures of *Arabidopsis* (Steeghs et al., 2004). The TPS26 gene (TER) in *Zea mays* was expressed in roots and additionally in seedling leaf sheaths (Lin et al., 2008). The α-terpineol synthase in the hemi-parasitic tropical tree *Santalum album* was found in leaf and wood tissue but the terpenes produced by this enzyme could not be found in the distilled oil of sandalwood (Jones et al., 2008).

Often, the emission of floral scents occurs at different times during the day with different intensities or different compositions; high levels during the day or night are referred to as diurnal or nocturnal emissions, respectively (Piechulla and Effmert, 2010). Here, we show that the TER enzyme activities in petal extracts of *N. alata* exhibited an oscillation pattern with highest activity at the transition from day to night. This correlates well with the higher 'cineole cassette' monoterpene emission during the day (Raguso et al., 2003). The floral emission of *N. langsdorfii* flowers was not definite. One publication referred to diurnal monoterpene emission (Raguso et al., 2006) while another set of experiments indicated no difference between day and night (Raguso et al., 2003). Our determinations of the TER enzyme activities of *N. langsdorfii* did not support preferred diurnal or nocturnal synthesis. The results obtained with *N. langsdorfii* contrasted the oscillations observed at the transcriptional, translational, and post-translational levels of the CIN of *N. suaveolens*, while the emission and enzyme activity oscillations were alike with *N. alata* (Roeder et al., 2007). However, further experiments will be necessary, such as determination of the mRNA and protein levels and measurements under free running conditions to deduce whether a circadian clock regulates the temporal expression of the *N. alata* terpineol synthase.

Our investigations demonstrated significant discrepancies regarding the cineole/α-terpineol ratios observed under *in vitro* or *in planta* conditions. The overexpressed enzyme capabilities of *N. alata* and *N. langsdorfii* revealed other product quantities than those obtained from floral emissions. These quantitative discrepancies may indicate the existance of regulatory processes that mediate between *in vitro* capabilities of these enzymes and *in planta* floral emission. The *in vitro* situation reflects the maximum capacity of the enzyme measured under non-substrate limitations and optimized assay conditions (Roeder et al., 2007). However, it cannot be excluded that other metal co-factors and concentrations in combination with different pH values would result in the formation of different product quantities (Crock et al., 1997; Lee and Chappell, 2008). The emission profile *in planta* is influenced by several other conditions (e.g. substrate availability, cytosolic pH) and greenhouse growth conditions (e.g. temperature, humidity, biotic and abiotic stress). Alternatively, it is also possible that another monoterpene synthase is present in these species that preferentially produce 1,8-cineole in *N. alata* and *N. langsdorfii*. Another reason for this discrepancy could be that, *in planta*, the monoterpenes are subject to storage by differential glycosylation and hence the ratio of the emitted terpenes would get changed compared to the ratios produced by the enzymes. Altogether, several parameters may influence the process from synthesis until emission and it is presently not understood whether the emission is only based on diffusion or whether transport processes are involved. It will be the task of future work to unravel the processes and mechanisms that influence and control the monoterpene synthesis and volatiles emission *in planta*.

METHODS

Plant Growth

Nicotiana alata (TW7) and *Nicotiana langsdorfii* (TW 74) 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). A quicksilver vapourlamp (Osram, München, Germany) was used for illumination. Plants were watered with Hoaglands solution (Hoagland and Aronon, 1938).

Volatile Collection of Flowers from *N. alata* and *N. langsdorfii*

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 glass 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

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

of 1.4 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°C min^{-1}. Mass spectra were obtained by using the scan modus (total ion count, 40–280 mz^{-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) and (2) comparison of obtained spectra with spectra in the library of the National Institute of Standards and Technology (NIST147).

Crude Protein Extracts from Petals

Petals were harvested every 3 h 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 $Na_2S_2O_5$, 2 mM DTT, 5 mM ascorbate, 2 μl mercaptoethanol, 0.1 g PVPP (polyvinyl polypyrrolidone), and protease inhibitor cocktail tablets (Roche, Mannheim, Germany). The crude extracts were centrifuged for 15 min at 13 000 *g* (4°C). The supernatant was collected. After addition of 10% glycerol, the crude extract was stable at –70°C until used for Western blots, enzyme activity tests, or for long-term storage.

