Localization of Methyl Benzoate Synthesis and Emission in *Stephanotis floribunda* and *Nicotiana suaveolens* Flowers

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Abstract: **The emission of fragrances can qualitatively and quantitatively differ in different parts of flowers. A detailed analysis was initiated to localize the floral tissues and cells which contribute to scent synthesis in *Stephanotis floribunda* (Asclepiadaceae) and *Nicotiana suaveolens* (Solanaceae). The emission of scent compounds in these species is primarily found in the lobes of the corollas and little/no emission can be attributed to other floral organs or tissues. The rim and centre of the petal lobes of *S. floribunda* contribute equally to scent production since the amount of SAMT (salicylic acid carboxyl methyltransferase) and specific SAMT activity compensate each other in the rim region and centre region. *In situ* immunolocalizations with antibodies against the methyl benzoate and methyl salicylate-synthesizing enzyme indicate that the adaxial epidermis with few subepidermal cell layers of *S. floribunda* is the site of SAMT accumulation. In *N. suaveolens* flowers, the petal rim emits twice as much methyl benzoate due to higher total protein concentrations in the rim versus the petal centre; and, both the adaxial and abaxial epidermis house the BSMT (salicylic acid/benzoic acid carboxyl methyltransferase).**

Key words: **Scent emission, carboxyl methyltransferase, *Stephanotis floribunda, Nicotiana suaveolens,* cellular localization, methyl benzoate, methyl salicylate.**

Introduction

Flowers are a major source of scent volatiles. More than 2000 divergent volatile organic compounds are released (Knudsen et al., 1993 a; Knudsen, personal communication) as mediators for inter- and intra-organismic communication, either to attract or repel organisms. The low-molecular weights, low polarities, and low vapour pressures facilitate the volatility of the respective carbon compounds (volatile organic compound, VOC). Often, the last steps in the biosynthetic pathways of VOC synthesis promote volatility, e.g., the formation of esters due to methylations or acetylations. Esters are major components of fruit aromas, e.g., more than 100 different esters have been detected in ripe strawberry fruits (Zabetakis and Golden, 1997) and esters are also synthesized in vegetative parts of plants, either constitutively or in response to stress or insect infestation (Mattiacci et al., 2001; Dicke and Bruin, 2001; Shulaev et al., 1997). In floral fragrances, esters such as methyl salicylate, methyl benzoate, and benzyl acetate are often present (Knudsen et al., 1993 b; Effmert et al., 2005 b).

The process of volatile emanation depends either on the specific features of the chemical compounds and/or the morphology, anatomy, and cellular properties. Defined floral organs which possess fragrance release properties, called osmophores, were first described for *Arum ithalicum* (Arcangeli, 1883) and were extensively studied in a number of plant species, primarily Araceae, Aristolochiaceae, and Orchidaceae (Vogel, 1962). The osmophores (scent glands) can be found in the whole inflorescence as part of the perianth, bracts, appendices of peduncles or anthers, and vary in shape and appearance (plane-, whip-, brush-, club-, or palp-shape), usually face towards the adaxial side of the perianth and have a bullate, rugose, pileate, conical, or papillate epidermis. Respective subepidermal cell layers often comprise a dispersed vacuome, and the cells contain enlarged nuclei, abundant rER, large starch deposits, and, sometimes, lipoid droplets (summarized in Effmert et al., 2005 c).

Furthermore, flower organs contribute to a different extent to the complex bouquet of a flower. Usually, petals are the main source of fragrance emission, but stamens, pistils, and sepals can also present specific VOC compositions. For example, the petal scent of *Rosa rugosa* (hedgehog rose) contains the dominating compounds (citronellol, nerol, geraniol, 2-phenylethanol) while sesquiterpenes are only found in sepals and the gyneocium (Dobson et al., 1990), and in *Crysanthemum coronarium* (garland crysanthemum), camphor, and *cis*-crysanthenyl acetate are primarily released from tubular and ligulate florets, while ocimene and myrcene are emitted from bracts (Flamini et al., 2003). Pollen also emits scents, however, the constituents are remarkably different to those emitted from other flower parts and seem to contribute little to the whole flower bouquet; nevertheless, it cannot be excluded that they are ecologically relevant (Dobson et al., 1990; Bergström et al., 1995; Dobson et al., 1996).

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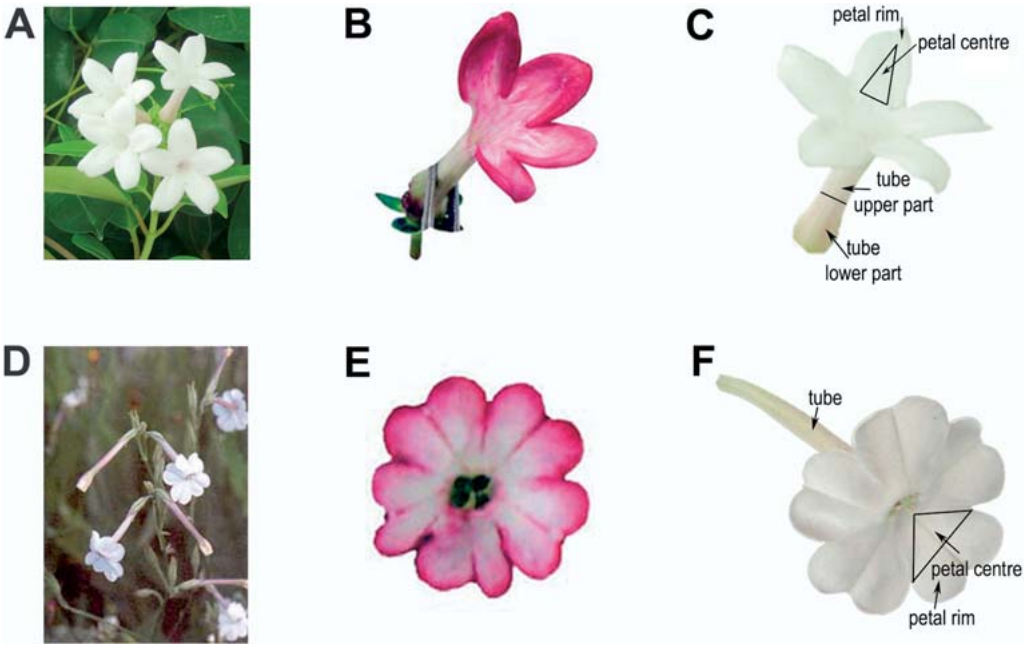


Fig. 1 *Stephanotis floribunda* and *Nicotiana suaveolens* flowers. (**A**) An umbel of *S. floribunda*. (**B**) *S. floribunda* flower (day 1 after opening) was stained for 1 h with neutral red. (**C**) *S. floribunda* petal areas are assigned: petal rim, petal centre, upper and lower tube. (**D**) Inflorescence of *N. suaveolens*. (**E**) *N. suaveolens* flower (day 2 after opening) was stained for 24 h with neutral red. (**F**) *N. suaveolens* petal areas are assigned: petal rim, petal centre, and tube.

In many flowering scent-emitting plants, osmophores or other scent-synthesizing and emitting glands, tissues or morphological structures are not obvious and have so far remained elusive because of technical limitations. Since progress was made in the elucidation of the biosynthetic pathways of volatiles, the respective enzymes and genes now provide very good tools for detailed localization experiments and are presently the best indicator for VOC synthesis.

