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Evening specific oscillations of scent emission, SAMT enzyme activity, and *SAMT* mRNA in flowers of *Stephanotis floribunda*

Marcella B. Pott[1], Eran Pichersky[2], Birgit Piechulla[1] *

[1] University of Rostock, Department of Molecular Physiology and Biotechnology, Albert-Einstein-Str. 3, 18051 Rostock, Germany

[2] Molecular, Cellular and Developmental Biology (MCDB), University of Michigan, 830 N. University Street, Ann Arbor, MI 48109-1048, USA

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Summary

Stephanotis floribunda (Asclepediaceae), a plant that grows in Madagascar and is now a common house plant, emits a number of volatiles from its flowers. Its floral scent has been noted to increase in intensity during the night. At this time prominent scent compounds include benzyl alcohol, benzyl acetate, benzyl benzoate, eugenol, α-farnesene, linalool, linalool oxide, methyl benzoate (MBA), methyl salicylate (MSA), β-ocimene, phenylethyl alcohol, and 1-nitro-2-phenylethane. To investigate the level(s) at which the variation in fragrance emission is controlled, we isolated a gene encoding the enzyme salicylic acid carboxyl methyltransferase (SAMT), which catalyses the synthesis of methyl salicylate from salicylic acid and *S*-adenosyl-L-methionine. The expression of SAMT is petal-specific and developmentally regulated. Under light/dark conditions, *SAMT* mRNA levels and SAMT enzyme activities oscillate and reach the maxima in the first half of the night. These patterns correlate well with the emission of MSA. We conclude that the daily fluctuation in emission of MSA in *S. floribunda* likely involves *de novo* volatile synthesis resulting from time-regulated *SAMT* mRNA accumulation and SAMT enzyme activity.

Key words: Enzyme activity oscillations – scent emission oscillations – S-adenosyl-L-methionine : salicylic acid carboxyl methyl transferase (SAMT) – *SAMT* mRNA oscillations – *Stephanotis floribunda*

Abbreviations: BA = benzoic acid. – BAMT = S-adenosyl-methionine : benzoic acid methyltransferase. – LD = light/dark conditions. – LL = continuous illumination. – MBA = methyl benzoate. – MSA = methyl salicylate. – SA = salicylic acid. – SAMT = *S*-adenosyl-L-methionine : salicylic acid methyltransferase. – SPME = solid phase micro extraction

* E-mail corresponding author:
birgit.piechulla@biologie.uni-rostock.de

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Introduction

Many species that are insect-pollinated have flowers that emit scent (Dudareva and Pichersky 2000). In some cases, such emission of scents occurs primarily during the day, for example *Citrus medica, Odontoglossum constrictum* (Matile and Altenburger 1988) and *Platanthera chlorantha* (Nilsson 1983). In other cases, the emission is primarily during the night, for example *Cestrum nocturnum* (Overland 1960), *Hoya carnosa* and *Stephanotis floribunda* (Matile and Altenburger 1988, Altenburger and Matile 1988). Furthermore, in *H. carnosa, S. floribunda, C. nocturnum, Nicotiana suaveolens* and *N. sylvestris*, oscillations of scent emission were observed even under constant conditions, indicating that a circadian clock is involved in controlling the timing process (summarized in Dudareva et al. 2000 a). Yet in other cases, emission does not vary between the night and day periods. In cases where emission does vary, it has been interpreted as an adaptation to maximise resources and to advertise to specific pollinators whose activity is temporally limited, for example night-flying moths.

Several enzymes that act in flowers to catalyse the formation of scent volatiles have recently been described, including enzymes for the synthesis of linalool, methyl salicylate (MSA), and methyl benzoate (MBA) (Pichersky et al. 1994, 1995, Ross et al. 1999, Dudareva et al. 2000 b). The availability of such enzymes and the genes which encode them makes it possible to examine i) whether oscillation in floral scent emission is controlled at the levels of synthesis or release, ii) when *de novo* synthesis is the rate limiting step, or iii) whether this process is controlled at the protein and/or nucleic acid levels.

Stephanotis floribunda (Asclepiadaceae), whose flowers produce a strong fragrance at night, is native to Madagascar, but is also now grown as a common house plant. The scent components that had previously been identified in its headspace include benzyl alcohol, α-farnesene, 1-nitro-2-phenylethane, linalool and methyl benzoate (Matile and Altenburger 1988). The latter three volatiles were shown to be emitted at higher levels at night and less so during the day (Altenburger and Matile 1990). However, the mechanisms responsible for such evening specific oscillations have not been examined. We have therefore used *S. floribunda* to study the underlying control mechanisms for the heightened evening emission of its floral scent.

Materials and Methods

Plant material and growth conditions

Stephanotis floribunda (Brongn.) plants were grown in a greenhouse with additional light. Plants were illuminated at least 16 hours (from 6 am to 10 pm).

Determination of the volatile compounds

The Solid Phase Micro Extraction (SPME) device (Sigma-Aldrich, Taufkirchen, FRG) contained a fused silica fiber coated with 100 μm poly(dimethylsiloxane). Exactly 10 min before sampling, the whole plant, including stocks of open flowers, was wrapped in a plastic bag. For volatile collection, the syringe of the SPME device was inserted in the plastic bag and the fiber was exposed for exactly 5 min. Next, the SPME fiber was inserted into the GC injection port, and the sample was eluted and chromatographed on a DB 5MS column (122–5532; 30 m; I.D. 0.25 mm; film 0.25 μm; J and W Scientific, Folsom, CA). To determine volatile composition at different time points during the day, measurements were carried out with two plants during a period of 48 hours. The volatiles were identified through comparison of mass with known standards [retention time of benzyl alcohol (26.65), benzylacetate (23.45), benzyl benzoate (40.24), eugenol (32.32), α-farnesene (23.95), linalool (19.35), linalool oxide (furan, 23.77), methyl benzoate (20.97), methyl salicylate (MSA) (24.42), β-ocimene (11.51), phenylethyl alcohol (27.39), and 1-nitro-2-phenylethane (31.34)].

