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Transcriptional and post-translational regulation of S-adenosyl-L-methionine : salicylic acid carboxyl methyltransferase (SAMT) during *Stephanotis floribunda* flower development

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Summary

Methyl salicylate (MeSA) and a number of other volatiles are primarily emitted in the evening/night by *Stephanotis floribunda* leading to attraction of night active pollinators. A second minor emission peak for MeSA occurs in the morning/day. To understand these emission patterns, we have studied in detail the temporal regulation of the last step of the biosynthetic pathway of MeSA, the convertion of salicylic acid (SA) to MeSA catalysed by S-adenosyl-L-methionine : salicylic acid carboxyl methyltransferase (SAMT). We observed that in young flowers a maximum in SAMT activity occurs in the night, and that in flowers which were open longer than 4 days, two SAMT activity maxima occurred per day. These maxima correlated well with dawn and dusk and the previously detected MeSA emission peaks. The SAMT mRNA levels, however, have a broad maximum during the dark phase, while the SAMT protein levels continuously increase during floral development without showing daily rhythms. Furthermore, under continuous illumination (LL) the SAMT mRNA levels and activity patterns oscillate, suggesting the involvement of a circadian clock in the regulation network. Taken together, this analysis clearly demonstrates that regulation of MeSA emission occurs both at the transcriptional and post-translational levels, indicating that control at more than one level is necessary to guarantee the precise timing of volatile emission in flowers of *S. floribunda*.

Key words: *Stephanotis floribunda* – S-adenosyl-L-methionine : salicylic acid carboxyl methyltransferase (SAMT) – circadian clock – transcriptional and post-translational regulation

Abbreviations: SA = salicylic acid. – BA = benzoic acid. – MeSA = methyl salicylic acid. – SAMT = S-adenosyl-L-methionine : salicylic acid carboxyl methyltransferase

Introduction

Flowers emit scent to attract specific pollinators to improve fruit and seed production. The bouquet emitted by the flowers can vary in respect to their chemical composition (e.g. terpenoids, phenylpropanoids, benzenoids, fatty acid derivatives), their absolute levels (concentrations) as well as their ratios. These three parameters can be used by the pollinator as cues for plant or flower selection. Since the biosynthesis of these secondary metabolites in plants is energetically expensive, flower scent compounds are often only synthesised in

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specific tissues (petals, stigma, sepals), or during certain times of floral development, when the flowers are sexually mature. In a number of plant species, volatiles are primarily emitted at specific times during the day or night (summarised in Dudareva et al. 2000 b). This emission is usually correlated with the activity of the pollinator, e.g. flowers emitting scent during the day are pollinated by day-active bees, bumble bees and butterflies, while those plants that emit primarily during the night are pollinated by evening- or night-active organisms (e.g. bats, moths).

Stephanotis floribunda (Asclepiadaceae), a native plant from Madagascar, emits its fragrance primarily during the night. Twelve compounds (methyl benzoate, methyl salicylate, benzyl benzoate, benzyl acetate, benzyl alcohol, phenethyl alcohol, 1-nitro-2-phenylethane, beta-ocimene, linalool, linalool oxide, alpha-farnesene, eugenol), identified by GC-MS analysis, exhibit a nocturnal emission pattern (Pott et al. 2002). The maxima of emission were reached between 6 pm and 9 pm while lowest levels were around noon. Due to the precise timing of scent emission of compounds, synthesised by different biosynthetic pathways (e.g. the phenyl propanoid and the terpenoid pathway), the mechanism that coordinates the synthesis and emission is of interest. Altenburger and Matile (1990), who demonstrated oscillations of methyl benzoate, 1-nitro-2-phenylethane and linalool under constant illumination, suggested the involvement of a circadian clock.

Methyl salicylate (MeSA), the major component of wintergreen oil, is a prominent component of volatiles of many plants, including *S. floribunda*. It is derived from salicylic acid (SA), which is an important component of the signal transduction pathway of the pathogen related systemic aquired resistance reaction (SAR) (Dong 2001, Glazebrook 2001). The methylation of salicylic acid is performed by the S-adenosyl-L-methionine : salicylic acid carboxyl methyltransferase (SAMT). Flower-specific SAMTs, and the genes that encode them were previously characterised from *Clarkia breweri* (Ross et al. 1999) and *S. floribunda* (Pott et al. 2002). The *C. breweri* and the *S. floribunda* SAMT enzymes share 56 % sequence identity at the amino acid level, and only 43 % sequence identity to a benzoic acid methyl transferase (BAMT) from *Anthirrinum majus* (Dudareva et al. 2000 a). Although both compounds, MeSA and MeBA, are present in the flower scent of *S. floribunda*, enzyme assays indicate the presence of SAMT but not of BAMT, suggesting that the SAMT enzyme also accepts BA.