The major floral scent compounds are terpenoids, phenylpropanoids, and fatty acid derivatives. Oxidoreductases, dehydrogenases, acyltransferases, methyltransferases, and terpene synthases are the pronounced enzymes involved in fragrance formation (Dudareva et al., 2004). A new class of O'methyltransferases, the carboxyl methyltransferases, was recently shown to be involved in the synthesis of volatile esters such as methyl salicylate and methyl benzoate. These VOCs are synthesized from phenylalanine via the phenylpropanoid pathway, to form benzoic acid and 2-hydroxybenzoic acid (salicylic acid). Depending on their biochemical properties (K_m value and catalytic efficiency), these enzymes prefer salicylic acid and/or benzoic acid and are therefore named SAMT, BSMT, or BAMT.

Flowers of *S. floribunda* and *N. suaveolens* emit complex scents, primarily in the evening or at night (nocturnal emission) (Pott et al., 2002; Raguso et al., 2003). Methyl benzoate and methyl salicylate are common VOC compounds of both species and the respective methyltransferase genes/enzymes of these species have previously been cloned and analyzed by our group. *S. floribunda* (Asclepiadaceae) is native to Madagascar and its umbel contains approximately 12 white flowers, each fused from five petals comprising a *ca.* 5-cm tube with five lobes, five fused sepals, and a gynostegium. *N. suaveolens* (Solanaceae) is native to Australia and the white flowers have a similar appearance (tube and five lobes) to *S. floribunda*. To investigate the process of floral scent emanation of these two species, i) scent profiles of various petal areas were obtained, ii) petal morphologies were investigated, and iii) antibodies against the SAMT and BSMT enzymes were used to localize the enzymes in the floral tissue.

Methods

Plant material and growth conditions

Nicotiana suaveolens (Lehmann) plants were raised in a growth room (18 ± 1 °C; photoperiod: 14 h; illumination 160 µE m^{-2} s^{-1}) on vermiculite, and watered with Hoagland solution. *Stephanotis floribunda* (Brongn.) plants were kept in soil in the greenhouse with supplemental light between 6.00 a.m. and 10.00 a.m. (200 µE m^{-2} s^{-1}) during autumn and winter and at ambient temperatures (25 ± 10 °C).

Analysis of head space volatiles from flower parts

Flowers of defined age were harvested at the time of maximum volatile release (Pott et al., 2002), immediately dissected into parts such as petal rim, petal centre, and tube (Figs. **1 C, F**) and separately sealed into 20 mL head space vials. In order to standardize continued volatile emission into the vial, the separated flower part was directly compared with a correspondingly cut whole flower. GC/MS analysis was immediately performed with a QP5000 (70 eV; Shimadzu, Kyoto, Japan) equipped with a head space autosampler AOC5000 (CTC Analytics, Zwingen, Switzerland). After equilibration (10 min, 35 °C), 500 µL of head space air were directly transferred into the injection port (200 °C) and applied to a DB5MS column (60 m × 0.25 mm × 0.25 µm; J&W Scientific). After sampling for 2 min, the temperature programme commenced with a gradient (10 °C/min) at 35 °C up to 280 °C, with a final hold for 5 min. The carrier gas helium had a flow rate of 1.1 mL/min and a linear velocity of 28 cm/s. Mass spectra obtained using the scan mode (total ion chromatogram, mass range 40 – 300) were

compared with spectra from the National Institute of Standards and Technology library (NIST147) and/or compared with spectra and retention times of standards. Experiments were replicated twice. The emission of volatiles was estimated per square centimetre.

Neutral red staining

Neutral red is a weak cationic dye that selectively penetrates intact tissue and thus indicates different permeability of the plant tissue. One- and 2-d-old flowers were submersed in a 0.1% (w/v, tap water) aqueous solution of neutral red. The staining was performed in the dark for 24 h for *N. suaveolens* and 1 h for *S. floribunda* flowers. After rinsing with tap water, images were taken using an Olympus C-3030 Zoom camera (Tokyo, Japan) and the appending Camedia processing software.

Scanning electron microscopy

Flowers of *N. suaveolens* and *S. floribunda* were harvested and immediately fixed in 4% (v/v) glutaraldehyde in Tris-HCl (0.1 mM; pH 8.0) for 15 to 30 min. Samples were dehydrated with acetone and, after critical-point drying and sputter coating with gold, they were investigated using a digital scanning electron microscope (DSM 960A; Carl Zeiss, Oberkochen, Germany) equipped with digital image processing software package (DIPS 2.5).

Preparation of antibodies

The *bsmt* (*N. suaveolens*) and *samt* (*S. floribunda*) genes were amplified by RT-PCR and cloned into the vectors pET101/D-Topo® and pCR®T7/CT-TOPO (Invitrogen, Karlsruhe, Germany), respectively, which carry a C-terminal polyhistidine (6 × His) tag. Plasmids were transformed into the *E. coli* over-expression strain HM174[D3]. The over-expressed proteins were purified by Ni-NTA affinity chromatography according to the manufacturer's recommendations (Qiagen, Hilden, Germany). As the final purification step, SAMT was quantitatively separated by SDS-PAGE and recovered from the gel using an electroeluter (Bio-Rad Laboratories, Hercules, CA). BSMT was purified to homogeneity via Ni-NTA chromatography. The polyclonal antibodies were produced by Biotrend (Cologne, Germany) and Davids Biotechnology (Regensburg, Germany) and were highly specific to the *S. floribunda* SAMT and *N. suaveolens* BSMT.

Immunolocalization of BSMT and SAMT

Flowers of *N. suaveolens* and *S. floribunda* of different age were harvested at the time of maximum enzyme activity (Pott, 2003; Pott et al., 2003), at 1.00 a.m. and 7.00 a.m., respectively. Tissue pieces (2 × 3 mm) from defined areas of the petal rim, petal centre and tube (Figs. **1 C, F**) were cut and immediately submersed in 4% (w/v) paraformaldehyde supplemented with 0.1% (v/v) 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). In order to completely remove air from the parenchyma, samples were vacuum-infiltrated and the final fixation was then performed at room temperature for 2 h. After washing with PBS, the tissue pieces were stepwise dehydrated to 100% ethanol, underwent an ascending ethanol/polyethylene glycol (25, 50, 75% PEG) series, and were finally embedded in a mixture of PEG 1500 and PEG 4000 (2 : 1, v/v). Sections of 3-µm thickness were prepared with a sliding microtome (Jung, Heidelberg, Germany) and collected on poly-L-lysine-coated slides. After rinsing with PBS, the sections were treated with NH_4Cl (0.1 M) to block aldehydes, rinsed again with PBS, and incubated for 30 min with 5% (w/v) BSA in PBS to reduce unspecific binding. The incubation with adequate BSMT- or SAMT-specific antibody (dilution 1 : 500 in 5% [w/v] BSA in PBS) was performed overnight at 4 °C. After washing with 0.1% (w/v) and 1.0% (w/v) BSA in PBS, the sections were incubated with the secondary antibody, goat-anti-rabbit IgG-Alexa Fluor®488 (dilution 1 : 500 in PBS; Molecular Probes, Invitrogen, Karlsruhe, Germany), for 90 min at 37 °C. Sections were rinsed with PBS, counterstained with DAPI (1 mg/mL; Merck, Darmstadt, Germany), and covered with Citifluor-Glycerol (Plano, Wetzlar, Germany) and a cover slide, which was sealed with nail varnish. Sections used as controls were treated in the same way but the primary antibody was replaced by the appropriate preimmune serum.

Fluorescence imaging

Microscopy was performed using the inverse microscope Diaphot 300 (Nikon, Düsseldorf, Germany) with a 100 × 1.4 NA or 60 × 1.4 NA objective lens. Images were recorded with a cooled CCD camera (SenSys; Photometrics, Munich, Germany) equipped with a B-2A filter (Nikon, Düsseldorf, Germany) and Shutter Uniblitz D122 (Vincent Assoc., Rochester, NY), and processed using the IPLab Spectrum 3.2 software (Scanalytica, Fairfax, VA). The intensity of fluorescence was quantified using the software Metamorph software version 6.1 (Molecular Devices, Sunnyvale, CA).