Cloning the SAMT gene RT-PCR

Four flowers of *S. floribunda* (each flower weighs approximately 400 mg) were harvested at 6 : 30 pm on the first day after flower opening, and total RNA was extracted with CsCl purification according to Sambrook et al. (1989). RT-PCR was performed with 500 ng total RNA with ‹Access RT-PCR› kit (Promega, Mannheim, FRG) following the manufacturer's instructions. Primers for RT and PCR were TpsA and C as described in Steele et al. (1995). The PCR product was analysed by gel electrophoresis and the fragments were recovered from the agarose gel with a gel extraction kit (Qiagen, Hilden, FRG). The purified fragments were re-amplified with the same primers and cloned with the ‹pGEM-T cloning› kit (Promega, Mannheim, FRG). Both strands were sequenced using the ‹SequiTherm Excel II DNA Sequencing› kit with IRD-800 labelled primers and a LI-COR automated sequencer (MWG, Ebersberg, FRG). The amino acid sequences encoded by these fragments were compared with the amino acid sequences of known proteins of the databases using the BLAST Search System (NCBI).

5′-RACE and full length cDNA cloning

The PCR fragment with similarity to the *SAMT* gene of *Clarkia breweri* (366 bp) was incomplete at the 5′ end. A 5′-RACE according to Eyal et al. (1999) with the following modifications was performed: 4 μg of total RNA were used together with the phosphorylated primer SF4/7-AS1 (MWG) (5′-TATTGGAACAGCAACG CAGTGG-3′) and 200 U MMLV and supplied buffer (Promega, Mannheim, FRG) for RT. After inactivation of the enzyme for 10 min at 70 °C, the RNA was degraded by incubation with 0.4 units RNase H at 37 °C for 20 min. After heat-inactivation for 3 min at 94 °C, the ss cDNA was purified with a PCR purification kit (Qiagen, Hilden, FRG), and 16 μL of the 40 μL eluate were used for self-ligation with T4-RNA ligase (MBI Fermentas, St. Leon-Rot, FRG) following the manufacturer's instructions. Inverse PCR was carried out with 3 μL of the self-ligated cDNA with 3 units Pfu-Polymerase (Promega, Mannheim, FRG) according to the manufacturer's manual, using primers Sf4/7-S1 (5′-CCACTGCGTTGCTGTTCCAATA-3′) and Sf4/7-AS2 (5′-CTCTTCATGGAGACGGTGACAT-3′). The PCR products

were again separated by gel electrophoresis and the fragment with the expected length of 1.1 kb was cloned and sequenced as before.

To obtain a full-length copy of the coding region of *S. floribunda SAMT* for expression in *E. coli*, new primers were designed, STSAMT Forward and STSAMT Reverse (5′-AATGGAAGTTGTTGAAGTTCTTC-3′ and 5′-TAATTAAACCCTTCTCTTCAT-3′, respectively). PCR was performed on the ss cDNA from the 5′-RACE. The resulting fragment was cloned in an expression vector with the ‹TOPO Cloning› kit (Invitrogen, Groningen, NL) without further purification, and after verification by sequencing the recombinant plasmid was mobilised into *E. coli* BL-21 cells.

Sequence alignments of the SAMT and BAMT proteins were performed with an on-line ClustalW sequence alignment program.

Protein extractions

The crude extracts were prepared as described in Wang et al. (1997), with following modifications: Flower parts (⅕ of the petals, ½ of the gynostegia) or a 3 cm in diameter circle from a leaf were cut and immediately submerged in ice-cold extraction buffer (5 µL per mg FW). The plant material was ground on ice in an 1.5 mL reaction tube or a small mortar, centrifugated for at least 15 min, and the supernatant transferred to a new tube. Glycerol (¼ volume) was added and the crude extract stored at −20 °C.

Enzyme assay

The assay was performed according to Wang et al. (1997). Assay solution contained 12.5 µL of crude extract, 10 µL of assay buffer (250 mmol/L Tris-HCl, pH 7.5, 25 mmol/L KCl), 1 µL of 50 mmol/L salicylic acid or benzoic acid dissolved in ethanol (or 1 µL of pure ethanol as control), 1 µL [methyl-^{14}C] *S*-adenosyl-L-methionine (58 mCi/mmol, in 9 : 1 (v/v) mixture of sulfuric acid (pH 2.0) and ethanol, Hartmann, Braunschweig), add H_2O to a final volume of 50 µL. The samples were incubated at 24 °C for 60 min before 100 µL ethyl acetate and then 3 µL concentrated HCl were added to stop the reaction. The tubes were vortexed, briefly centrifuged, and 30 µL of the organic phase (on top) was transferred to a scintillation vial, mixed with 2 mL scintillation fluid (emulsifier-safe, Canberra Packard, Dreieich, FRG) and counted in a scintillation counter (Tri-Carb 2100 TR, Canberra Packard).

RNA extraction

The RNA was extracted as described in Sambrook et al. (1989). For each extraction, the petals of at least 5 flowers or half of the gynostegia of 8 flowers per time point were used. Two young leaves also were harvested.

Hybridisation

RNA samples (3 µg each) were separated on a formaldehyd denaturing gel and transferred to a Nylon membrane (Roche Diagnostics, Mannheim, FRG). For time-specific expression, the RNA (3 µg in 16 X SSC) was directly i) spotted onto the membrane via a dotblot apparatus or ii) transferred to Northern Blots. The *SAMT* and the 18S rDNA probes were labeled by PCR using dig-dUTP (Roche Diagnostics, CH), according to the manufacturer's instructions. Primers for the *SAMT* probe were STSAMT Forward (see paragraph: 5′ RACE) and STSAMT 18 Reverse (5′-ATCTGAAAATGGTGTTGAAATCAT-3′). The resulting fragment was 322 bp long. PCR was performed on the TOPO vector with the *SAMT* gene. The 18S rDNA primers were Pa-rDNA1 and Pa-rDNA2 (5′-GGTCGCAAGGCTGAAACTT-3′ and 5′-TTATTGCC-TCAAACTTCC-3′, respectively – corresponding to the 18S rRNA sequence of *Picea abies*. A fragment of ca. 300 bp was obtained from genomic DNA of *S. floribunda*.