Immunolocalization of Cineole Synthase in Petals

The flowers of *N. alata* were harvested at 9 pm (1 h before darkness) and flowers of *N. langsdorfii* were harvested at 12 am, the time points of highest enzyme activity. The petals were cut into 2 × 3-mm pieces and were transfered into fixation solution, which consisted of 4% (wv^{-1}) paraformaldehyde with 0.1% (vv^{-1}) Triton-X 100 in phosphate buffered saline (PBS, 540 mM NaCl, 12 mM KCl, 6 mM KH_2PO_4, 32 mM Na_2HPO_4; pH 7.0–7.5). To assure that the parenchym of the flowers was also fixed, the samples were vacuum-infiltrated and again incubated with fixation buffer for 2 h. After incubation, the samples were washed with PBS and stepwise dehydrated with 100% ethanol. Then, the samples were passed through ascending ethanol/polyethylene glycol solutions (25, 50, 75% PEG) and, afterwards, embedded in a mixture of PEG 1500 and PEG 4000 (2:1, vv^{-1}). A sliding microtome (Jung, Heidelberg, Germany) cut pieces of 3-μm thickness, which were collected on poly-L-lysine-covered slides. After hardening overnight, the slices were washed with 0.1 M NH_4Cl to block aldehyd groups. Unspecific binding was additionally prevented by saturation with PBS (1) and 5% (wv^{-1}) bovine serum albumin (BSA) in PBS. The thin sections were then incubated with a specific antibody (anti-*N. suaveolens* CIN from rabbit (Davids Biotechnology, Regensburg, Germany) and diluted in 1:500 5% (wv^{-1}) BSA in PBS overnight at 4°C. The surplus of antibody was removed by washing with 0.1% (wv^{-1}) and 1.0% (wv^{-1}) BSA in PBS. Subsequently, the thin sections were incubated with the secondary antibody (Alexa Fluor® 488 goat anti-rabbit IgG, Invitrogen, Karlsruhe, Germany) for 90 min at 37°C. Afterwards, excess of the secondary antibody was removed by washing with 1 PBS. The slices were evaluated, using a transmission light fluorescence microscope equipped with the IP-Lab Scanlytics software.

RNA Extraction

RNA from *N. langsdorfii* (TW74) and *N. alata* (TW7) was 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 μl mercaptoethanol and 15 ml CTAB buffer (2% CTAB, (hexadecyltrimethylammoniumbromide), 2% PVP (polyvinylpyrrolidone K40), 100 mM Tris-HCl, 25 mM EDTA, 2 M NaCl add H_2O to 500 ml). Samples were extracted with 15 ml of chloroform:isoamyl alcohol (24:1) added twice and centrifugated for 20 min (10 000 *g*). The samples were resuspended in 0.25 M LiCl buffer to allow precipitation overnight at 8°C. RNA was centrifuged for 30 min at 4°C and the pellet was resuspended in 700 μl SSTE 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°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. The RT reaction was performed with the ThermoScript™ Reverse Transcriptase (Invitrogen, Karlsruhe, Germany) according to the manufacturer's recommendation. For cDNA synthesis, 2 μg RNA, 25 pmol Oligo dT (GAC TGG TCA ATC AGT TAC $(T)_{16}$) primer, 10 mM dNTPs, 0.1 M DTT, 5 μl 5× cDNA synthesis buffer were incubated at 58°C for 1 h. The reaction was terminated by heating at 85°C for 5 min. Subsequently, the PCR reaction was performed using the following components: 1 μg first-strand cDNA, 5 μl 10× *Pfu* buffer, 1.25 units *Pfu* DNA polymerase (Invitrogen, Karlsruhe Germany), 25 pmol of each antisense/sense primer and sterile H_2O was added to a final volume of 50 μl. To amplify the monoterpene synthase of *N. alata*, the primer combination CINS1 (start codon, 5′ATG AAC CAT CAC CTA ATC ATT ACT CCG3′) and R2 (5′GAC TGG TCA ATC AGT TAC3′), which binds to the adapter sequence of oligo dT, was used and resulted in a 1.8-kb fragment. For *N. langsdorfii*, the primer pair CINS6 (5′AGA CGT TCG GGG GAA A3′) and R2 binds to the $RR(X)_8W$ motif and resulted in a 1.6-kb fragment. The PCR reactions were performed at standard conditions: 98°C 2 min (1×), 98°C 30 s, 54°C 30 s, 68°C 1 min kb^{-1} (30×), and 10 min 72°C. After seperating the products on a 1.2% agarose gel, the nucleic acid fragments were extracted with the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany). For sequencing, the full-length monoterpene synthase cDNAs were ligated into the vector pBluescript II KS+ 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 mg ml^{-1} ampicillin, the transformants were analyzed by colony PCR using the vector primers M13 forward (5′GTA AAA CGA CGG CCA GT3′) and

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

M13 reverse (5′GGA AAC AGC TAT GAC CAT G3′) under standard conditions by using mastermix (Qiagen, Hilden Germany).

