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N-METHYLTRANSFERASE ACTIVITIES IN CAFFEINE BIOSYNTHESIS: BIOCHEMICAL CHARACTERIZATION AND TIME COURSE DURING LEAF DEVELOPMENT OF *COFFEA ARABICA*

SIMONE S. MÖSLI WALDHAUSER, JOSEF A. KRETSCHMAR and THOMAS W. BAUMANN*

Institute of Plant Biology, University of Zurich, CH-8008 Zürich, Switzerland

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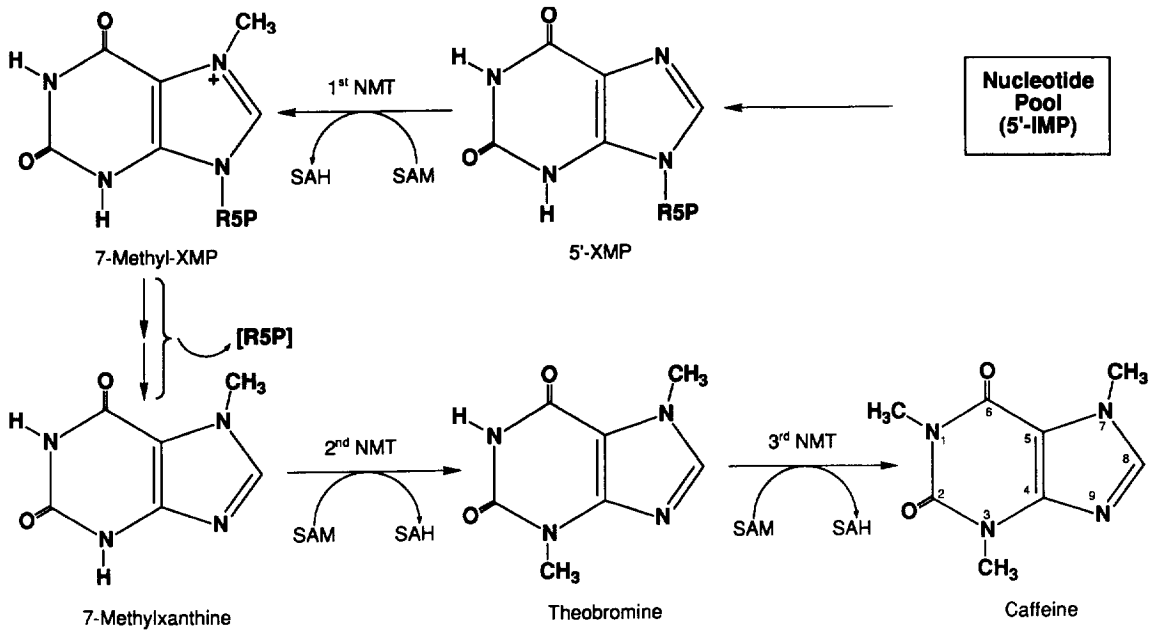
Key Word Index—*Coffea arabica*; Rubiaceae; caffeine; 7-methylxanthine; theobromine; purine alkaloids; SAM; *N*-methyltransferase; chemical defence; enzyme assay; leaf development.

Abstract—In order to accurately measure and characterize the very labile methyltransferase activities catalysing the two last steps in caffeine biosynthesis (from 7-methylxanthine to theobromine, and from theobromine to caffeine by the 2nd and 3rd NMT activity, respectively), an assay was designed that renders the use of radioactivity superfluous. Since in the assay maximum velocity was achieved by sufficiently high concentrations of both the methyl donor and acceptor, sensitivity was equal to the conventional protocols using minute substance quantities of [methyl-^{3}H]- or [methyl-^{14}C]SAM of high specific radioactivity. For the above mentioned NMT activities of the coffee plant a maximum pH stability was found at pH 7.5. Temperature stability tests revealed an almost linear decrease by increasing temperature with the exception of the 3rd NMT that was relatively stable between 30 and 50°. The isoelectric point was located at pH 4.5 to 5.0 (2nd NMT), and the pH optima at 7 to 8 and 8.5 for the 2nd and 3rd NMT activity, respectively. The temperature optimum is broad and around 35° for both activities. Interestingly, the 2nd NMT activity has a lower K_m for the methyl acceptor than the 3rd one, and this inversely for the methyl donor, SAM. Maximum relative and absolute methyltransferase activities coincide with leaf emergence, when, as expected, the chemical defence has highest priority and the purine alkaloids (PuA) are allocated at peak concentrations. The 2nd NMT activity was always considerably higher than the 3rd one. However, they frequently paralleled each other as they were components of one entity. The only transient accumulation of theobromine in the young leaflets suggestedly results from the competition of the two NMT activities for SAM and/or from a reduced supply of purine precursors in the preceding methylations. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