Bright field microscopy

Sections of flower tissue collected on poly-L-lysine slides were rinsed with PBS and stained with 0.1% (w/v) toluidine blue in PBS for 30 min. Sections were covered with PBS and a cover slide, sealed with nail varnish, and analyzed using a Zeiss Axioplan imager (Jena, Germany). Images were processed with AxioVision (Zeiss, Jena, Germany).

Enzyme extraction and activity assay

Crude extracts were prepared as described by Wang et al. (1997). Flowers of *N. suaveolens* and *S. floribunda* of different age were harvested at the time of maximum enzyme activity (Pott, 2003; Pott et al., 2003) at 1.00 a.m. and 7.00 a.m., respectively, dissected into petal rim, petal centre, and tube as shown in Figs. **1 C** and **F**, and immediately submersed in freshly prepared ice-cold extraction buffer (50 mM BisTris-HCl, pH 6.9; 14 mM β-mercaptoethanol, 1% [v/w] PVP-40, and 5 mM $Na_2S_2O_5$; 5 µL/mg FW). The tissue was homogenized in a chilled mortar with 1/10 volume of quartz sand, centrifuged for 15 min at 12 000 × g, and, after addition of 25% (v/v) glycerol, the supernatant was immediately used for enzyme assays or stored at – 20 °C. Protein quantification was done according to Bradford (1976) or Esen (1978).

The enzyme assay was performed according to Wang et al. (1997). The reaction contained 10 µL crude enzyme extract, 10 µL assay buffer (250 mM Tris-HCl, pH 7.5, 25 mM KCl), 1 µL benzoic acid (50 mM in ethanol), 1 µL S(methyl-^{14}C)-adeno-

syl-L-methionine (20 µCi/mL; 52 mCi/mmol; Hartmann Analytics, Braunschweig, Germany), and water to a final volume of 50 µL. Samples were incubated for 40 min at 25 °C. The reaction was terminated by adding 3 µL HCl and the product was extracted with 100 µL ethyl acetate. 30 µL ethyl acetate were mixed with 2 mL scintillation liquid (PerkinElmer, Boston, MA) and radioactivity was measured in a liquid scintillation counter (Tri-Carb 2100TR, Packard, Canberra).

SDS-PAGE and Western blot analysis

20 µg of the crude protein extract were loaded onto a polyacrylamide gel (10%) and electrophoretically separated (Miniprotean, Bio-Rad Laboratories, Hercules, CA). Proteins were transferred to a nitrocellulose membrane (Optitran BA-S 83, Schleicher and Schüll, Dassel, Germany) using a mini-tank blotting gel cassette (XCell II, Novex, San Diego, CA). The membranes were placed for at least 2 h in Tris-buffered solution (TBS; 20 mM) with 0.05% (v/v) Triton X-100, 4% (w/v) skimmed milk, 1% (w/v) BSA (blocking solution). After incubation with the appropriate BSMT- or SAMT-specific antibody (diluted 1 : 1000 in blocking solution) for 2 h and repeated washing with TBS containing 0.05% (v/v) Triton-X100 (TBS-Triton), the membrane was incubated with the secondary antibody anti-rabbit alkaline phosphatase conjugate (diluted 1 : 20 000 in TBS-Triton; Sigma-Aldrich, St. Louis, MS) and washed again with TBS-Triton and TBS. The membrane was equilibrated in detection buffer (100 mM Tris-HCl, 150 mM NaCl, 50 mM $MgCl_2$), incubated with CSPD (Roche, Mannheim, Germany; 0.25 µM in detection buffer) in darkness, and exposed to the Luminescent Image Analyzer LAS-1000 (Fujifilm, Japan). The luminescence was read for 15 – 30 min and quantification was done with the Fujifilm Image Gauge software. Additional staining was performed with nitroblue tetrazolium salt and 5-bromo-4-chloro-3-indolyl phosphate (50 mg/mL in dimethylformamide; Roche, Mannheim, Germany; 2 : 1).

Results

Tissue-specific scent emission in petals

The human nose was for a long time the most important instrument used to determine scent emitting organs or tissues in flowers. During his investigations, S. Vogel (1962) observed a correlation between the presence of osmophores and the stainability of the tissue with neutral red (Vogel, 1962; Pridgeon et al., 1985; Stern et al., 1987). Apparently, intact osmophore tissue is selectively able to take up and retain this vital dye due to increased permeability of the cell walls of osmophores and the storage capacity of the vacuoles. The nonionic form of neutral red (aqueous solution, 0.1%, pH 8) penetrates the tonoplast and enters the vacuole. Due to the slight acidic environment of the vacuole, the neutral red cation remains there. With the intention to localize the scent emission areas in flowers of *S. floribunda* and *N. suaveolens* (Figs. **1A, D**), the flowers were incubated for 1 – 24 h in neutral red solution. Differential staining of the floral tissue was observed. The five adaxial petal lobes of *S. floribunda* were stained red (Fig. **1B**), the rims being intensively red while in the centre pointing towards the tube, less staining was observed. The abaxial epidermis (not shown) and the tube remained unstained. Similarly, the five petal lobes of *N. suaveolens* exhibit intensive red staining on the petal rims, which vanishes towards the centre and the tube (Fig. **1E**).

This neutral red staining of the flowers of *S. floribunda* and *N. suaveolens* may be indicative for emitting and non-emitting tissue of the petals. Based on this assumption, the petals of *S. floribunda* and *N. suaveolens* were dissected into petal rim, petal centre, and upper and lower tube tissue regions (Figs. **1C, F**), and the emitted volatiles of respective floral sections were separately determined. The GC-MS profiles from *S. floribunda* petal parts showed six pronounced peaks, corresponding to benzyl alcohol, (E)-β-ocimene, β-linalool, methyl benzoate, α-farnesene, and one unidentified compound ("e") (Fig. **2A**). Ocimene, methyl benzoate, and α-farnesene are emitted primarily from the petal rim and centre, while the tube tissues of *S. floribunda* contribute very little to total floral emanation. The emission of methyl benzoate per petal area (cm^{-2}) is almost equal from the petal centre (44%) and the petal rim (51%) (Fig. **2B**). About 5% is emitted from the upper part of the tube. Two dominant volatiles are emitted from *N. suaveolens* flower parts, methyl benzoate, and benzyl benzoate (Fig. **2C**). The emission of methyl benzoate per petal area (cm^{-2}) is twice as high from the petal rim compared to the petal centre (Fig. **2D**).

This analysis demonstrates that those petal parts of the *S. floribunda* and *N. suaveolens* flowers which face the open air are the primary source of methyl benzoate and other volatiles. Emission of VOCs from petal lobes rather than from the tube tissue allows unhindered volatile distribution in all directions and optimizes the attraction and guidance of pollinators over a long distance, together with orientation within the flower.

Petal morphology and anatomy

The petal lobes of *S. floribunda* and *N. suaveolens* flowers are very important for the emission of volatiles, in particular of methyl benzoate. To further characterize scent synthesis at the cellular level, the anatomy of the petal lobes was analyzed in detail. Thin sections of lobe tissue were investigated by either light or scanning electron microscopy. The petals of *S. floribunda* exhibit a resistant and rigid appearance. They are approximately 150 µm thick, with tight flat to bullate epidermal cells (Figs. **3A, B, 4A, B**). The waxy cuticle of the adaxial epidermis seals the surface. Wax layers of the abaxial epidermis are even thicker and a flat and rugose surface is apparent (Figs. **3B, 4B**). Abaxial subepidermal multi-cell layers contain small, tightly connected round cells, while only one additional uniform cell layer is found under the adaxial epidermis (Fig. **3B**). The density and tight packaging of these cell layers are the basis for the rigid appearance of the *S. floribunda* corolla. Towards the interior of the petal, the mesophyll cells become larger, have a less uniform shape, and are separated by large intercellular spaces (Figs. **3A, B**). The *N. suaveolens* petals, in contrast, are soft and fragile and approximately 100 µm in diameter. The thin section of the lobe has bullate to rugose epidermal cell layers on both sides of the petal (Fig. **3C**). A distinct cuticle is not detectable and supporting subepidermal cell layers are not present, but adjacent to the epidermis, a characteristic loose mesophyll cell system with large intercellular spaces is apparent (Fig. **3C**).