Hybridisation was performed with high SDS-buffer at 50 °C overnight (Roche Diagnostics). Membranes were washed twice for 5 min with 2 X SSC, 0.1 % SDS at room temperature and twice for 15 min with 0.1 X SSC and 0.1 % SDS at 50 °C. After blocking, the membranes were incubated with anti-Dig AP. CSPD (Roche Diagnostics) was used as a substrate and the chemiluminescence signal detected and quantitated with the LAS-1000 (Raytest, software: Image Gauge) for 10–60 min. *SAMT* mRNA transcript levels were normalised to rRNA levels which were determined by succeeding hybridisations and the SAMT mRNA/rRNA ratio was calculated. For all blots, the signal of the sample harvested at 6 : 30 pm 2 days after flower opening was chosen as a standard and was set to 100 %.

Results

Temporal oscillation in emission of specific floral volatiles in *S. floribunda*

In an earlier study of *S. floribunda* floral scent, the oscillations in emission of linalool, methyl benzoate, and 1-nitro-2-phenylethane were followed (Matile and Altenburger 1988, Altenburger and Matile 1990). We have used a SPME approach (see Materials and Methods) to identify a total of 12 volatiles in the *S. floribunda* scent (Fig. 1): benzyl alcohol, benzylacetate, benzyl benzoate, eugenol, α-farnesene, linalool, linalool oxide (furan), methyl benzoate (MBA), methyl salicylate (MSA), β-ocimene, phenylethyl alcohol, and 1-nitro-2-phenylethane. In addition, relative daily fluctuations in the emission of these components were followed over a period of 24 hours at 3 hour intervals for two plants (Fig. 2). All investigated compounds were emitted at higher levels in the evening than during the day, although the degree of variation between trough and peak was not uniform (e.g., 5-fold for linalool oxide, benzyl acetate and eugenol, 3-fold for MBA and linalool, and 2-fold for MSA, phenethyl alcohol and benzyl alcohol). The evening emission patterns of the 12 compounds can be grouped into two categories: class 1 comprises volatiles that reach highest levels approximately at 9 pm (e.g. β-ocimene, linalool, MBA, benzyl acetate and linalool oxide) while scent compounds of class 2 peak additionally between 6 am and 9 am. The second peak, however, is significantly smaller. Since all 12 volatiles reach maximum emission at 9 pm it seems very likely that a time coordinated regulation is underlying.

Isolation and characterisation of a putative *SAMT* cDNA from *S. floribunda*

We attempted to isolate a cDNA encoding linalool synthase from *S. floribunda* by the RT-PCR technique, using mRNA de-