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. For expression of monoterpene synthases, the truncated terpene synthases without targeting sequence was amplified to avoid the formation of inclusion bodies by *E. coli* (Bohlmann et al., 1998). The 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. The PCR products were purified according to the manufacturer's recommendation (Qiagen, Hilden, Germany) ligated into the Champion™ pET SUMO vector (Invitrogen, Karlsruhe, Germany), and was sequenced to assure the correctness of the sequence. The plasmid was transformed into *E. coli* HMS 174 (DE3) (Novagene, Darmstadt, Germany). The bacteria were cultivated in 5 ml LB medium supplemented with 50 μg ml^{-1} kanamycin overnight at 37°C. 1 ml of an overnight preculture was inoculated into 50 ml LB medium containing 50 μg ml^{-1} kanamycin and 1% glucose and the culture was grown at 37°C to an OD_{600} 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 centifugation 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^{-1} lysozyme for 1 h on ice. After centrifugation at 4°C for 30 min (8 000 *g*), the resulting supernatant was either transferred into a Ni-NTA column or used for enzyme assays.

The 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 at 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, 1976).

SDS–Polyacrylamide Gel Electrophoresis and Western Blot Analysis

Samples (15 μg) of the crude protein extract or the purified recombinant enzyme was loaded onto polyacrylamide gels (12.5%), electrophoretically separated (Miniprotean, Bio-Rad Laboratories, Hercules, CA, USA), and finally transferred to PVDF (polyvinyldifluoride) 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* (diluted 1:3000 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 luminescent image analyser (LAS-1000, Fujifilm, Japan). Its luminescence (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. Enzyme assay was optimized and performed as described by Roeder et al. (2007). 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 synthase was incubated with 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 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, 5 ng internal standard were added (*cis*-nerolidol or nonanyl acetate, Roth, Karlsruhe, Germany, 5 ng μl^{-1}). The products were extracted by vortexing for 2 min and followed by a centrifugation for 2 min at 2000 *g*. Aliquots of the hexane phase were analyzed by GC–MS.

Sequence Analysis and Phylogenetic Tree Construction

The genes were sequenced using the ABI 3730xl sequencer (Roche/454 GS FLX) 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 (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 neighbor-joining 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:

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

Agastache rugosa LIM AY055214, *Arabidopsis thaliana* MYR/OCI AF178535, *Arabidopsis thaliana* CIN AY691947, *Citrus limon* PIN AF514288, *Citrus unshiu* OCI AB110642, *Citrus unshiu* CIN BAD91045, *Citrus unshiu* TS AB110639, *Magnolia grandiflora* TER ACC66282, *Mentha spicata* LIM AAC37366, *Nicotiana suaveolens* CIN EF175166, *Ocimum basilicum* TES AY693650, *Ocimum basilicum* MYR AY693649, *Perilla frutescens* var. *hirtella* LIS ACN42013, *Perilla frutescens* var. *crispa* LIS DQ897973, *Perilla frutescens* var. *frutescens* LIM AAG3148, *Perilla frutescens* GES ABB30218, *Quercus ilex* PIN AM283099, *Rosmarinus officinalis* CIN DQ839411, *Rosmarin officinalis* PIN ABP01084, *Rosmarin officinalis* LIM ABD77416, *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.

3-D Modeling

To build the homology-based 3-D structure of the *N.alata* TER, the limonene synthase (LIM) of *Mentha spicata* (Colby et al., 1993) was chosen because of the high sequence similarity of 96% (recommendation ⩾ 30%). Using ClustalW or BioEdit version 7.0.5. (as described above) as alignment tools, the cDNA sequence of *N. alata* TER was adjusted to the *M.s.* LIM sequence. The program SPDBV 3.7. (Swiss Institute of Bioinformatics) was used to perform and visualize the final 3-D structure. To carry out autodocking of the substrate in the active pocket, the GOLD program (Jones et al., 1997) was applied.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online.*

FUNDING

We thank the DFG (Pi 153/22) and University of Rostock for financial support to B.P. and A.F.