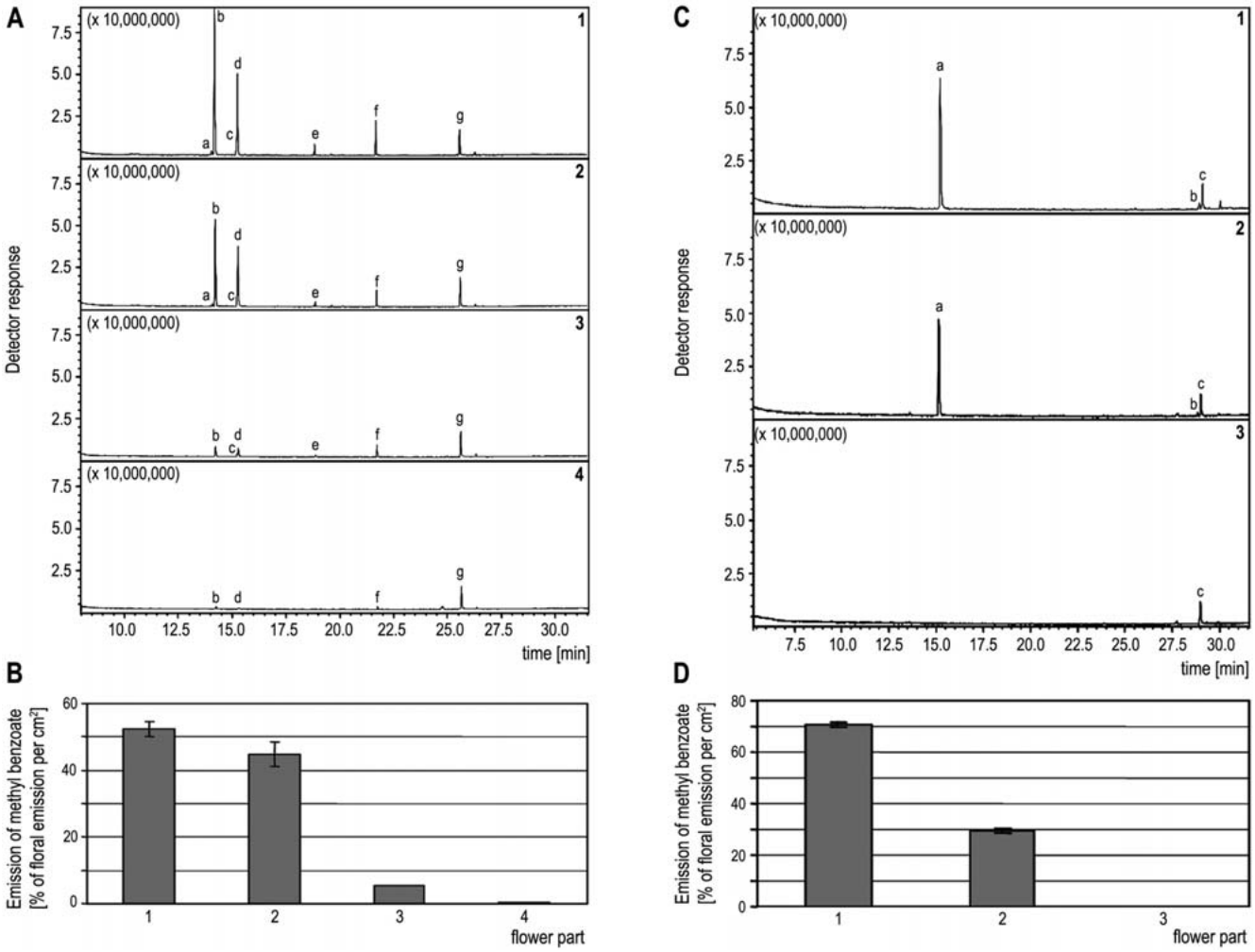


Fig. 2 VOC analysis of *Stephanotis floribunda* and *Nicotiana suaveolens* different corolla regions. (**A**) *Stephanotis floribunda* flowers (3 days after opening, at 12.00 p.m.) were cut in petal rim (1), petal centre (2), upper tube (3), and lower tube (4). VOCs of corolla parts were separately harvested and analyzed by GC-MS. The following VOCs were identified: benzaldehyde (a), *trans*-β-ocimene (b), β-linalool (c), methyl benzoate (d), unidentified (e), α-farnesene (f), standard (g). (**B**) Quantification of emission of methyl benzoate from different corolla parts (per area, cm^{-2}) of *Stephanotis floribunda* (petal rim and centre, n = 2; upper and lower tube n = 1). (**C**) *Nicotiana suaveolens* flowers (2 days after opening, at 12.00 p.m.) were cut in petal rim (1), petal centre (2), and tube (3). VOCs of corolla parts were separately harvested and analyzed by GC-MS. The following VOCs were identified: methyl benzoate (a), benzyl benzoate (b), standard (c). (**D**) Quantification of emission of methyl benzoate from different corolla parts (per area, cm^{-2}) of *Nicotiana suaveolens* (n = 2).

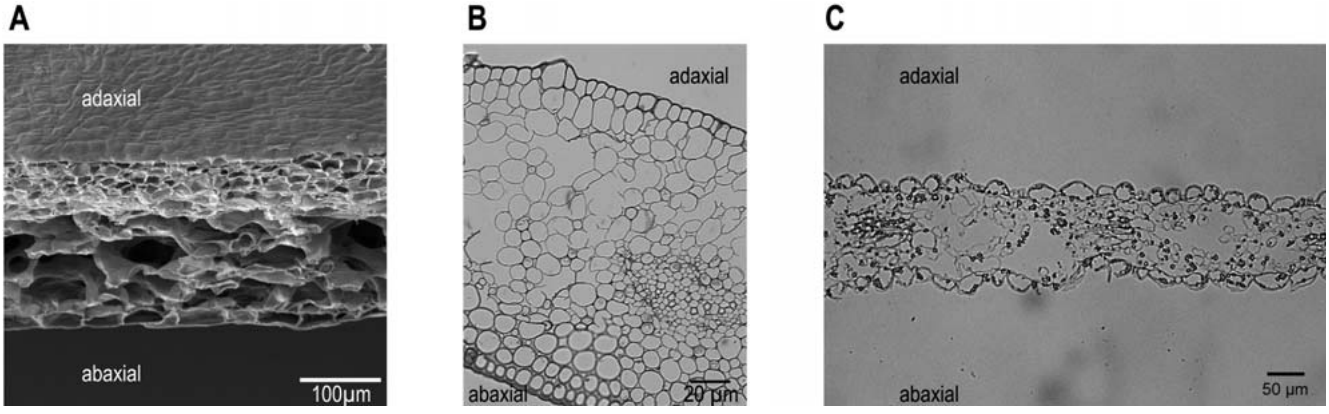


Fig. 3 Anatomy of petal lobe tissue from *Stephanotis floribunda* and *Nicotiana suaveolens*. (**A**) Scanning electron micrograph of the petal lobe of a 5-day-old *S. floribunda* flower. (**B**) Toluidine blue staining of a thin section of a *S. floribunda* bud (light microscopy). (**C**) Light microscopic thin section of the petal lobe of a 2-day-old *N. suaveolens* flower.