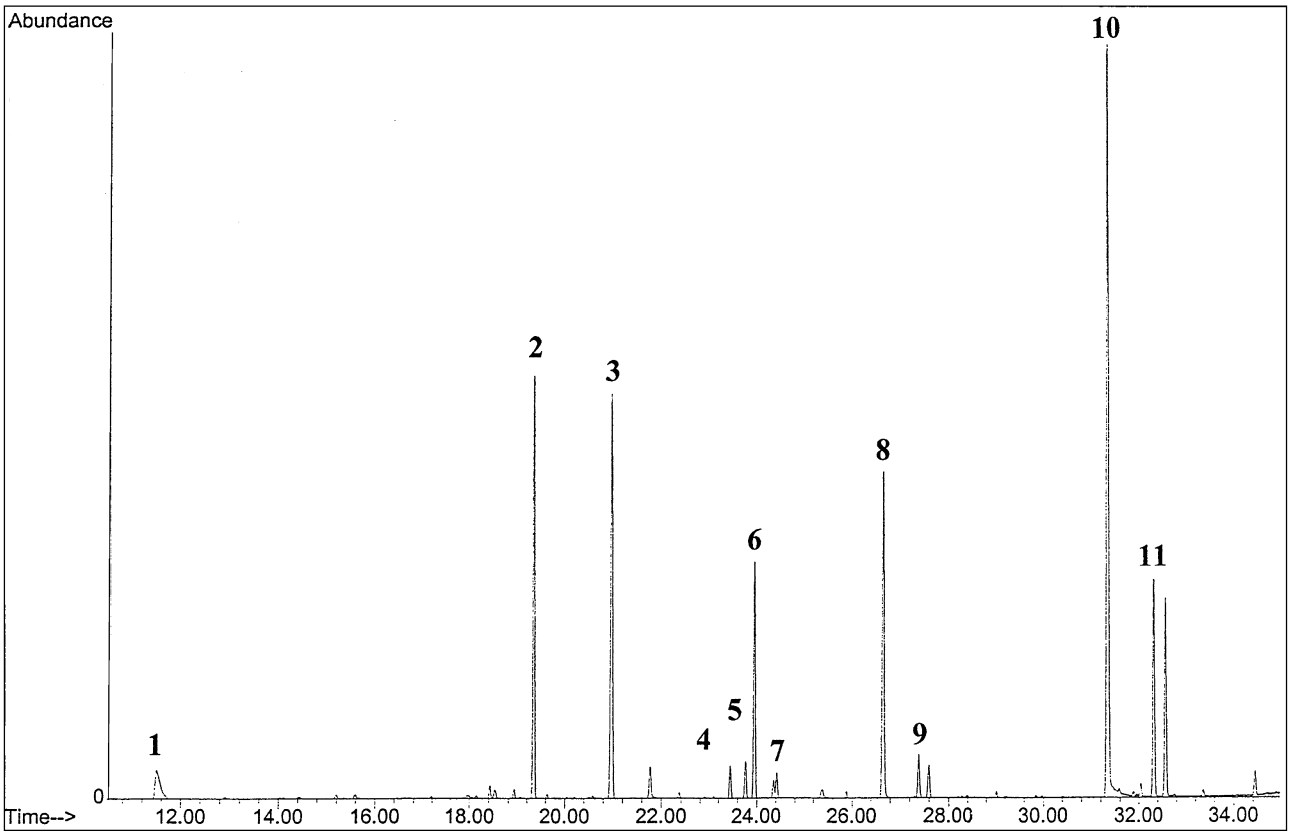

Figure 1. Volatiles emitted from *S. floribunda*. *S. floribunda* plants were transferred to a container and the emitted volatiles were absorbed on a SPME (solid phase micro extraction) fiber and analysed by GC/MS. The following compounds were identified: (1) β-ocimene, (2) linalool, (3) methyl benzoate, (4) benzyl acetate, (5) linalool oxide, (6) α-farnesene, (7) methyl salicylate, (8) benzyl alcohol, (9) phenethyl alcohol, (10) 1-nitro-2-phenylethane, (11) eugenol. Benzyl benzoate at RT 40.24 is not shown in this chromatograph.

rived from flowers and two oligonucleotides that were based on conserved motifs in terpene synthases (Steele et al. 1995). One of the DNA fragments thus generated proved to encode a protein with high similarity to *Clarkia breweri* SAMT, since one of the primers also exhibited sequence similarity to the carboxyl-methyltransferase coding region. The SAMT enzyme is responsible for the synthesis of the floral scent component MSA (Ross et al. 1999). Upon close inspection of the sequence of the isolated fragment, it appears that the oligonucleotides apparently hybridised to one segment located close to the end of the coding region, and to another sequence in the 3′ non-coding region of the mRNA. Taking advantage of this fortuitous result, the sequence of this fragment was used to generate additional oligonucleotides which were used in a 5′-race experiment to get a full-length cDNA clone of this putative *S. floribunda SAMT*. The complete cDNA comprises 1101 nt (accession number AJ308570) that encodes a protein of 366 amino acids (Fig. 3). The amino acid sequence encoded by this gene was aligned with the previously published sequence of SAMT from *Clarkia breweri* (Ross et al. 1999) and the sequence of BAMT from *Anthirrinum majus* (snapdragon, family Scrophulariaceae) (Fig. 3). The *S. floribunda* protein sequence is overall 56 % identical to the *C. breweri* SAMT and 43 % identical to the snapdragon BAMT. The sequence alignments also revealed four regions that are highly conserved among all three sequences (numbered region I through IV in Figure 3). These regions share 78 % or more identical amino acids.

Determination of substrate specificity of the recombinant SAMT

To verify that the isolated cDNA clone encodes SAMT, the gene was cloned into an expression vector, expressed in *E. coli* and the soluble protein was produced and tested in an *in vitro* enzyme assay with benzoic acid (BA) and salicylic acid (SA) as previously described (Ross et al. 1999). The extracts from the induced *E. coli* culture carrying the *S. floribunda SAMT* gene had a 3-fold higher activity with SA than with BA. Extracts from a culture of *E. coli* carrying only the plasmid had no activity with the substrate (Ross et al. 1999). *E. coli* cultures that harbor the *S. floribunda* cDNA were also grown in the presence of BA and SA in the medium (5 mg/mL), as