ACKNOWLEDGMENTS

We thank the two anonymous reviewers for their helpful comments 865 on an earlier version of the manuscript. The authors thank Dr Uta Effmert (University of Rostock) for many helpful suggestions. Elke Michalsky and Dr Robert Preissner (Charite, Berlin) gave us advice for the homology modeling. Sabine Werner and Dr Milton Stubbs (Martin-Luther-University, Halle-Wittenberg) helped with the docking experiments and Bettina Peters (formerly University of Rostock) prepared the antibodies against CIN of *N. suaveolens*. No conflict of interest declared.

REFERENCES

Aharoni, A., Giri, A.P., Verstappen, F.W.A., Bertea, C.M., Sevenier, R., Sun, Z., Jongsma, M.A., Schwab, W., and Bouwmeester, H.J. (2004). Gain and loss of fruit flavor compounds produced by wild and cultivated strawberry species. Plant Cell. **16,** 3110–3131.

Altschul, S.F., Gish, W., Miller, W., Byers, E.W., and Lipman, D.J. (1990). Basic local alignment search tool. J. Mol. Biol. **215,** 403–410.

Arimura, G.I., Kopke, S., Kunert, M., Volpe, V., David, A., Brand, P., Dabrowska, P., Maffei, M.E., and Boland, W. (2008). Effects of feeding *Spodoptera littoralis* on lima bean leaves: IV. Diurnal and nocturnal damage differentially initiate plant volatile emission. Plant Physiol. **146,** 965–973.

Arimura, G.I., Ozawa, R., Kugimiya, S., Takabayashi, J., and Bohlmann, J. (2004). Herbivore-induced defense response in a model legume: two spotted spider mites induce emission of (*E*)-β-ocimene and transcript accumulation of (*E*)-β-ocimene synthase in *Lotus japonicus*. Plant Physiol. **135,** 1976–1983.

Back, K., and Chappell, J. (1996). Identification functional domains within terpene cyclase using a domain-swapping strategy. Proc. Natl Acad. Sci. U S A. **93,** 6841–6845.

Bohlmann, J., Martin, D., Oldham, N.J., and Gershenzon, J. (2000). Terpenoid secondary metabolism in *Arabidopsis thaliana*: cDNA cloning, characterization, and functional expression of a myrcene/(*E*)-β-ocimene synthase. Arch. Biochem. Biophys. **375,** 261–269.

Bohlmann, J., Meyer-Gauen, G., and Croteau, R.B. (1998). Plant terpenoid synthases: molecular biology and phylogenetic analysis. Proc. Natl Acad. Sci. U S A. **95,** 4126–4133.

Bohlmann, J., Phillips, M., Ramachandiran, V., Katoh, S., and Croteau, R.B. (1999). cDNA cloning, characterization, and functional expression of four new monoterpene synthase members of the *Tpsd* gene family from grand fir (*Abies grandis*). Arch. Biochem. Biophys. **368,** 232–243.

Bohlmann, J., Steele, C.L., and Croteau, R.B. (1997). Monoterpene synthases from grand fir (*Abies grandis*). J. Biol. Chem. **272,** 21784–21792.

Bouwmeester, H.J., Gershenzon, J., Koning, M.C.J.M., and Croteau, R.B. (1998). Biosynthesis of the monoterpenes limonene and carvone in the fruit of caraway. 1. Demonstration of enzyme activities and their changes with development. Plant Physiol. **117,** 901–912.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Biochem. **72,** 248–254.

Byun-McKay, A., Godard, K., Toudefallah, M., Martin, D.M., Alfaro, R., King, J., Bohlmann, J., and Plant, A.L. (2006). Wound-induced terpene synthase gene expression in Sitka spruce that exhibit resistance or susceptibility to attack by the white pine weevil. Plant Physiol. **140,** 1009–1021.

Chang, S., Puryear, J., and Caiurney, J. (1993). A simple and efficient method for isolating RNA from pine trees. Plant Mol. Biol. Reporter. **11,** 113–116.

Chen, F., Ro, D.-K., Petri, J., Gershenzon, J., Bohlmann, J., Pichersky, E., and Tholl, D. (2004). Characterization of a root-specific *Arabidopsis* terpene synthase responsible for the formation of the volatile monoterpene 1,8-cineole. Plant Physiol. **135,** 1956–1966.

Chen, F., Tholl, D., D'Auria, J.C., Farooq, A., Pichersky, E., and Gershenzon, J. (2003). Biosynthesis and emission of terpenoid volatiles from *Arabidopsis* flowers. Plant Cell. **15,** 481–494.

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

Colby, S.M., Alonso, W.R., Katahira, E.J., McGarvey, D.J., and Croteau, R.B. (1993). 4S-limonene synthase from the oil glands of Spearmint (*Mentha spicata*). J. Biol. Chem. **268,** 23016–23024.