Careful measurements of daily MeSA emission from *S. floribunda* revealed two maxima, one with a high amplitude in the evening/night (6 to 9 pm) and one with a significantly smaller amplitude in the morning (6 to 9 am). It was also shown that at this time in leaves, the *SAMT* mRNA levels and SAMT enzyme activities were below detection level, suggesting that the morning/day peak was created by the flowers. Since it is accepted that *S. floribunda* is a moth-pollinated species (personal communication, Dr. Gottsberger and Dr. Wessjohann), it appears clear that the function of the evening/night MeSA emission is to attract the pollinator. However, the function of the smaller morning/day MeSA emission remains unclear. To get a better understanding of the MeSA emission in *S. floribunda* flowers, we examined the regulation of SAMT expression, with particular interest in the precise timing of volatile emissions.

Material and Methods

Plant material and growth conditions

Stephanotis floribunda (Brongn.) plants were grown in a greenhouse with additional light. Plants were illuminated for a minimum of 16 hours (from 6 am to 10 pm) artificial light (160 μmol/m^2 $\times$ sec). Continuous light conditions (LL) were performed in winter, and artificial light was the main light source during the day.

Enzyme extraction

All extraction steps were carried out on ice. Harvested petals of *Stephanotis floribunda* (minimum of two flowers were pooled) were immediately submerged in freshly prepared ice-cold extraction buffer containing 50 mmol/L BisTris-HCl, pH 6.9, 14 mmol/L β-mercaptoethanol, 1 % (v/w) polyvinyl-pyrrolidone (PVP-40), and 5 mmol/L $Na_2S_2O_5$, 10 % glycerol (ratio buffer : tissue was 4 : 1) (Wang et al. 1997). Protease inhibitors (1 mmol/L PMSF or proteinase inhibitor cocktail (Roche) were added prior to petal extractions. The tissue was homogenised in a chilled mortar with 1/10 volume of quartz sand. The homogenate was centrifuged for 15 min at 12,000 $\times$ g. 1/4 th of the volume glycerol was added to the supernatant and then stored at $-20\,^{\circ}$C.

Enzyme assay

All assays were performed immediately after enzyme extraction. One reaction contained crude enzyme extract (10 μL), assay buffer (250 mmol/L Tris-HCl, pH 7.5, 25 mmol/L KCl; 10 μL), salicylic acid (50 mmol/L in ethanol, 1 μL), and S[methyl-^{14}C]-adenosyl-L-methionine (58 μCi/mmol; Hartmann, Braunschweig, Germany; 1 μL). Water was added to a final volume of 50 μL. Samples were mixed and incubated for 50 min at 25 $^{\circ}$C. The reaction was terminated by the addition of 3 μL HCl, and methyl salicylate was extracted with 100 μL ethylacetate. 30 μL of the ethylacetate layer was mixed in 2 mL scintillation liquid (Packard Bioscience, Meriden, CT) and the radioactivity was measured in a liquid scintillation counter (Tri-Carb 2100TR, Packard, Canberra). All assays were conducted in duplicates.

Protein expression and preparation of antibodies

The *SAMT* gene was amplified and cloned into the vector pCR®T7/CT-TOPO which carries a C-terminal polyhistidine tag (6 $\times$ His). The vector was transformed into *E. coli* (HM174 [D3]). An overnight culture was

inoculated into 1 L LB medium. Cells were grown to an OD of ca. 0.3–0.5 and IPTG was added to a final concentration of 1 mmol/L. The protein was purified by Ni-NTA affinity chromatography following the standard protocol (Qiagen, Hilden, Germany). As the final purification step, the overexpressed protein was quantitatively separated by SDS-PAGE and recovered from the gel using an electroeluter (Bio-Rad Laboratories, Hercules, CA). A polyclonal antibody was produced by Biotrend (Cologne, Germany).

SDS-PAGE and Western blot analysis

Twenty micrograms of protein was loaded onto a polyacrylamid gel (12 %) and electrophoretically separated (Miniprotean, Bio-Rad Laboratories, Hercules, USA). Proteins were transferred to a nitrocellulose membrane (Optitran BA-S 83, Schleicher & Schüll, Dassel, Germany) using a mini tank blotting gel cassette (XCell II, Novex, San Diego, USA). The blotting buffer consisted of 12 mmol/L Tris, 96 mmol/L glycine, 20 % methanol (v/v) which results in a pH of 8.3. Protein immobilisation on the membrane was checked by staining with Ponceau red (0.5 % w/v in 1 % v/v in acetic acid). For blot development, the membrane was placed in blocking solution (TBS, 0.05 % Triton X-100, 4 % skim milk, 1 % BSA, w/v) and then exposed to the primary antibody (in TBS buffer). After repeated washing (3 × 20 min) with TBS-Triton, the membrane was treated with the secondary antibody (anti-rabbit alkaline phosphatase conjugate; Sigma-Aldrich, St. Louis, USA) and washed again with TBS-Triton (3 × 20 min) and TBS (1 × 10 min). Incubations and washings were all done under agitation. For visualisation of SAMT protein, the membrane was equilibrated in detection buffer (100 mmol/L Tris-HCl, 150 mmol/L NaCl, 50 mmol/L $MgCl_2$) and incubated with CSPD (Roche Molecular Biochemicals, Mannheim, Germany; 0.25 μmol/L in detection buffer) for 20 min under darkness. The membrane was exposed to the Luminescent Image Analyzer LAS-1000 (Fujifilm, Japan) and luminescence was read for 10 min. Assessment was done with Fujifilm Image Gauge software.