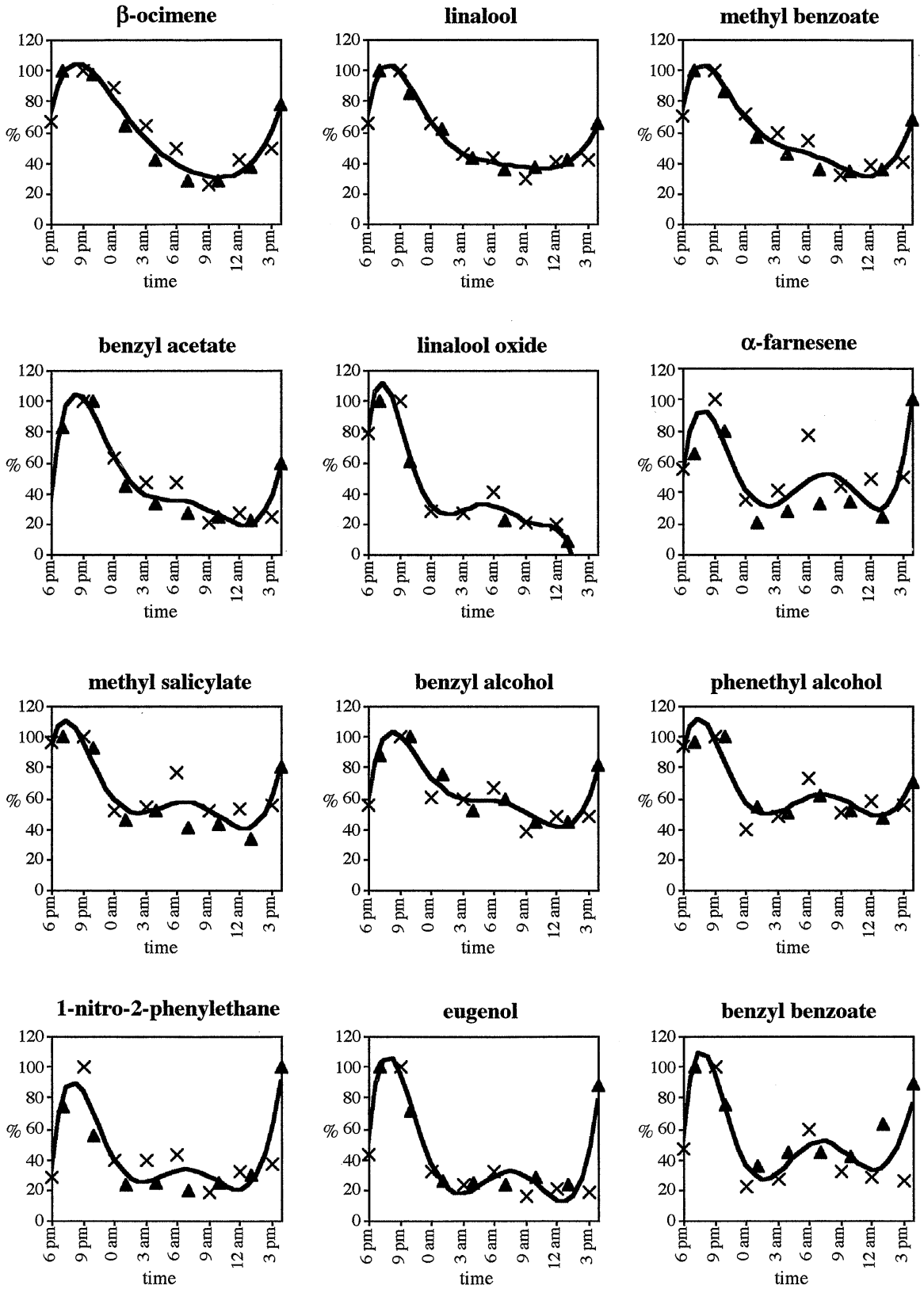

Figure 2. Temporal patterns of volatile emission from *S. floribunda*. The emission of 12 volatiles of two plants was individually followed over a time course of 24 hours (plant 1: x; plant 2: ▲) (light: 6 am till 10 pm). At 3-hour intervals, samples were withdrawn, (0 am: midnight, 12 am: noon), analysed on a GC/MS and relative levels (%) were determined. Lights on: 6 am till 6 pm.

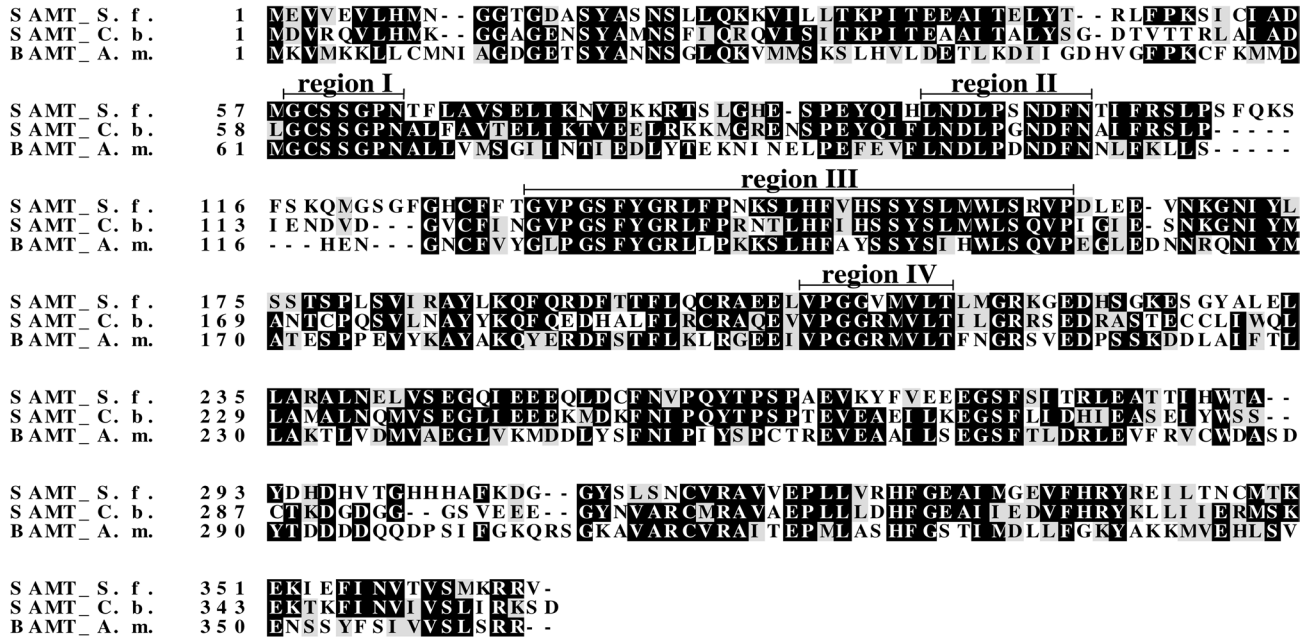

Figure 3. Sequence alignment of the *S. floribunda* SAMT with other plant carboxyl-methyltransferases. The SAMT amino acid sequence of *S. floribunda* was aligned with SAMT from *Clarkia breweri* (SAMT C.b., accession No: AF133053) and BAMT from *Anthirrinum majus* (BAMT A.m., accession No: AF198492). Alignment was performed with the program Clustal X. Identical and similar amino acids are on black or gray background, respectively. The four regions of high sequence identity are indicated.

previously described (Ross et al. 1999). After induction and growth, the spent media were extracted with hexane, and the extracts analysed on GC-MS. When the cells were grown in the presence of SA, the spent medium contained 74 ng/mL MSA, and when the cells were grown in the presence of BA, the spent medium contained 45 ng/mL MBA. We conclude from these results that the *S. floribunda* cDNA encodes an SAMT enzyme that is also active (although to a lesser degree) with BA, thus being similar to *C. breweri* SAMT (Ross et al. 1999), but different from the snapdragon BAMT, which is active only with BA (Dudareva et al. 2000 b).

Levels of transcript and enzyme activities in different organs and at different developmental stages

RNA or crude protein extracts were prepared from the petals and gynostegia (the fused female and male flower parts) as well as from green leaves. Six- to nine-fold higher levels of *SAMT* transcript were found in petals than in leaves or in gynostegia (Fig. 4 A). This result correlated well with levels of enzymatic activities, which were found to be high in petals and very low in leaves (Fig. 4 B). The crude protein extract was also tested for methylation activity with BA, and the results indicate 11-fold lower activity with BA than with SA.