Crock, J., Wildung, M., and Croteau, R. (1997). Isolation and bacterial expression of a sesquiterpene synthase cDNA clone from peppermint (*Mentha x piperita*, L.) that produces the aphid alarm pheromone (*E*)-β-farnesene. Proc. Natl Acad. Sci. U S A. **94,** 12833–12838.

Croteau, R., Alonso, W.R., Koepp, A.E., and Johnson, M.A. (1994). Biosynthesis of monoterpenes: partial purification, characterization, and mechanism of action of 1,8-cineole synthase. Arch. Biochem. Biophys. **15,** 184–192.

Crowell, A.L., Williams, D.C., Davis, E.M., Wildung, M.R., and Croteau, R.B. (2002). Molecular cloning and characterization of a new linalool synthase. Arch. Biochem. Biophys. **405,** 112–121.

Degenhardt, J., Köllner, T.G., and Gershenzon, J. (2009). Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. Phytochem. **70,** 1621–1637.

Dudareva, N., Martin, D., Kish, C.M.K., Gorenstein, N., and Fäldt, J. (2003). (*E*)-β-ocimene and myrcene synthase genes of floral scent biosynthesis in snapdragon: function and expression of three terpene synthase genes of a new terpene synthase subfamily. Plant Cell. **15,** 1227–1241.

Effmert, U., Buβ, D., Rohrbeck, D., and Piechulla, B. (2005). Localization of the synthesis and emission of scent compound within the flower. In Biology of Floral Scent, Dudareva N. and Pichersky E., eds (Taylor and Francis Group, CRC, Boca Raton), pp. 105–123.

Effmert, U., Dinse, C., and Piechulla, B. (2008). Influence of green leaf herbivory by *Manduca sexta* on floral volatile emission by *Nicotiana suaveolens*. Plant Physiol. **146,** 1996–2007.

El Tamer, M.K., Lücker, J., Bosch, D., Verhoeven, H.A., Verstappen, F.W.A., Schwab, W., van Tunen, A.J., Voragen, A.G.J., de Maagd, R.A., and Bouwmeester, H.J. (2003). Domain swapping of *Citrus limon* monoterpene synthases: impact on enzymatic activity and product specificity. Arch. Biochem. Biophys. **411,** 196–203.

Fäldt, J., Arimura, G.-I., Gershenzon, J., Takabayashi, J., and Bohlmann, J. (2003a). Funtional identification of AtTPSO3 as (*E*)-β-ocimene synthase: a monoterpene synthase catalyzing jasmonate- and wound-induced volatile formation in *Arabidopsis thaliana*. Planta. **216,** 745–751.

Fäldt, J., Martin, D., Miller, B., Rawat, S., and Bohlmann, J. (2003b). Traumatic resin defense in norway spruce (*Picea abies*): methyl jasmonate-induced terpene synthase gene expression, and cDNA cloning and functional characterization of (+)-3-carene synthase. Plant Mol. Biol. **51,** 119–133.

Fischbach, R.J., Zimmer, W., and Schnitzler, J.P. (2001). Isolation and functional analysis of a cDNA encoding a myrcene synthase from holm oak (*Quercus ilex L.*). Eur. J. Biochem. **268,** 5633–5638.

Greenhagen, B.T., O'Maille, P.E., Noel, J.P., and Chappell, J. (2006). Identifying and manipulating structural determinates linking catalytic specificities in terpene synthase. Proc. Natl Acad. Sci. U S A. **103,** 9826–9831.

Günnewich, N., Page, J.E., Köllner, T.G., Degenhardt, J., and Kutchan, T.M. (2007). Functional expression and characterization of trichome-specific (–)-limonene synthase and (+)-α-pinene synthase from *Cannabis sativa.* Nat. Prod. Commun. **2,** 223–232.

Hammer, K.A., Carson, C.F., and Riley, T.V. (2003). Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil. J. Appl. Micobiol. **95,** 853–860.

Heath, R.R., and Manukian, A. (1994). An automated system for use in collecting volatile chemicals released from plants. J. Chem. Ecol. **20,** 593–608.

Hoagland, D.R., and Aronon, D.L. (1938). The water-culture method of growing plants without soil. California Agriculture Experimental Station, Circular. **374,** 1–39.

Hosoi, M., Ito, M., Yagura, T., Adams, R.P., and Honda, G. (2004). cDNA isolation and functional expression of myrcene synthase from *Perilla frutescens.* Biol. Pharm. Bull. **27,** 1979–1985.