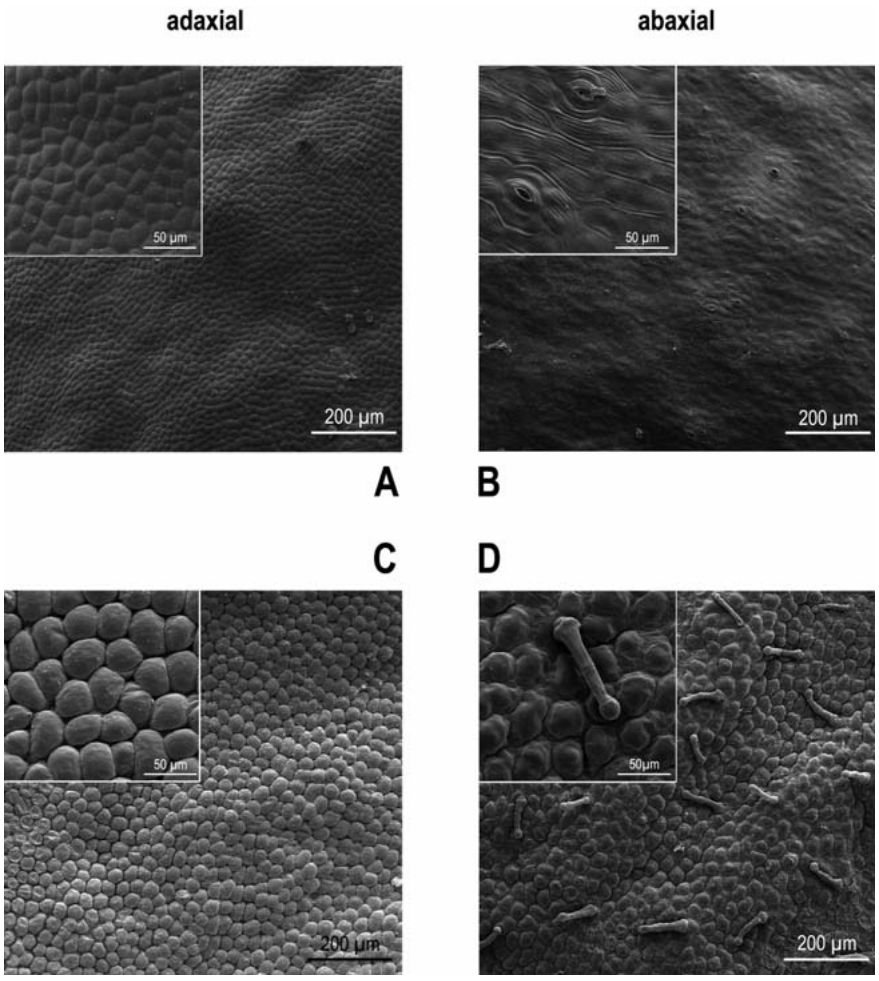


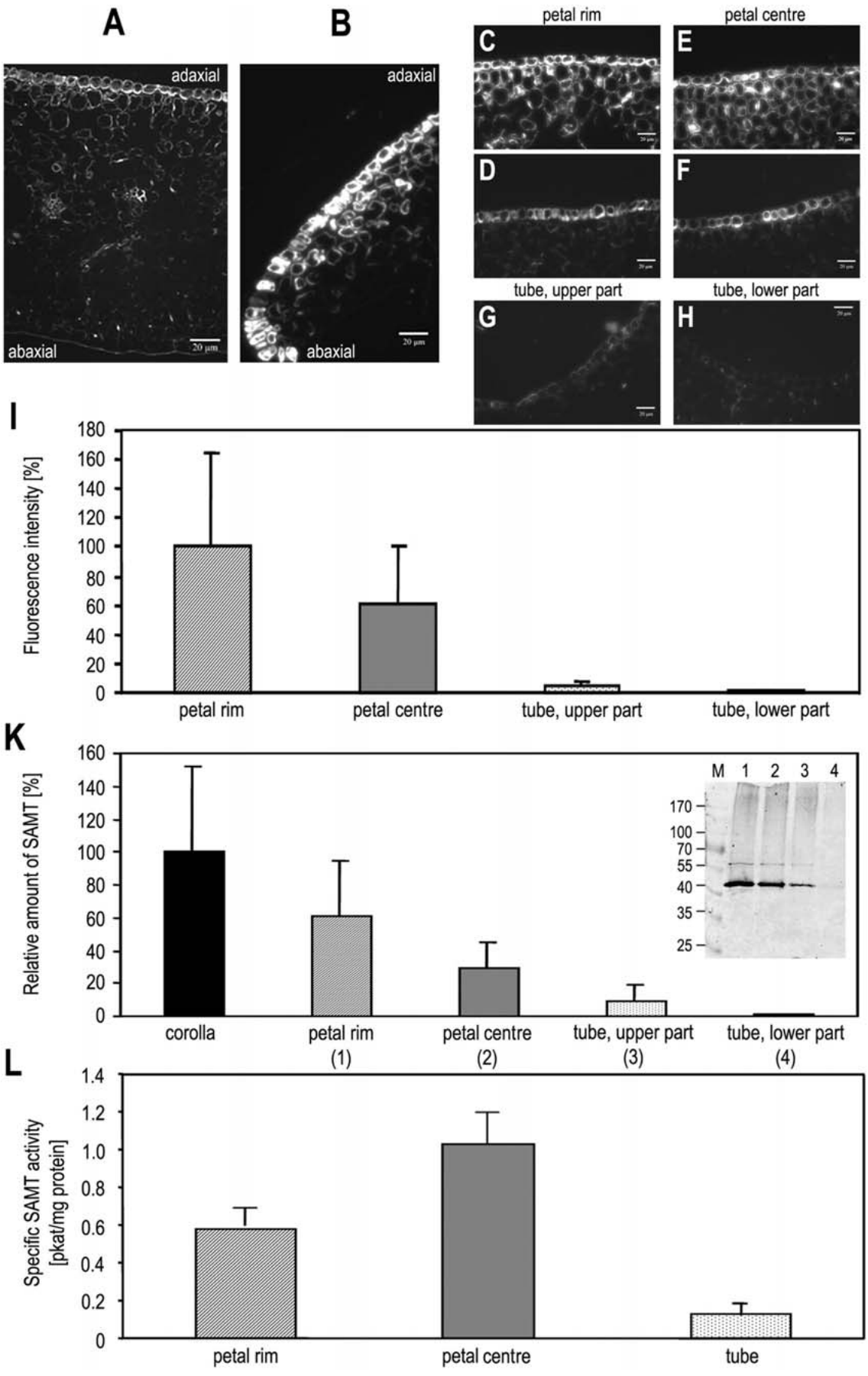
Fig. 4 Scanning electron micrographs of the adaxial and abaxial epidermis of *Stephanotis floribunda* and *Nicotiana suaveolens* petals. (**A**) Adaxial epidermis of the petal lobe of a 1-day-old *S. floribunda* flower. (**B**) Abaxial epidermis with stomata of the petal lobe of a 1-day-old *S. floribunda* flower. (**C**) Adaxial epidermis of the petal lobe of a 3-day-old *N. suaveolens* flower. (**D**) Abaxial epidermis with trichomes of the petal lobe of a 3-day-old *N. suaveolens* flower. Inserts are magnifications.