Having observed that most of the SAMT activity and *SAMT* mRNA were found in the petals, we examined *SAMT* transcript levels and SAMT enzymatic activities for five consecutive days after the flowers opened, at the same time point for each day (Fig. 5 A and B). Levels of mRNA did not vary much over the 5 days, while SAMT enzymatic activity peaked on the first day and thereafter decreased continuously.

Evening specific oscillations in steady-state SAMT transcript levels and SAMT activities

Since our headspace analysis demonstrated that MSA is a (previously unidentified) component of the floral scent of *S. floribunda* (Fig. 1), and that MSA emission was higher in the evening and lower during the day (Fig. 2), we examined SAMT enzyme activities and SAMT mRNA levels at different time points over a 72-hour period. *SAMT* mRNA transcript levels were observed to oscillate, with maximum levels in the dark phase (Fig. 6 A, C). SAMT enzymatic activity also oscillated, again with highest enzyme activities occurring in the evening/night (Fig. 6 D).

Discussion

Characterisation of a *SAMT* cDNA from *S. floribunda*

MSA is synthesised from SA and SAM by the enzyme *S*-adenosyl-L-methionine : salicylic acid carboxyl methyltransferase (SAMT), which was previously characterised in flowers of *Clarkia breweri* (Ross et al. 1999). The cDNA isolated by RT-

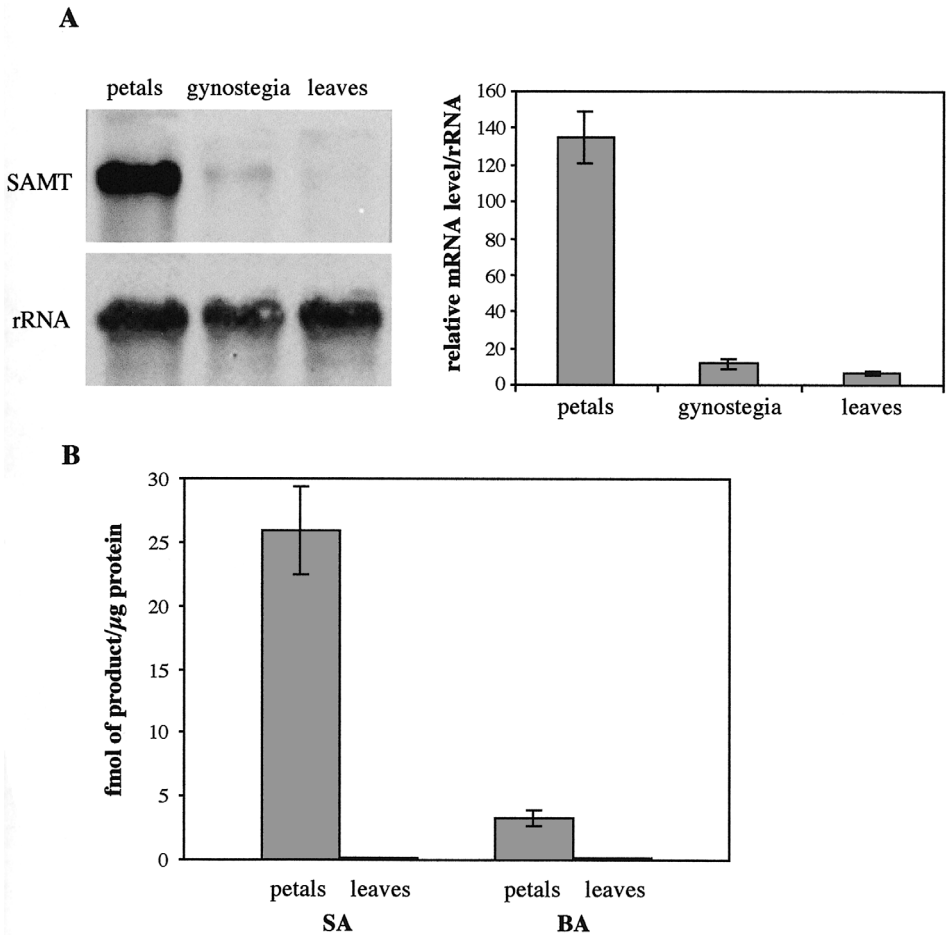

Figure 4. Determination of *SAMT* mRNA levels and SAMT enzymatic activity. (A) Transcript levels and enzyme activities were determined in different organs of *S. floribunda*. Petals, gynostegia and leaves were harvested at 0 : 30 am (midnight). Total RNA was isolated and hybridised on a Northern Blot using the *SAMT* probe from *S. floribunda*. Results were calculated from three hybridisation experiments and normalised to 18S rRNA levels (error bars indicate standard deviation). (B) Petals and leaves were harvested at 0 : 30 am (midnight) and protein extracts were prepared. Enzyme activity was determined with salicylic acid (SA) and benzoic acid (BA) as substrates.

PCR from *S. floribunda* encodes a protein of 366 amino acid length with 84.2 % similarity and 56.3 % identity to *C. breweri* SAMT (Fig. 3). The expression of this cDNA in *E. coli* and the *in vitro* activity assays demonstrated that it has similar activity to SAMT from *C. breweri*, also being active (but to a lesser extent) with BA. Interestingly, crude extracts of *S. floribunda* petals exhibit significantly higher activity with SA than with BA (Fig. 4), even though MBA emission from *S. floribunda* flowers is much higher than MSA emission (Fig. 1). This observation suggests that *S. floribunda* does not have a methyltransferase that is specific to BA, as snapdragon flowers do (Dudareva et al. 2000 b), but that the *S. floribunda* SAMT also synthesises MBA, and that the higher levels of MBA produced may be due to higher levels of BA in the tissue.