Huber, D.P.W., Philippe, R.N., Godard, K.A., Sturrock, R.N., and Bohlmann, J. (2005). Characterization of four terpene synthase cDNAs from methyl jasmonate-induced Douglas-fir, *Pseudotsuga menziesii*. Phytochem. **66,** 1427–1439.

Hyatt, D.C., and Croteau, R.B. (2005). Mutational analysis of a monoterpene synthase reaction: altered catalysis through directed mutagenesis of (–)-pinene synthase from *Abies grandis*. Arch. Biochem. Biophys. **439,** 222–233.

Hyatt, D.C., Younn, B., Zhao, Y., Santhamma, B., Coates, R.M., Croteau, R.B., and Kang, C.H. (2007). Structure of limonene synthase, a simple model for terpenoid cyclase catalysis. Proc. Natl Acad. Sci. U S A. **104,** 5360–5365.

Iijima, Y., Gang, D.R., Fridman, E., Lewinsohn, E., and Pichersky, E. (2004). Characterization of geraniol synthase from the peltate glands of sweet basil. Plant Physiol. **134,** 370–379.

Ito, M., and Honda, G. (2006). Geraniol synthases from perilla and their taxonomical significance. Phytochem. **68,** 446–453.

Jia, J.W., Crock, J., Croteau, R., and Chen, X.-Y. (1999). (3R)-linalool synthase from *Artemisia annua L.*: cDNA isolation, characterization, and wound induction. Arch. Biochem. Biophys. **372,** 143–149.

Jones, C.G., Keeling, C.I., Ghisalberti, E.L., Barbour, E.L., Plummer, J.A., and Bohlmann, J. (2008). Isolation of cDNAs and functional characterization of two multi-product terpene synthase enzymes from sandalwood, *Santalum album L*. Arch. Biochem. Biophys. **477,** 121–130.

Jones, G., Willet, P., Glen, R.C., Leach, A.R., and Taylor, R. (1997). Development and validation of a genetic algorithm for flexible docking. J. Mol. Biol. **267,** 727–748.

Kampranis, S.C., et al. (2007). Rational conversion of substrate and product specificity in a *Salvia* monoterpene synthase: structural insights into the evolution of terpene synthase function. Plant Cell. **19,** 1994–2005.

Knudsen, J.T., Eriksson, R., Gershenzon, J., and Stahl, B. (2006). Diversity and distribution of floral scent. Botanical Review. **72,** 1–120.

Landmann, C., Fink, B., Festner, M., Dregus, M., Engel, K.H., and Schwab, W. (2007). Cloning and functional characterization of three terpene synthases from lavender (*Lavandula angustifolia*). Arch. Biochem. Biophys. **465,** 417–429.

Lee, S., and Chappell, J. (2008). Biochemical and genomic characterization of terpene synthese in *Magnolia grandiflora*. Plant Physiol. **147,** 1017–1033.

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

Lin, C., Shen, B., Xu, Z., Köllner, T.G., Degenhardt, J., and Dooner, H.K. (2008). Characterization of the monoterpene synthase gene tps26, the ortholog of a gene induced by insect herbivory in maize. Plant Physiol. **146,** 940–951.

Lu, S., Xu, R., Jia, J.-W., Pang, J., Matsuda, S.P.T., and Chen, X.-Y. (2002). Cloning and functional characterization of a β-pinene synthase from *Artemisia annua* that shows a circadian pattern of expression. Plant Physiol. **130,** 477–486.

Lücker, J., El Tamer, M.-K., Schwab, M.W., Vertappen, F.W.A., van der Plas, L.H.W., Bouwmeester, H.J., and Verhoeven, H.A. (2002). Monoterpene biosynthesis in lemon (*Citrus limon*) cDNA isolation and functional analysis of four monoterpene synthases. Eur. J. Biochem. **269,** 3160–3171.

Martin, D.M., and Bohlmann, J. (2004). Identification of *Vitis vinifera* (–) α-terpineol synthase by in silico screening of full-length cDNA ESTs and functional characterization of recombinant terpene synthase. Phytochem. **65,** 1223–1229.

Martin, D.M., Fäldt, J., and Bohlmann, J. (2004). Functional characterization of nine Norway spruce TPS genes and evolution of gymnosperm terpene synthases of the TPS-d subfamily. Plant Physiol. **135,** 1908–1927.

Maruyama, T., Ito, M., Kiuchi, F., and Honda, G. (2001). Molecular cloning, functional expression and characterization of δ-limonene synthase from *Schizonepeta tenuifolia*. Biol. Pharm. Bull. **24,** 373–377.