Scanning electron microscopy (SEM) of the epidermis of the petal lobes generates further interesting differences between the petals of the two plant species. The *S. floribunda* adaxial epidermis appears very flat and smooth, with an irregular cell pattern (Fig. **4A**). The cells are connected without cavities or niches between cells. The abaxial epidermis appears even smoother, and wax wrinkles are observed. Numerous stomata are found on the abaxial epidermis of the *S. floribunda* petal lobe (Fig. **4B**). The SEM of the adaxial and abaxial epidermis of *N. suaveolens* exhibits bullate cell types (Figs. **4C,D**). The epidermis appears bumpy and the cells are clearly separated by gaps. Stomata were not detectable on the abaxial side of the petal lobe, but the random distribution of approximately 100 µm long capitate trichomes is significant.

Although, in both *S. floribunda* and *N. suaveolens,* the petal lobes are the primary area of scent emission, the morphological and anatomical structures are significantly different. While the *S. floribunda* epidermal cells are smooth and flat, covered with wax layers, have abaxial stomata and several subepidermal cell layers that form the basis for the rigid structure, the *N. suaveolens* epidermal cells are rugose, apparently without wax layers, have abaxial capitate trichomes and a loose cellular system that supports the fragile petal lobes. These investigations demonstrate the different anatomies of the petals of

Fig. 5 Immunofluorescence localization of SAMT in various petal regions of *Stephanotis floribunda.* Localization of the SAMT with specific polyclonal antibodies and Alexa Fluor®488-labelled goat-anti-rabbit secondary antibodies. (**A**) In a petal cross section of a 1-day-old flower, (**B**) at the edge of the petal rim of a 5-day-old flower, (**C**) at the petal rim of a 2-day-old flower, (**D**) at the petal rim of a 4-day-old flower, (**E**) at the petal centre of a 3-day-old flower, (**F**) at the petal centre of a 4-day-old flower, (**G**) in the upper part of the tube of a 1-day-old flower, (**H**) in the lower part of the tube of a 2-day-old flower. (**I**) Quantification of fluorescence intensity of defined areas (n = 30, average from 1- to 6-day-old flowers; error bars indicate standard deviation) using the software Metamorph Offline (Molecular Devices, Sunnyvale, CA). (**K**) Detection and quantification of SAMT protein in *S. floribunda* protein extracts from corolla, petal rim (1), petal centre (2), and upper (3) and lower (4) part of the tube (n = 6, average from 2- to 6-day-old flowers, error bars indicate standard deviation). Insert: Western blot performed with specific primary SAMT antibodies and anti-rabbit alkaline phosphatase-labelled secondary antibodies which allow visualization with NBT/BCIP (5-day-old flowers). M: protein marker with indicated molecular masses. (**L**) Methylation activity with benzoic acid in extracts from petal rim, petal centre and the tube of *S. floribunda* flowers. Preparation of the protein extract and conditions of the enzyme assay are described in methods (n = 5, average from 2- to 6-day-old flowers, error bars indicate standard deviation).

Fig. 5


A
B
adaxial
abaxial
20 µm
petal rim
petal centre
C
E
D
F
tube, upper part
tube, lower part
G
H
I
Fluorescence intensity [%]
180
160
140
120
100
80
60
40
20
0
petal rim
petal centre
tube, upper part
tube, lower part
K
Relative amount of SAMT [%]
160
140
120
100
80
60
40
20
0
corolla
petal rim (1)
petal centre (2)
tube, upper part (3)
tube, lower part (4)
M 1 2 3 4
170
100
70
55
40
35
25
L
Specific SAMT activity [pkat/mg protein]
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
petal rim
petal centre
tube

the two species, and no common morphological structures responsible for scent synthesis and emission could be observed with this methodology.

Cellular localization of scent synthesis/emission

Previously we isolated the carboxyl O'methyltransferase genes/enzymes, SAMT and BSMT, catalyzing the last step in the synthesis of methyl salicylate and methyl benzoate in *S. floribunda* and *N. suaveolens,* respectively. Antibodies against these enzymes are excellent tools to localize the enzyme in floral tissues and cells. Thin sections of petal lobes of *S. floribunda* and *N. suaveolens* were incubated with the specific antibodies (anti S.f. SAMT, anti N.s. BSMT) and immunofluorescence-labelled antibodies (Figs. **5, 6**).

Stephanotis floribunda

The S.f. SAMT enzyme is found in the adaxial epidermis and up to approximately 20 µm deep into the subepidermal cell layers of *S. floribunda,* while no immunofluorescence is detectable in the abaxial epidermis (Fig. **5A**). Some autofluorescence was found in cells surrounding the phloem and the wax layers on the abaxial side. The clear difference between adaxial and abaxial SAMT localization is particularly well documented in a thin section of the petal rim (Fig. **5B**). Cells facing upwards and sidewards express the SAMT enzyme, while in downward (abaxial)-pointing epidermal cells, SAMT could not be detected.

A detailed investigation and quantification of SAMT localization in different petal regions clearly demonstrates the substantial presence of SAMT in epidermal and subepidermal cell layers in the petal rim and centre, respectively (Figs. **5A–C, E**). In the petal centre, less SAMT accumulation was observed compared to the petal rim (Fig. **5I**). Local immunofluorescence pattern variability was also detected. In older flowers (4 days after opening), SAMT was exclusively present in the epidermal cells of the petal rim and centre (Figs. **5D, F**). Compared to the petal lobe, very little SAMT was found in the upper and lower petal tube (Figs. **5G–I**).

The presence of SAMT enzyme in petal lobes rather than in the tube is further confirmed in the respective protein extracts obtained by Western blot analysis and enzyme activity measurements. Proteins were extracted from the complete petals and from different regions of the *S. floribunda* petal tissue, separated by SDS-PAGE and SAMT was identified with specific polyclonal antibodies. A prominant protein band with an approximate molecular mass of 40 kD was detected. The highest level of SAMT protein is found in the petal rim, less in the petal centre, and small amounts in the tube of the *S. floribunda* flower (Fig. **5K**). The lower amounts of SAMT found in the petal centre compared to the petal rim tissue correlate well with results obtained by immunofluorescence quantification (Fig. **5I**). Regarding the specific enzyme activities in different petal tissue areas, it was observed that the methylation activity of benzoic acid in row extracts is approximately 60% in the petal rim (0.6 pkat/mg protein) compared to the petal centre (1 pkat/mg protein) (Fig. **5L**). Low activities were determined in the petal tube (0.1 pkat/mg protein). These investigations indicate that higher specific activity of SAMT is present in the centre compared to the rim, which is partly due to a 1.3-fold higher total protein concentration per unit area (cm^{-2}) in the petal rim versus the petal centre (data not shown). Furthermore, although SAMT might be the predominant enzyme for methylating benzoate, it cannot be totally excluded that other methyltransferases contribute to the formation and emission of methyl benzoate.

In summary, the petal lobes with the adaxial epidermis and subepidermal cell layers are the primary regions of scent synthesis and emission in *S. floribunda* flowers. SAMT protein determinations (immunolocalization and Western blot analysis, Figs. **5I, K**, respectively) indicated that less SAMT protein is in the centre, but since the activity of the enzyme is approximately twice as high in the petal centre (Fig. **5L**), the rim and centre contribute equally to the methyl benzoate synthesis and emission (Fig. **2B**).