RNA extraction and hybridisation

The RNA was extracted as described in Sambrook et al. (1989). For each extraction the petals of at least 5 flowers per time point were used. RNA samples (3 μg each) were separated on a formaldehyde denaturing gel and transferred to a nylon membrane (Roche Diagnostics, Mannheim, FRG). For time-specific expression, the RNA (3 μg in 16× 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 (5′-AATGGAAGTTGTTGAAGTTC TTC-3′) and STSAMT 18 Reverse (5′-ATCTGAAAATGGTGTTGAAATC AT-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′-TTATTGCCTCAAACTTCC-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× SSC, 0.1 % SDS at room temperature and twice for 15 min with 0.1× 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 choosen as a standard and was set to 100 %.

Results

SAMT activities in different developmental stages of the flowers

We have previously shown that two peaks were observed in the daily emission of methyl salicylate (MeSA) from whole *S. floribunda* plants, which included several inflorescences and vegetative tissues (Pott et al. 2002). One prominent maximum in the evening/night (6 to 9 pm), and a smaller maximum occurred in the morning (6 to 9 am). In contrast to these two peaks, only one peak of SAMT activity and *SAMT* mRNA accumulation occurred during a 24-hour light/dark period in flower petals of *S. floribunda*. Both parameters correlated well with the evening/night-specific emission maximum. To examine the discrepancy between the occurance of two peaks of emission, but only one peak of enzyme and mRNA per 24 hours, we performed a detailed study of enzyme activities in petal extracts harvested from flowers of different developmental stages. The *Stephanotis* inflorescences are usually composed of 5–12 individual flowers which open their petals independently and desynchronised. For each individual flower, the day of opening was marked, to allow harvest of flowers of defined developmental stages. Flowers scenescence normally begins on day 7, when the petals start to turn brown and wilt. In our experiments, flowers of five developmental stages were collected at indicated time points during the day and night and SAMT activities were determined in these extracts. In petals of flowers that were opened for one and two days, the SAMT activity reached one maximum during the night, while two SAMT activity peaks were detected in flowers that were opened for five days (Fig. 1). During the first three days after flower opening, the maximum shifts from shortly after midnight, to about sunset. On the fourth to the sixth day, two peaks are present and are separated by approximately 12 hours, one peak was around 6 pm and the other around 6 am. Therefore during development a shift from one to two peaks of elevated SAMT activities per day occurs. This temporal pattern of SAMT activities correlates well with the temporal pattern of MeSA emission (Pott et al. 2002).