Sequence alignments (Fig. 3) of the SAMT from *S. floribunda* with *Clarkia breweri* SAMT and *Anthirrinum major* BAMT revealed 4 regions of very high sequence identity (higher than 78 %). However, these regions are distinct from the conserved regions found in other plant *O*-methyltransferases such as caffeic acid OMTs and caffeoyl-CoA OMTs (Ibrahim et al. 1998, Joshi and Chiang 1998). This strengthens the conclusion reached by Ross et al. (1999) and Dudareva et al. (2000 b) that the SAMT/BAMT type of methyltransferases evolved independently.

Characterisation of the scent emitted by *S. floribunda*

Matile and Altenburger (1988) identified benzyl alcohol, methyl benzoate, linalool, 1-nitro-2-phenylethane and α-farnesene as scent compounds of attached flowers from *S. floribunda*. However, several volatiles remained unidentified. We used the SPME method to identify additional volatiles such as eugenol, benzyl benzoate, linalool oxide, benzyl acetate, phenethyl alcohol, trans-β-ocimene and methyl salicylate as components that appear in the scent mixture of *S. floribunda*.

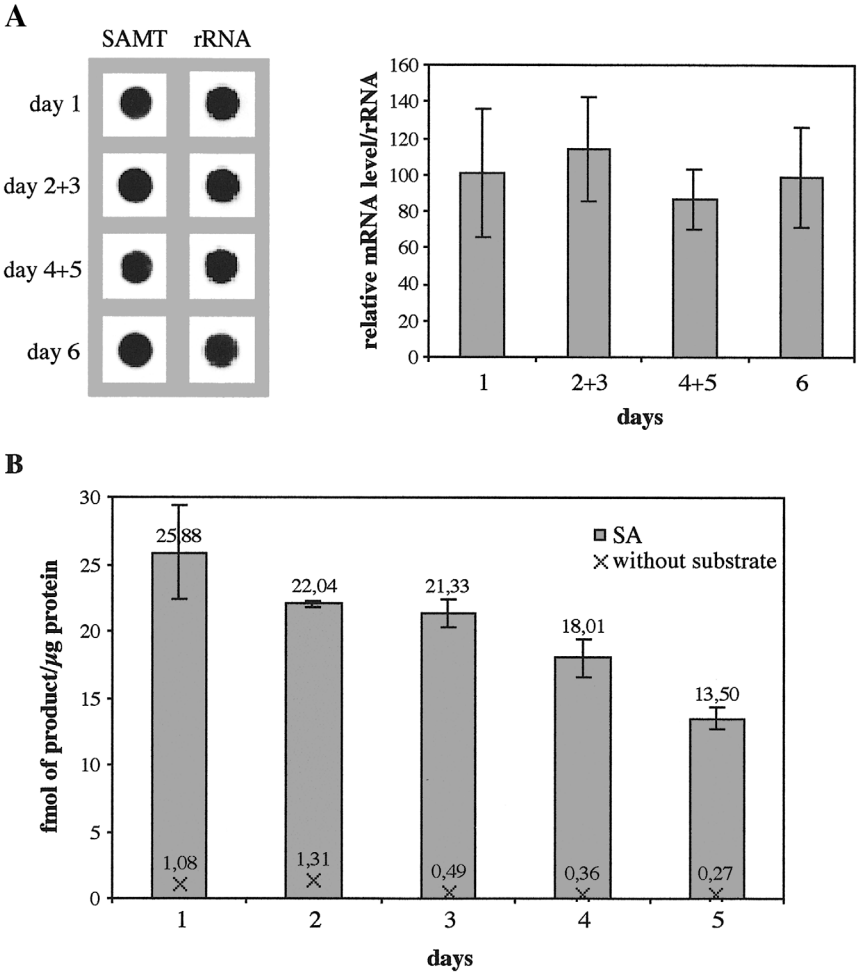

Figure 5. Determination of transcript levels and enzyme activities in petals of *S. floribunda* at different developmental stages. (A) Petals from flowers of different age (2+3: samples of age 2 and 3 post flower opening were combined; 4+5: respectively) were harvested at 0 : 30 am (midnight), and total RNA was isolated and hybridised on a dot blot device using the *SAMT* probe from *S. floribunda*. Results were calculated from three hybridisation experiments and normalised to 18S rRNA levels (error bars indicate standard deviation). (B) Petals of different age (days post flower opening) were harvested at 0 : 30 am (midnight) and protein extracts were prepared. Enzyme activity was determined providing salicylic acid (SA) as substrate.

(Fig. 1). To allow comparison of volatile levels emitted at different time points during the day, two plants were individually examined at 3-hour intervals during a 24 hour period. All twelve volatiles show synchronised emission with higher levels in the evening versus the day, the fold increase, however, varies among the different scent compounds. These results add to the results previously obtained by Matile and Altenburger (1988) and Altenburger and Matile (1990), who observed robust oscillations in emission of methyl benzoate, 1-nitro-2-phenylethane and linalool under both LD and LL conditions.

Spatial and temporal expression of *SAMT* in *S. floribunda*

The time of day-specific emission of fragrance can be a consequence of either time-specific release of stored volatiles or *de novo* synthesis. The latter could be achieved either by increasing enzyme activity through post-translational modification or by new synthesis of active enzyme molecules. The latter option can in turn be regulated either transcriptionally or post-transcriptionally.

In S. *floribunda,* both *SAMT* mRNA and SAMT activity are found in high levels in the petals on the first day when the flowers open. While the mRNA levels do not change significantly during the following days, the levels of enzyme activity decrease continuously (Fig. 5). This result is different from the observations with the scent genes of *C. breweri*. In *C. breweri*, where mRNA for scent genes always peak 1–2 days after anthesis, and while some enzyme activities also decline, others persist at high levels (Dudareva and Pichersky 2000). Thus the persistence of *SAMT* mRNA in *S. floribunda* flowers with the concomitant decline in SAMT enzyme activity levels is the opposite of that observed for some *C. breweri* scent genes and enzyme, and might perhaps be related to the fact that *C. breweri* is an annual plant with short-lived flowers while *S. floribunda* is a perennial plant with long-lived flowers.