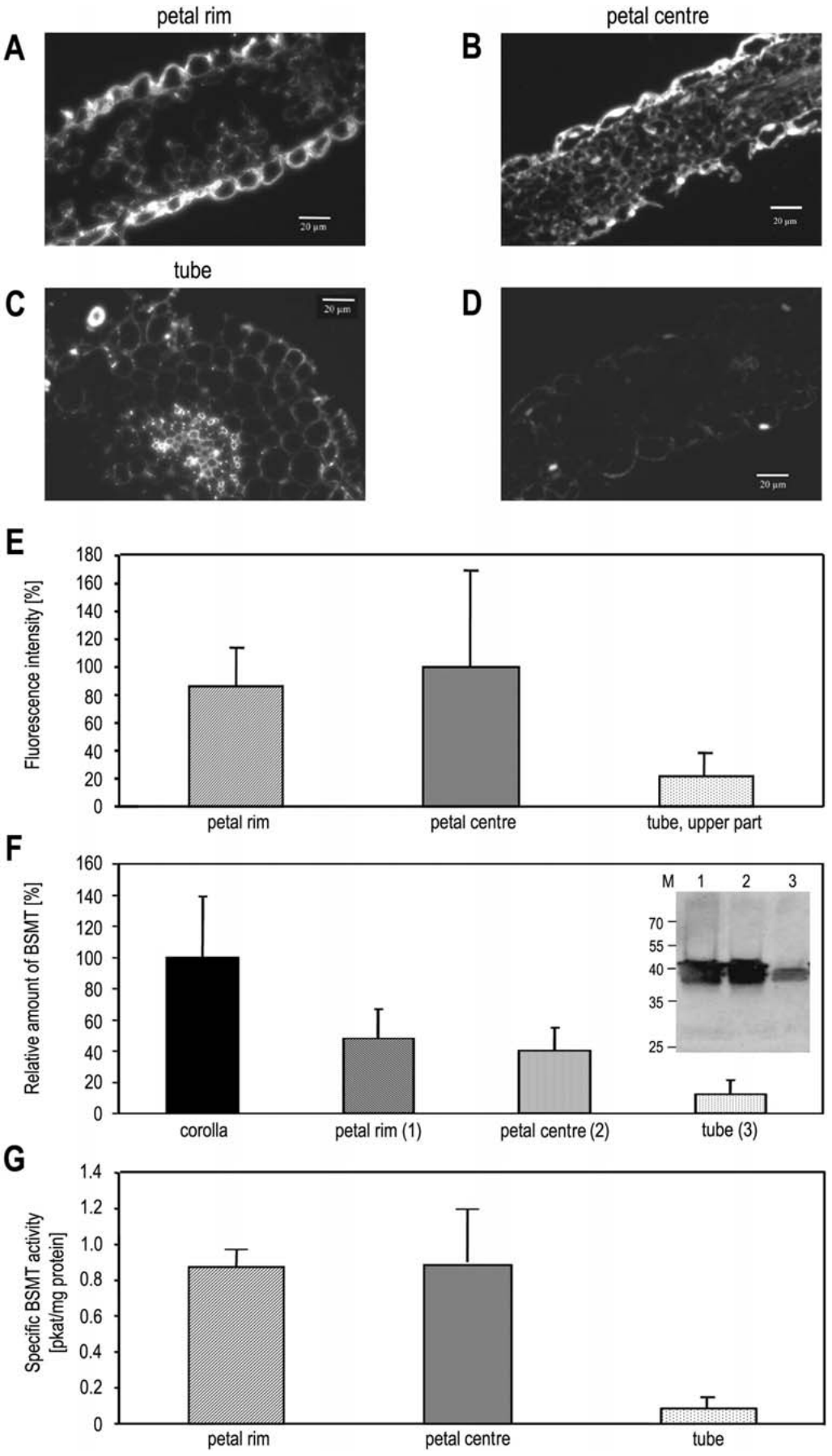
Nicotiana suaveolens

The immunolocalization experiments with the BSMT antibody in *N. suaveolens* flowers clearly demonstrate that the enzyme is present in the adaxial and abaxial epidermis (Figs. **6A, B**), therefore, apparently both sides of the petal are involved in methyl benzoate synthesis and emission. This observation is clearly different to that found with *S. floribunda* flowers. BSMT is primarily found in epidermal cells of the petal rim, while the enzyme is also detectable in the mesophyll cells of the petal centre (Fig. **6B**). In a cross section of the petal tube, little BSMT is found in epidermal cells, and some autofluorescence surrounding the phloem can also be recognized (Fig. **6C**). Background autofluorescence obtained with pre-immune serum is presented in Fig. **6D**.

The levels of BSMT protein in the rim and centre of the petal lobes are very similar and very little enzyme is detected in the tube tissue (Western blot analysis, Fig. **6F**). The enzyme activity measurements support this result, reaching specific activities of about 0.9 pkat/mg protein with benzoic acid in the petal centre and rim, and less than one tenth of this in the tube tissue (Fig. **6G**).

Fig. 6 Immunofluorescence localization of BSMT in various petal regions of *Nicotiana suaveolens*. Localization of BSMT with specific polyclonal antibodies and Alexa Fluor®488-labelled goat-anti-rabbit secondary antibodies. (**A**) In the petal rim of a 1-day-old flower, (**B**) in the petal centre of a 1-day-old flower, (**C**) in a cross section of the tube of a 3-day-old flower. (**D**) Cross section of the petal rim (3-day-old flower) incubated with preimmunserum revealing the level of autofluorescence. (**E**) Quantification of fluorescence intensity of defined areas (n = 18, average from 1-, 3-, 5-day-old flowers; error bars indicate standard deviation) using the software Metamorph version 6.1 (Molecular Devices, Sunnyvale, CA). (**F**) Detection and quantification of BSMT protein in *N. suaveolens* protein extracts from corolla, petal rim (1), petal centre (2), and tube (3) (n = 6, average from 2- to 6-day-old flowers, error bars indicate standard deviation). Insert: Western blot performed with specific primary BSMT antibodies and anti-rabbit alkaline phosphatase-labelled secondary antibodies which allows visualization with CSPD (2-day-old flowers). M: protein marker with indicated molecular masses. (**G**) Methylation activity with benzoic acid in extracts from petal rim, petal centre, and the tube of *N. suaveolens* flowers. Preparation of the protein extract and conditions of the enzyme assay are described in methods (n = 5, average from 2- to 6-day-old flowers, error bars indicate standard deviation).

Fig. 6


petal rim
A
20 µm
petal centre
B
20 µm
tube
C
20 µm
D
20 µm
E
Fluorescence intensity [%]
180
160
140
120
100
80
60
40
20
0
petal rim
petal centre
tube, upper part
F
Relative amount of BSMT [%]
160
140
120
100
80
60
40
20
0
M 1 2 3
70
55
40
35
25
corolla
petal rim (1)
petal centre (2)
tube (3)
G
Specific BSMT activity [pkat/mg protein]
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
petal rim
petal centre
tube

In summary, the adaxial and abaxial epidermal cells of the petal lobes of *N. suaveolens* are the source of methyl benzoate synthesis. Immunofluorescence localisation of BSMT (Fig. **6E**), steady state protein levels (per mg protein, Fig. **6F**), and specific enzyme activities (Fig. **6G**) indicate that the rim and centre of the petal lobes should contribute equally to methyl benzoate synthesis and emission in *N. suaveolens*. However, the emission of methyl benzoate is 2.5-fold higher from the rim compared to the centre (Fig. **2D**), which can partly be explained by the 3.9-fold higher total protein concentration in the rim versus the centre (data not shown).