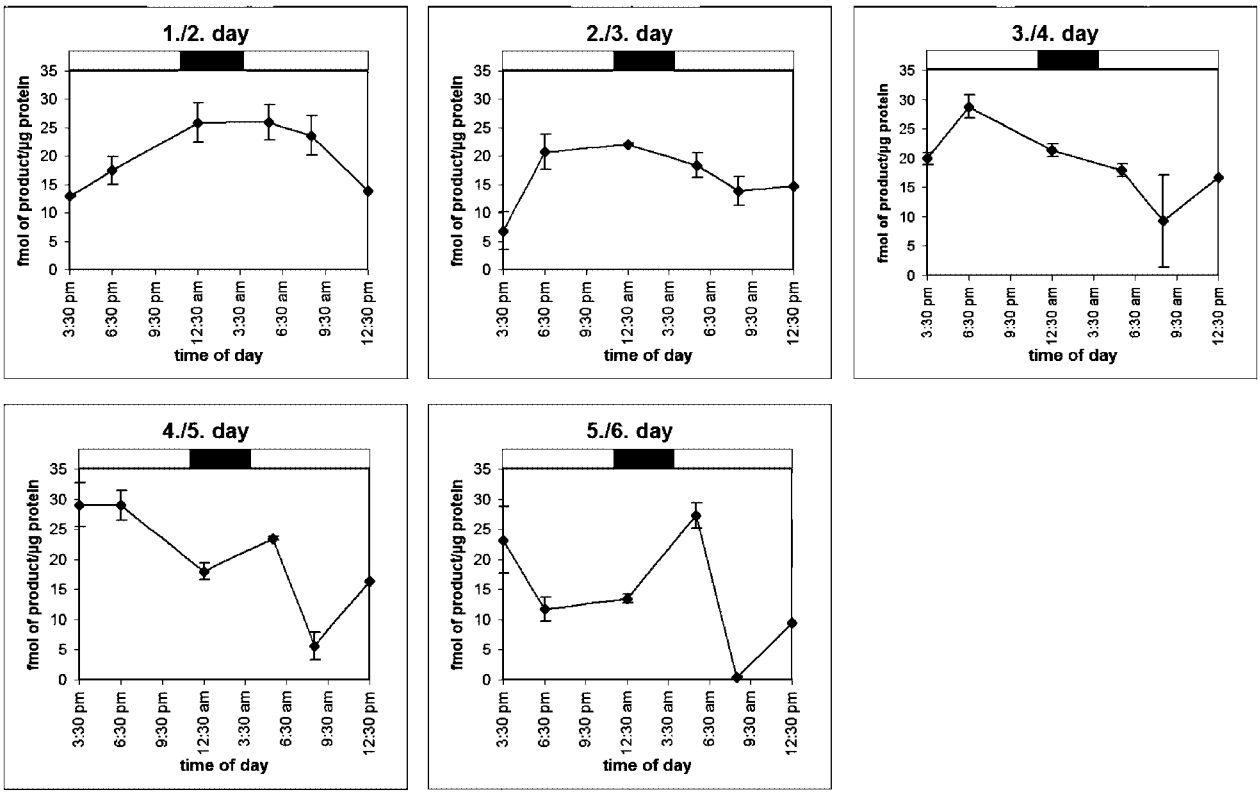

Figure 1. SAMT activities in *Stephanotis floribunda* petals during flower development. At indicated times after flower opening (days) petals were harvested at different time points during the day. Tissue was extracted and methylation activity was determined with crude petal extracts using salicylic acid (SA) as substrate (average values calculated from 2 independent assays, error bars: min/max values). Open bar: day/light, black bar: night/dark.

Temporal correlation between SAMT activity and protein levels

The daily oscillations of SAMT activities in flowers of *S. floribunda* can be the result of changes in the amount of enzyme, or alterations of the enzyme activity. To discriminate between these two possibilities, we performed Western blots with polyclonal antibodies made against *E. coli*-produced *S. floribunda* SAMT protein. The antibodies recognised a major band of 40 kD in the overexpressed *E. coli* and DE53 and MonoQ purified protein solution (Fig. 2 A), a protein of the same size was detected in crude flower extracts. In three day old petal extracts, an additional band at 30 kD appears (Fig. 2 A). The calculated molecular mass of the SAMT protein is 40.2 kD. This correlates very well with the *E. coli* purified protein and the upper band in the flower extracts. The identity of the ca. 30 kD protein is presently unknown, however the independent experiments show that it is primarily present in older petals (Fig. 2 A, B, C). Addition of proteinase inhibitors reduces the amount of the 30 kD protein indicating that it is most likely a degradation product of the 40 kD protein (Fig. 2 B). Fig. 2 C shows that in one day opened flowers, low levels of the 40 kD protein are detectable, while the 30 kD protein is almost undetectable. During flower development, both proteins accumulate, reaching high levels on the fourth day. It is interesting to note that significant levels of the 30 kD protein are reached from the third day on, which correlates approximately with the appearence of the second SAMT activity maxima per day (Fig. 1).

Analysis of the levels of the 30 and 40 kD proteins at different time points during the day (Fig. 2 C) revealed fluctuations but no ca. 24 hour-oscillations. Therefore it seems unlikely that the SAMT activity oscillations (Fig. 1) are due to rhythmic variations of enzyme accumulation.

SAMT transcript levels during flower development

SAMT RNA was isolated from pooled petals harvested from spring until summer of one to three day, and four to seven day old flowers, analysed on Northern blots hybridised with the *SAMT* specific probe (Fig. 3). In young flowers, the mRNA levels reach a maximum around midnight, which correlates well with the SAMT activity pattern at this developmental stage (Fig. 1). In older flowers, the *SAMT* mRNA pattern exhibits two peaks within 24 hours. The time points of the mRNA