Maruyama, T., Seaeki, D., Ito, M., and Honda, G. (2002). Molecular cloning, functional expression and characterization of δ-Limonene Synthase from *Agastache rugosa*. Biol. Pharm. Bull. **25,** 661–665.

Masumoto, N., Korin, M., and Ito, M. (2010). Geraniol and linalool synthases from wild species of perilla. Phytochem. **71,** 1068–1075.

McKay, S.A.B., Hunter, W.L., Godard, K.A., Wang, S.X., Martin, D.M., Bohlmann, J., and Plant, A.L. (2003). Insect attack and wounding induce traumatic resin duct development and gene expression of (–)-pinene synthase in Sitka spruce. Plant Physiol. **133,** 368–378.

Nagegowda, D., Gutensohn, M., Wilkerson, C.G., and Dudareva, N. (2008). Two nearly identical terpene synthases catalyze the formation of nerolidol and linalool in snapdragon flowers. Plant J. **55,** 224–239.

Navia-Giné, W.G., Gomez, S.K., Yuan, J., Chen, F., and Korth, K.L. (2009). Insect-induced gene expression at the core of volatile terpene release in *Medicago truncatula.* Plant Signal. Behavior. **4,** 639–641.

Peters, R.J., and Croteau, R.B. (2003). Alternative termination chemistries utilized by monoterpene cyclases: chimeric analysis of bornyl diphosphate, 1,8-cineole, and sabinene synthase. Arch. Biochem. Biophys. **417,** 203–211.

Peters, R.J., Flory, J.E., Jetter, R., Ravn, M.M., Lee, H.J., Coates, R.M., and Croteau, R.B. (2000). Abietadiene synthase from grand fir (*Abies grandis*): characterization and mechanism of action of the 'pseudomature' recombinant enzyme. Biochem. **39,** 15592–15602.

Phillips, M.A., Wildung, M.R., Williams, D.C., Hyatt, D.C., and Croteau, R.B. (2003). cDNA isolation, functional expression, and characterization of (+)-α-pinene synthase and (–)–αpinene synthase from loblolly pine (*Pinus taeda*): stereocontrol in pinene biosynthesis. Arch. Biochem. Biophys. **411,** 267–276.

Pichersky, E., Raguso, R.A., Lewinsohn, E., and Croteau, R.B. (1994). Floral scent production in *Clarkia* (Onagraceae). I. Localization and developmental modulation of monoterpene emission and linalool synthase activity. Plant Physiol. **106,** 1533–1540.

Piechulla, B., and Effmert, U. (2010). Biosynthesis and regulation of flower scent. In Plant Development Biology: Biotechnological Perspectives, Vol. 2, Pua, E.C. Davey, M.R., eds, (Springer, Heidelberg), pp. 189–203.

Pina-Vaz, C., Gonçalves, A., Rodrigues, P.E., Costa-de-Oliveira, S., Tavares, C., Salgueiro, L., Cavaleiro, C., Gonçalves, M.J., and Martinez-de-Oliveira, J. (2004). Antifungal activity of *Thymus* oils and their major compounds. Europ. Acta. Dermatol. Venereol. **18,** 73–78.

Raguso, R.A., Levin, R.A., Foose, S.E., Holmberg, M.W., and Mc Dade, L.A. (2003). Fragrance chemistry, nocturnal rhythms and pollination 'syndromes' in *Nicotiana*. Phytochem. **63,** 265–284.

Raguso, R.A., Schlumpberger, B.O., Kaczorowski, R.L., and Holtsford, T.P. (2006). Phylogenetic fragrance patterns in *Nicotiana* sections *Alatae* and *Suaveolentes*. Phytochem. **67,** 1931–1942.

Rajaonarivony, J.I.M., Gershenzon, J., and Croteau, R.B. (1992). Characterization and mechanism of (4S)-limonene synthase, a monoterpene cyclase from the glandular trichomes of Peppermint (*Mentha x piperita*). Arch. Biochem. Biophys. **296,** 49–57.

Roeder, S., Hartmann, A.-M., Effmert, U., and Piechulla, B. (2007). Regulation of simultaneous synthesis of floral scent terpenoids by the 1,8-cineole synthase of *Nicotiana suaveolens*. Plant Mol. Biol. **65,** 107–124.

Romagni, J., Duke, S., and Dayan, R. (2000). Inhibition of plant asparagine synthetase by monoterpene cineoles. Plant Physiol. **123,** 725–732.