Discussion

Beside visual cues, floral scents are efficient communication signals between plants and other organisms. For long-distance attraction, the whole flower is sufficient to define the destination for pollinators, but in close proximity to an inflorescence or for individual flower-specific navigation, cues are necessary. For many insects, a precise guidance and orientation to and within the flower is absolutely necessary. Therefore, it is of great interest to determine the organs or tissues in a flower where volatiles are synthesized and emitted. Furthermore, up to now, the precise process of small organic compound trafficking and emanation within the cell and from the cellular interior to the outside has not yet been thoroughly investigated. Contradictory opinions are presently found in the literature. A possible scenario could be that the emission profiles reflect the internal concentrations of volatiles (Goodwin et al., 2003). In this concept, the consequences of free VOCs, glycosylated forms, or VOCs sequestered in droplets, lipid bodies, or vacuoles remains to be investigated. Carrier or transporter mechanisms, as well as vesicle transport of phytochemicals, are also discussed (Grotewold, 2004; Grotewold et al., 1998). The plasma membrane-localized ABC transporter (NpABC1) is involved in the secretion of isoprenoids from *Nicotiana* cells (Jasinski et al., 2001), while volatile emanation from the infloresence of *Sauromatum guttatum* relies on the rough ER, special pockets of the plasma membrane, and the trans-Golgi network (TGN) (Skubatz and Kunkel, 1999). Alternative secretory pathways that bypass the need for the Golgi apparatus and the TGN are also suggested from experiments with maize cells (Lin et al., 2003), and immunolocalization of flavonoid biosynthetic enzymes in *Arabidopsis* root cells identified cytoplasmic electron-dense structures which might be ideal complexes taking advantage of the cellular protein secretion machinery (Saslowsky and Winkel-Shirley, 2001).

To obtain further detailed information about the process of scent emanation from *Stephanotis floribunda* and *Nicotiana suaveolens* flowers, we undertook comparative research to determine the scent-emitting tissue, studied the morphology and anatomy of petal tissue and, finally, localized a scent-synthesizing enzyme at the cellular level as an indicator for scent synthesis.

At first glance, the morphology of individual flowers appears similar for both plant species, e.g., they are white, composed of five petals which are fused at the bottom to form a long tube and display acute or emarginate lobes. However, the gyneocium and androecium differ, in *S. floribunda* the male and female organs are fused to a gynostegium, while they are separate in *N. suaveolens*. In both species, analysis of VOC emission from different organs revealed that the corollas emit scent compounds, while no emission was measured from sepals or the female and male organs. Petals are often the main source for floral fragrances, but other parts of the inflorescence influence the emission of certain compounds, for example the androecium (Lex, 1954; von Aufsess, 1960; Dobson et al., 1990; Bergström et al., 1995) and pistil of *Clarkia breweri* specifically releases linalool oxide (Pichersky et al., 1994), and styles and stamens also contribute to the floral odour (Dobson et al., 1990; Bergström et al., 1995; Pichersky et al., 1994; Raguso and Pichersky, 1999).

Cases are also known where only certain areas of floral organs emit scent. The basal region of *Ranunculus acris* is characterized by higher emission of alpha-farnesene and 2-phenylethanol and an increased diversity of volatiles are released from the apical region. The wings of *Spartium junceum* and the vexillum of *Lupinus cruckshanksii* are defined as emitting tissues, while lilac aldehydes are emitted from the anthophore of *Silene latifolia* (summarized in Effmert et al., 2005c; Doetterl and Jürgens, 2005). The VOC emission experiments on the corollas of *S. floribunda* and *N. suaveolens* defined the petal lobes as the major source of volatile release and very little scent is emitted from the tube tissue. A similar observation was made when flowers of *Mirabilis jalapa,* which have a similar trumpet-like appearance to the flowers of *S. floribunda* and *N. suaveolens,* were dissected and the emanation of the individual parts was determined, revealing that the petaloid lobes of the corolla-like calyx are the region of highest β-ocimene emission (Effmert et al., 2005a).

The VOC emission profile from the petal lobes of *S. floribunda* and *N. suaveolens* flowers is congruent with the presence of a scent-emitting enzyme, the salicylic acid/benzoic acid carboxyl methyltransferase (SAMT, BSMT), which was demonstrated by Western blot analysis, enzyme activity assays and *in situ* immunolocalizations. A local restriction of the appearance of scent-synthesizing enzymes was also shown in *Clarkia breweri* and *C. concinna,* demonstrating that the biosynthesis of volatile compounds occurs in the stigma, styles, filaments, and petals (Dudareva et al., 1996), while petals are the major source of the scent compound linalool. It was shown that the linalool synthase and the S-adenosyl-L-methionine : eugenol O'methyltransferase genes in *C. breweri* are almost exclusively expressed in the cells of the epidermal layers of petals and other floral organs (Dudareva et al., 1996; Wang et al., 1997; Dudareva and Pichersky, 2000). In *Antirrhinum majus,* the benzoic acid carboxyl methyltransferase (BAMT) was primarily found in the conical adaxial epidermis cells of the tube and in the yellow hairs present on this tube and, to a lesser extent, in the abaxial epidermis (Kolosova et al., 2001). In *S. floribunda* and *N. suaveolens,* the epidermal cells are also involved in scent production. In *S. floribunda* petals, the adaxial epidermis and subepidermal cells exclusively contain the SAMT enzyme, while both the adaxial and abaxial epidermal cells of the petal lobes of *N. suaveolens* contain the BSMT enzyme. The finding that SAMT is present in the epidermal as well as subepidermal cell layers in *S. floribunda* petals, the large intercellular spaces in the subepidermal and mesophyll layers and the rapid neutral red staining of mesophyll cells layers of the petal lobes (data not shown) are indications for an osmophore, according to Vogel's definition (1962), although cellular secretory structures could not be identified in *S. floribunda* petal tissue. The

investigations performed here show that the adaxial epidermis of the petals is involved in volatile synthesis and emission in *S. floribunda* and *N. suaveolens*. This tissue faces the atmosphere, which is apparently the best way for volatile spreading and consequent pollinator navigation to the flower. In *N. suaveolens,* scent synthesis and emission is also found in the abaxial side of petal lobes, while this apparently does not occur in *S. floribunda* flowers. This is most likely because the thick wax cuticle would prevent volatile diffusion from the abaxial petal of *S. floribunda*. Another plausible explanation may come from the different inflorescence types. *N. suaveolens* presents individual flowers which can be freely accessed by attracted pollinators, regardless of whether the scent compound is emitted from the adaxial or abaxial epidermis. *S. floribunda* flowers, however, form an umbel where the adaxial epidermis represents the surface of this compound inflorescence. Emission of the scent components only from the adaxial epidermis ensures that the scent is released not to the internal but to the surrounding space from where the pollinators approach the flowers.

An alternative possibility for volatile emission is found in vegetative tissues. Leaves of oregano and peppermint, for example, possess specialized secretory structures, such as oil glands, glandular trichomes, oil or resin ducts which store a mixture of VOCs that can be released upon mechanical destruction of the glands (Turner et al., 2000 a, b; Werker et al., 1985). Since floral organs are metamorphotic leaves, it can be speculated that such glands may also exist on floral organs. It was demonstrated here that the *S. floribunda* adaxial and abaxial epidermis of the petals do not possess any kind of glands, while the abaxial epidermis of *N. suaveolens* has many trichomes. However, during this study, it was not possible to determine whether these trichomes play a role in scent synthesis and emission. In *Salvia dominica,* peltate hairs on the abaxial side of the calyx were described to be responsible for secretion of neryl acetate, alpha-terpineol, alpha-terpinyl acetate, and other minor volatiles (Werker et al., 1985). Linalool and linalyl acetate appear in large quantities in abaxial hairs on calyxes, bracts, and peduncles of *S. sclarea* and floral uniserate and multicellular capitate trichomes present on the abaxial side of the *M. jalapa* petaloid lobes were also shown to contain volatiles (Effmert et al., 2005 a).

The investigations outlined here demonstrate that scent synthesis and emission in *S. floribunda* and *N. suaveolens* is primarily restricted to the petal lobes, which apparently exhibit the best position for volatile spreading. At the cellular level, significant differences are observed between these two plant species. An osmophore, in the sense of Vogel's definition (1962), seems to be responsible for methyl benzoate synthesis in *S. floribunda,* while synthesis is restricted to the petal epidermal cells of *N. suaveolens*. Knowing the precise location of scent synthesis allows further questions regarding the process and regulation of emission to be addressed in detail.

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