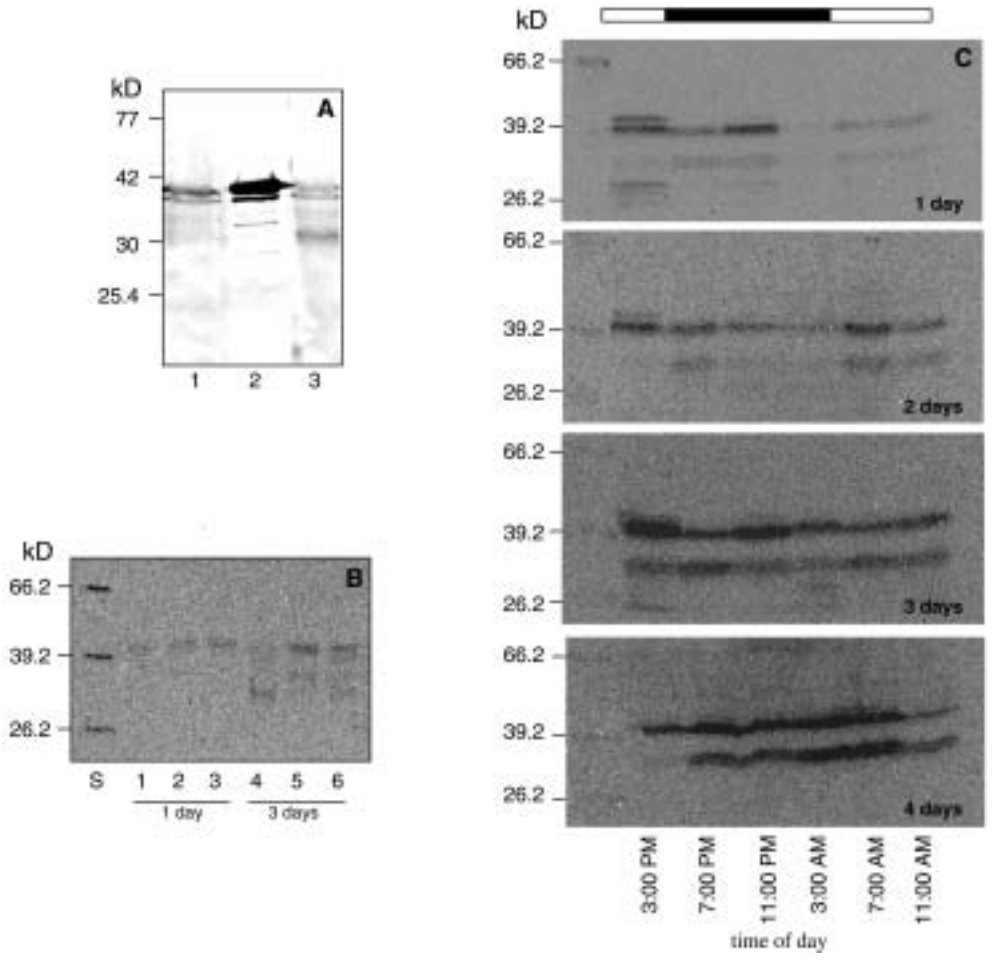

Figure 2. SAMT protein in *S. floribunda* petals during flower development. A) Extract from *E. coli* expressing the *S. f. SAMT* gene, purified by DE53 and MonoQ ion exchange chromatography (lane 2), and crude extract from 1 (lane 1) and 2 (lane 3) day old *S. floribunda* petals were separated by SDS-PAGE and incubated with S. f. SAMT specific polyclonal antibodies. B) Protease inhibitors (PMSF, lane 2 and 5) and inhibitor cocktail (lane 3 and 6) were added to the extraction buffer prior to extraction of 1 day (lane 1–3) and 3 day (lane 4–6) old flowers. C) At indicated times after flower opening (days) petals were harvested at different time points during the day. Protein was extracted, separated by SDS-PAGE and identified by SAMT specific polyclonal antibodies (Western blot). Open bar: day/light, black bar: night/dark.

maxima preceed the time points of SAMT activity maxima by about 6 hours.

Temporal correlation of SAMT activity with temperature and light quality changes

During SAMT activity determination of environmental parameters were additionally recorded (Fig. 4). No correlation between light intensity and temperature was observed, but changes of light quality that occur during dusk and dawn precisely relate to the time points of elevated SAMT activities. Around sunrise and sunset, the wavelength of 500 to 690 nm (red light) are significantly decreased and the far red/red light ratio was increased.

SAMT mRNA and SAMT activity under continuous illumination

Flowers, one to five days after opening, and flower buds were analysed for *SAMT* mRNA and SAMT activity at different time points during the day under continuous illumination (LL). As in the previous experiment, the SAMT activity pattern of young flowers, as well as in buds, changes from one peak to two peaks per 24 hours (Fig. 5 A).

At the same time, steady-state *SAMT* transcript levels in flowers were determined under continuous illumination (Fig. 5 B). Oscillations, however, with a small amplitude were determined. Under these growth conditions a broad mRNA peak per 24 hours was visible, but it was not clear from this analysis, whether two clear defined peaks were present in the older flowers.