Shelton, D., Zabaras, D., Choha, N., Wyllie, S.G., Baverstock, P., Leach, D., and Henry, R. (2004). Isolation and partial characterization of a putative monoterpene synthase from *Melaleuca alternifolia*. Plant Physiol. Biochem. **42,** 875–882.

Shimada, T., Endo, T., Fujii, H., Hara, M., and Omura, M. (2005). Isolation and characterization of (*E*)-β-ocimene and 1,8-cineole synthases in *Citrus unshiu* Marc. Plant Science. **168,** 987–995.

Shimada, T., Endo, T., Fujii, H., Hara, M., Ueda, T., Kita, M., and Omura, M. (2004). Molecular cloning and functional characterization of four monoterpene synthese genes from *Citrus unshiu* Marc. Plant Science. **166,** 49–58.

Singh, H.P., Batish, D.R., Kaur, S., Ramezani, H., and Kohli, R.K. (2002). Comparative phytotoxicity of four monoterpenes against *Cassia occidentalis.* Ann. Applied Biol. **14,** 111–116.

Starks, C.M., Back, K., Chappell, J., and Noel, J.P. (1997). Structural basis for cyclic terpene biosynthesis by tobacco 5-epi-aristolochene synthase. Science. **277,** 1815–1820.

Steeghs, M., Bais, H.P., Gouw, J., Goldan, P., Kuster, W., Northway, M., Fall, R., and Vivanco, J.M. (2004). Proton-Transfer-Reaction Mass Spectrometry as a new tool for real time analysis of root-secreted volatile organic compounds in *Arabidopsis*. Plant Physiol. **135,** 47–58.

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011

Tamura, K., Dudley, J., Nei, M., and Kumar, S. (2007). MEGA4: molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24,** 1596–1599.

Trapp, S.C., and Croteau, R.B. (2001). Genomic organization of plant terpene synthases and molecular evolutionary implications. Genetics. **158,** 811–832.

Tripathi, A.K., Prajapati, V., Aggarwal, K.K., and Kumar, S. (2001). Toxicity, feeding deterrence, and effect of activity of 1,8-cineole from *Artemisia annua* on progeny production of *Tribolium castanaeum* (Coleoptera: Tenebrionidae). J. Econ. Entomol. **94,** 979–983.

van Schie, C.C.N., Haring, M.A., and Schuurink, R.C. (2007). Tomato linalool synthase is induced in trichomes by jasmonic acid. Plant Mol. Biol. **64,** 251–263.

Wang, G., Tian, L., Aziz, N., Broun, P., Dai, X., He, J., King, A., Zhao, P.X., and Dixon, R.A. (2008). Terpene biosynthesis in glandular trichomes of hop. Plant Physiol. **148,** 1254–1266.

Wiggins, N.L., McArthur, C., McLean, S., and Boyle, R. (2003). Effects of two plant secondary metabolites, cineole and gallic acid, on nightly feeding patterns of the common brushtail possum. J. Chem. Ecol. **29,** 1447–1464.

Williams, D.C., McGarvey, D.J., Katahira, E.J., and Croteau, R.B. (1998). Truncation of limonene synthase preprotein provides a fully active 'pseudomature' form of this monoterpene cyclase and reveals the function of the amino-terminal arginine pair. Biochem. **37,** 12213–12220.

Wise, M.L., Savage, T.J., Katahira, E., and Croteau, R.B. (1998). Monoterpene synthases from common sage (*Salvia officinales*). J. Biol. Chem. **273,** 14891–14899.

Yang, T., Li, J., Wang, H.-X., and Zen, Y. (2005). A geraniol-synthase gene from *Cinnamomum tenuipilum*. Phytochem. **66,** 285–293.

Yoshikuni, Y., Ferrin, T.E., and Keasling, J.D. (2006). Designed divergent evolution of enzyme function. Nature. **440,** 1078–1082.

Yuan, J.S., Köllner, T.G., Wiggins, G., Grant, J., Degenhardt, J., and Chen, F. (2008). Molecular and genomic basis of volatile-mediated indirect defense against insects in rice. Plant J. **55,** 491–503.

Yuba, A., Yazaki, K., Tabata, M., Honda, G., and Croteau, R.B. (1996). cDNA cloning, characterrization, and functional expression of 4S-(–)-limonene synthase from *Perilla frutescens*. Arch. Biochem. Biophys. **332,** 280–287.

Downloaded from http://mplant.oxfordjournals.org/ at Universitaetsbibliothek Rostock on December 7, 2011