We were able to show that *SAMT* mRNA accumulates at higher levels in the evening when SAMT enzyme activity is also increased. This correlation suggests, but does not directly prove, that an important contribution to the early night increased emission of MSA is the increase in biosynthetic capacity that is brought about by increased synthesis of the SAMT mRNA and protein. Experiments to test these hypotheses are ongoing.

In *S. floribunda* flowers, at least 12 volatiles, including MSA, are synchronously produced, suggesting that the syn-

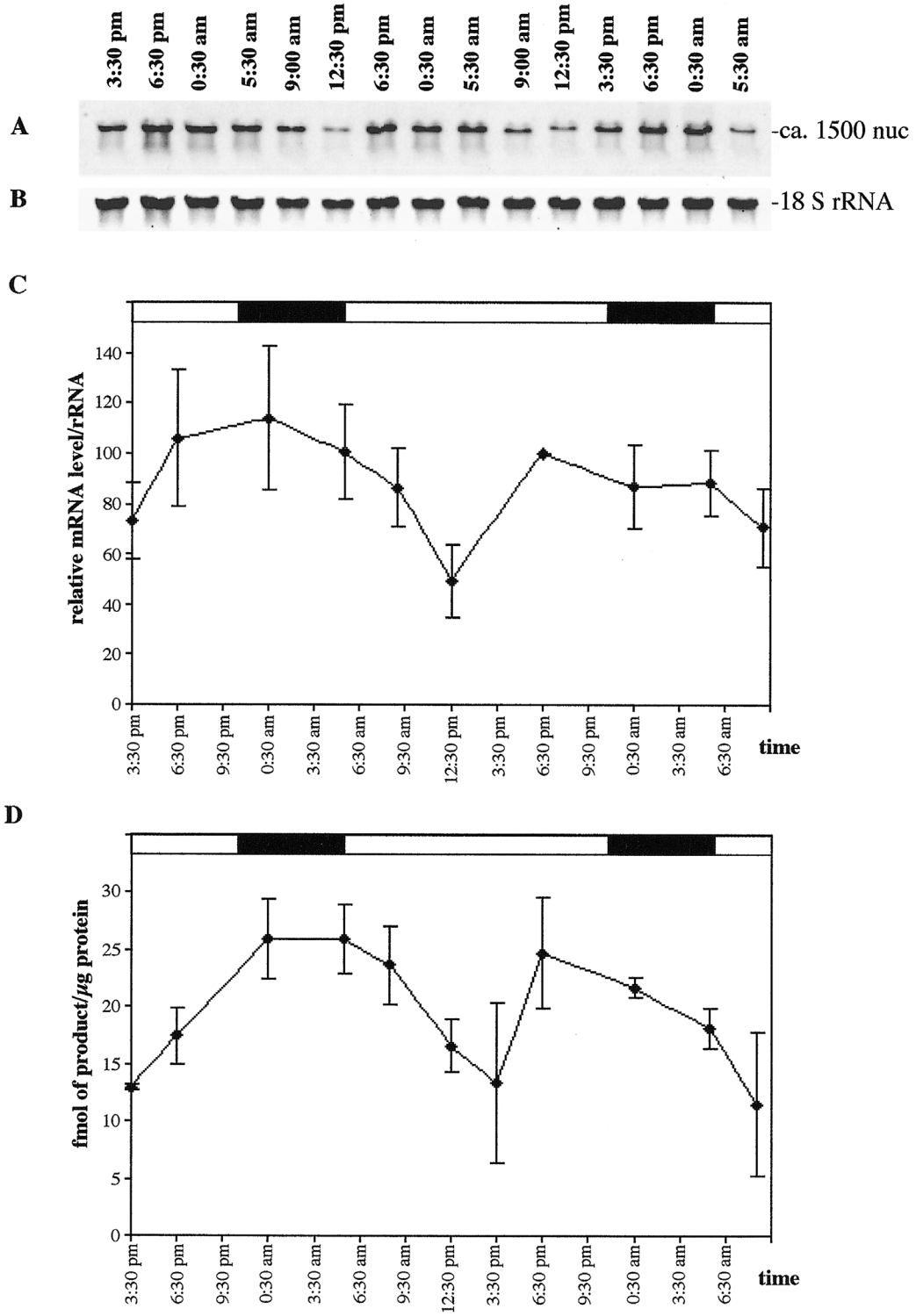

Figure 6. *SAMT* mRNA and enzyme activity oscillations in *S. floribunda* petals. (A) An example of RNA hybridisation experiments (Northern Blot) with RNA samples prepared from petals harvested at different time points at three consecutive days (0:30: midnight; 12:30 noon) and hybridised with the *S. floribunda* SAMT probe. (B) Some RNA samples were hybridised with the 18S rDNA probe. (C) mRNA oscillations at two consecutive days. Combined results of three RNA hybridisation experiments including the one presented above. (D) SAMT activity at two consecutive days. Sampling times were as in (A), except instead of 9:00 am samples were taken at 8:30 am. Protein extracts were immediately prepared at different time points during the first and second day after flower opening to determine enzyme activities with salicylic acid as substrate. Error bars indicate minimum and maximum of two measurements.

MSA, are synchronously produced, suggesting that the synthesis of these compounds is coordinately regulated. Furthermore, circadian oscillations of methyl benzoate, 1-nitro-2-phenylethane and linalool emission were observed under constant conditions (LL) also in tight synchrony with each other (Altenburger and Matile 1990). That a circadian clock, an endogenous regulatory circuit that usually is synchronised by external signals, is indeed involved in the regulation of MSA emission is suggested by the observation that after one day under constant conditions (LL), SAMT enzyme activity oscillations persist (Pott and Piechulla, unpublished results). Our data further suggest that one way in which this circadian clock controls the emission of MSA is by bringing about the oscillations in *SAMT* mRNAs and in SAMT enzymatic activity.

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