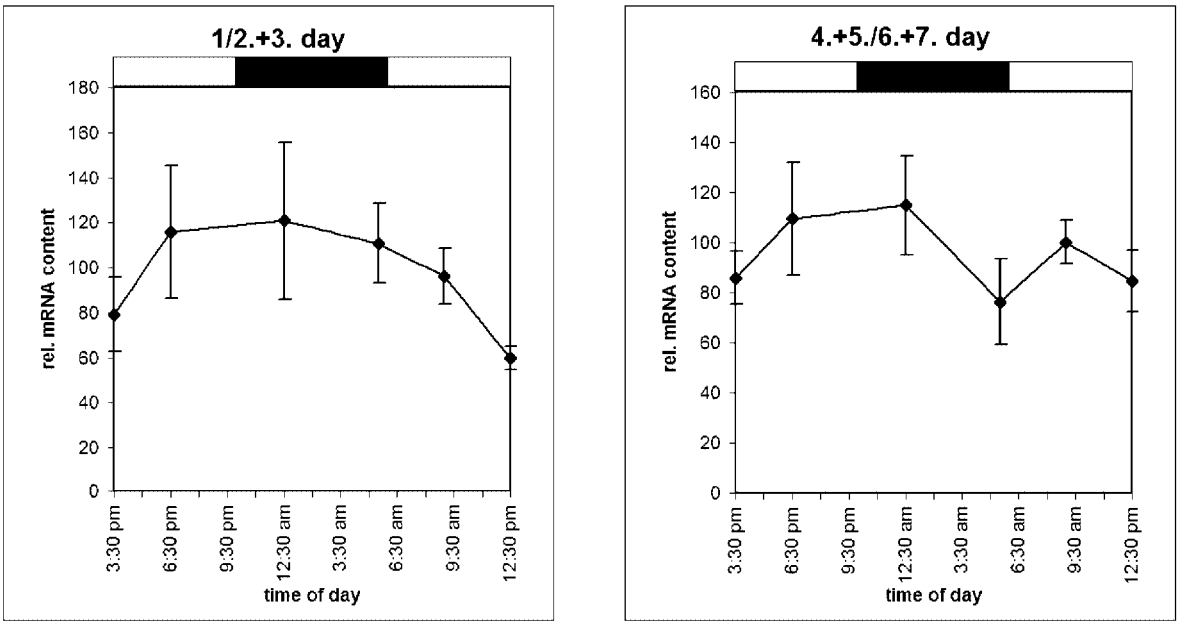

Figure 3. *SAMT* transcripts in *S. floribunda* petals during flower development. At indicated times after flower opening (days) petals were harvested at different time points during the day. Petal tissue was used for RNA isolation. Total RNA was hybridised with the *S. f. SAMT* probe and relative levels were calculated based on five RNA hybridisations (error bars: SE). Open bar: day/light, black bar: night/dark.

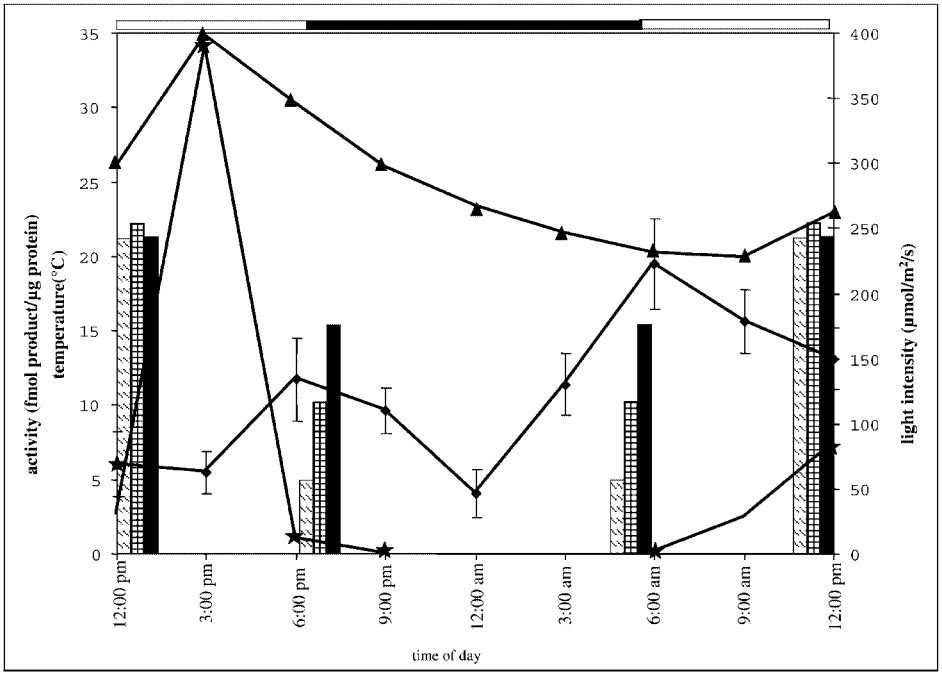

Figure 4. Correlation of SAMT enzyme activity with environmental alterations. SAMT activity in *S. floribunda* petals pooled from different developmental stages was determined at different time points during the day (black diamonds, 2 independent assays, error bars: min/max values). During the harvest temperature (triangles) and light intensity (stars) were registered. Changes of light quality and photon quantity at noon, dawn and dusk are indicated (600 nm hatched columns, 660 nm checkered columns, 690 nm filled columns). Open bar: day/light, black bar: night/dark.

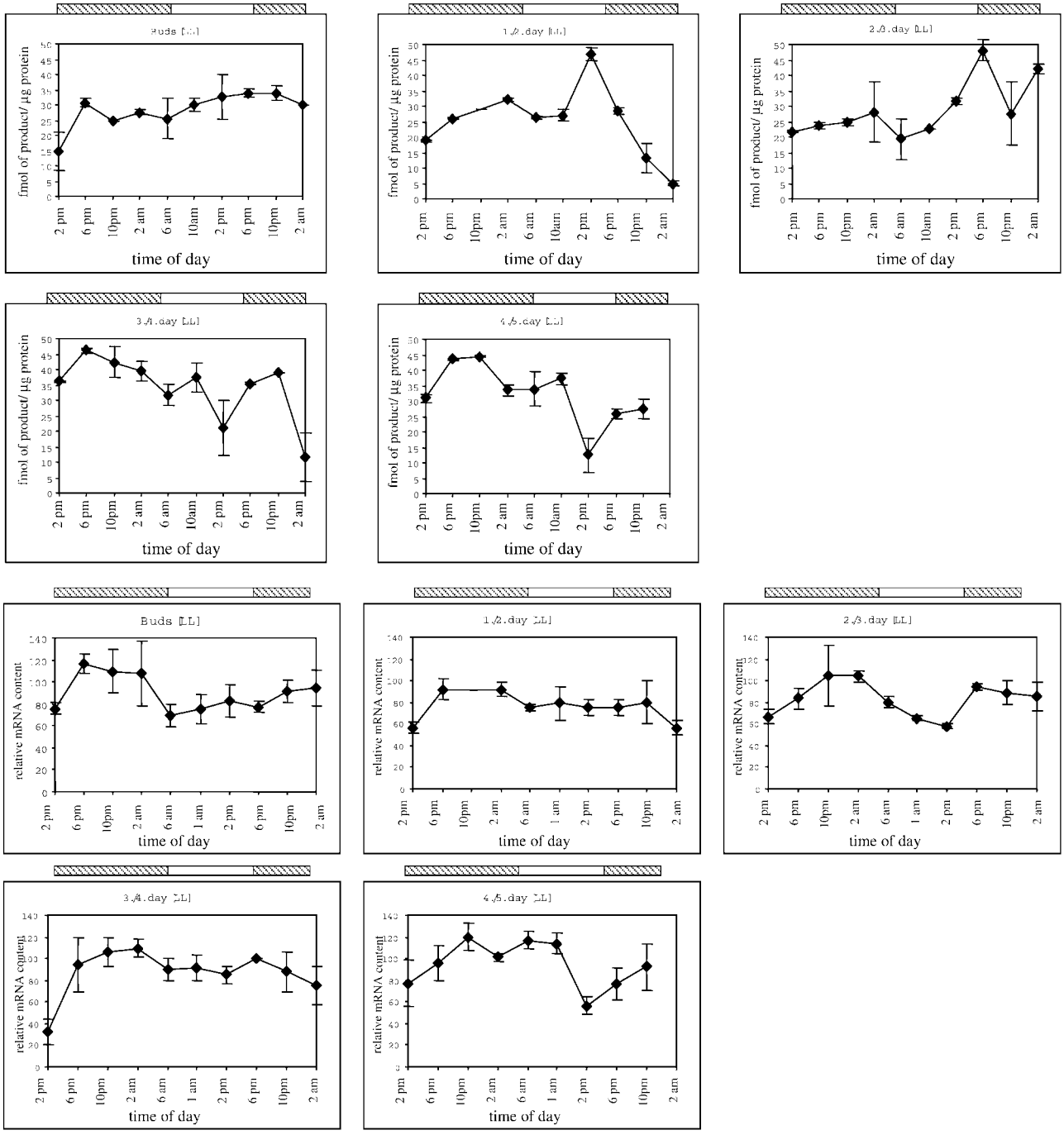

Figure 5. *SAMT* transcript and SAMT activity in *S. floribunda* petals during flower development under continuous illumination (LL). *S. floribunda* plants were continuously illuminated (160 µmol/m^2 × sec) for one week. At indicated times after flower opening (days) petals were harvested at different time points during the day. Tissue was extracted according to the respective methods to determine steady-state mRNA and enzyme activity levels. Open bar: subjective day/light phase, hatched bar: subjective night/dark phase. A) Methylation activity of petal extracts was determined using salicylic acid (SA) as substrate (2 independent assays, error bars: min/max values). B) Total RNA was hybridised with the *S. f. SAMT* probe and relative levels were calculated, based on three RNA hybridisations (3 hybridisations, error bars: SE).

Discussion

To understand the regulation of nocturnal flower scent emission, one has to take into account that each step of the biosynthetic pathway, as well as the volatile storage and emission process can be controlled. To unravel this complex process, we focussed on the last step of MeSA synthesis, the conversion of SA to MeSA catalysed by the SAMT. Our inves-

tigations revealed two key steps, which are likely to be responsible for the nocturnal MeSA emission of *Stephanotis* flowers, a) at the transcriptional and b) at the post-translational level. This conclusion is based on the fact that nocturnal *SAMT* transcript and SAMT activity oscillations were observed, while the protein levels do not show rhythmic alterations.

Furthermore, we were able to show that the SAMT activity pattern changes during flower development, from one to two activity maxima within 24 hours. Both amplitudes per day and at different developmental stages, reach approximately the same heights. Upon comparison it appears that the SAMT activity pattern in *Stephanotis* flowers is distinct from other methyltransferases in other flowers, e.g. increasing BAMT enzyme activities until seven days post-anthesis (thereafter activity levels decrease) were found in *Anthirrinum* flowers, while after anthesis decreasing levels were observed of the SAMT activities in *Clarkia breweri* (Kolosova et al. 2001, Dudareva et al. 1998). In respect to the daily temporal analysis only the BAMT from *A. majus*, *Petunia* cv. *Mitchell* and *Nicotiana suaveolens* have so far been investigated (Kolosova et al. 2001). In *N. suaveolens* the BAMT activity reaches a maximum at the end of the night phase (amplitude 10–20 %), in *Petunia* the maximum occurs at the end of the light phase (amplitude 30 to 40 %), and in three and four day old snapdragon flowers the BAMT activity increases continuously during the day, and lower levels are measured at night (amplitude 10–20 %). While the patterns of *Petunia* and snapdragon are similar in respect to the time point of activity maxima, the characteristic two-peak SAMT activity pattern of *S. floribunda* is distinct to all three BAMT patterns. Together, these results indicate that the regulation of the diurnal and nocturnal MeBA emission in snapdragon, *Petunia* and *N. suaveolens* is different to the MeSA emission of *S. floribunda*.

At present it is not known if the two SAMT activity peaks in *S. floribunda* flowers result from one or two enzymes expressed from independent genes. Our own experiments indicate the existance of two very related genes in *S. floribunda*, sharing 89 % sequence identity at the nucleotide level, revealing 17 amino acid alterations (Henning and Piechulla, unpubl. data). None of these changes are in the SAM or SA binding pocket. Alternatively, if the SAMT protein derives from one gene, the following scenarios may apply: The night peak present in young flowers is depressed/inhibited around midnight in older flowers, and only the flanking SAMT activities remain present, or the SAMT enzyme is activated twice a day. In this context it is also interesting to note that a 30 kD protein which is identified by the SAMT polyclonal antibody accumulates on the third day after flower opening which correlates with the time point of appearance of the second SAMT activity peak. It is intriguing to investigate whether the 30 kD protein has methylation activity and is responsible for the second SAMT peak appearing in older flowers.

Interestingly, the time points of enzyme activity occurs precisely at dawn and dusk, when light quality changes occur, and the far red/red light ratio increases. The biological importance of this parameter is well known and the temporal correlation between the low red light portion of the sun light and the increased SAMT activity seems to provide circumstantial evidence that phytochrome might be involved in the regulatory network. But to our knowledge, enzyme activation without the signal transduction chain leading to gene activation of a target gene has not yet been described. This possibility, however, should not be excluded since the structural similarities of phytochrome, with the bacterial sensory histidin protein kinase, has rejuvenated the idea, that phytochromes might be a light-dependent protein kinase (Hughes and Lamparter 1999).

Our investigations have also demonstrated a control at the transcriptional level. The *SAMT* transcripts oscillate during the day, reaching one or two maximum per night. Such rhythmic patterns imply that the mRNA half-life times have to be smaller than six hours otherwise such oscillations cannot be observed. This was recently demonstrated for the *Lhc* mRNAs from tomato, tobacco and spinach (T_H approximately 3 hours) while the turn-over times for the respective transcripts in *Pinus* and *Ginkgo* were 24 or 37 hours, and in the latter species no transcript oscillations could be detected (Brinker et al. 2001).

In contrast to the characteristic nocturnal mRNA oscillations, no rhythmic alterations of SAMT enzyme accumulations could be detected, indicating that the alterations of mRNA oscillations have no apparent impact on the protein level. A similar case was previously obtained for the light harvesting complex (Lhc) genes, where mRNA oscillations with large amplitudes did not reveal alterations of the steady-state protein levels, and only detailed studies showed that the synthesis of LHC proteins is indeed rhythmic (Riesselmann and Piechulla 1992).

The nocturnal and circadian emission pattern of scent compounds from *S. floribunda* flowers has suggested the involvement of a circadian clock as a regulatory mechanism (Altenburger and Matile 1990, Pott et al. 2002). Our analysis of *SAMT* transcript and SAMT activity levels under LD and continuous illumination (LL) support this hypothesis. Similar results were also obtained for the *BAMT* mRNA and BAMT enzyme regulation in *A. majus* (Kolosova et al. 2001). The recruitement of the circadian clock as a control mechanism has the advantage that even under poor light conditions volatiles can be emitted at the precise time point to achieve optimal pollination. It was, however, a surprise to see that the circadian clock seems to interact at two rather than one level. In other cases of circadian control, only one step was affected, e.g. for the luciferin binding protein (lbp) in *Gonyaulax polyedra*, the mRNA levels were constant while the protein accumulation exhibited a pronounced oscillation (Morse et al. 1989), and the mRNAs of the light harvesting complex genes (Lhc) in higher plants dramatically oscillate but the protein levels do not (Riesselmann and Piechulla 1992, Kellmann et al. 1993). The duplication of control in the case of *SAMT* gene

expression in *S. floribunda* has two advantages: i) several control steps are able to ensure the precise timing of volatile emission, and ii) control at more than one level allows fine tuning so that at each level no deficit or surplus of metabolites occurs, but rather balanced metabolic conditions in the flower tissue provide optimal pollination. Along this line a recent study, using microarray technology showed that about 6 % of the ca. 8000 genes investigated exhibited an oscillatory expression pattern in *A. thaliana* (Harmer et al. 2000). Particularly interesting was that 18 (putative) genes of the phenylpropanoid biosynthetic pathway, including PAL as the initial enzyme of this pathway showed coordinated maxima of steady-state transcript levels before subjective dawn. This survey demonstrates i.) that a precise timing of complete biosynthetic pathways is an efficient way to manage and coordinate metabolic activity, and ii) that one way to achieve this on the cellular level is to entangle the circadian clock in the regulatory network.

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