In chemical terms the purine alkaloid (PuA) caffeine is a three-fold methylated xanthine (1,3,7-trimethylxanthine). However, biogenesis most likely starts at the nucleotide level with a first methylation of XMP at position N7 (Scheme 1). After removal of the phosphoribose moiety, 7-methylxanthine is methylated in two steps to caffeine via theobromine [1–3]. Although much work has been done on caffeine biosynthesis, it is not yet clear how many enzymes catalyse these three methylations. On the one hand, early experiments with mixed substrates suggested the participation of two separate enzymes in the two final methylations [4]. This view may be supported by the fact that, for example, the leaves of the cocoa clone Pound 12 form theobromine but no caffeine (Fritz P. J. and Baumann T. W., unpublished). Similarly, the leaves as well as the flower buds of a wild tea plant (*Camellia irrawadiensis*) are virtually caffeine-free and accumulate the dimethylated precursor theobromine [5]. On the other hand, it is conceivable that the metabolite channel or enzyme complex, as it was recently postulated for caffeine synthesis [3], may contain together with hydrolytic enzymes one single methyltransferase furnished with all the three activities, and that 'metabolic tightness' of the channelled reactions eventually determines the ratio of the methylated products formed. As long as we do not know anything definite about the number of methyltransferases, we will use the term 'activity' and number it according to the methylating steps catalysed, i.e. '2nd NMT activity' signifies the second *N*-methyltransferase step leading from 7-methylxanthine to theobromine, and '3rd NMT activity' the last *N*-methyltransferase reac-

* Author to whom correspondence should be addressed.



Scheme 1. Caffeine biosynthesis as elucidated by isotope and enzymic studies with suspension-cultured cells and with leaves of coffee [1–3]. The methylations are catalysed by *N*-methyltransferases (NMT) using the methyl donor SAM (*S*-adenosylmethionine) that is demethylated to SAH (*S*-adenosylhomocysteine). In the present study a non-radioactive assay for the 2nd and 3rd NMT activity was established.

tion transforming theobromine into caffeine. The 1st NMT activity which catalyses the methylation most likely of XMP to 7-methylXMP [4] was not investigated in this study.

The aim was to characterize the NMT activities mentioned above and to study their time course during leaf development of *Coffea arabica*. The study was triggered by the fact of extreme lability of these NMT activities, and by the need to find an optimum source for later isolation work. We decided, prior to attempting the delicate isolation, to determine some crucial features such as, for example, the pH and temperature stability as well as the related optima, the pI and the optimum assay conditions. A striking example of a 'straightforward approach' ignoring basic enzymology is the work of Mazzafera *et al.* [6]. They claim to have purified the 3rd NMT activity to homogeneity. However, a close look to Table 1 of their publication, which shows decreasing specific activity during purification, clearly reveals the authors' failure. In contrast, encouraging progress has been achieved recently by a Scottish group [7], showing that purification goes along with increasing specific activity as it should be and as we recently experienced as well.

RESULTS AND DISCUSSION

Characterization of the N*-methyltransferase activities*

Maximum pH stability was around pH 7.5 for both NMT activities, whereas over the entire pH range of 5–9 the 2nd was significantly more stable than the 3rd one, and at pH 7.5 by 55%. Subsequently, a pH of 7.5 was routinely used for extraction and storage. With the exception of the 3rd NMT activity showing no further decline between 30 and 50°, stability decreased almost linearly by increasing temperature. Between 4 and 30° the 2nd NMT activity was slightly more stable. It would be most interesting to test for thermal stability of the 3rd NMT activity at temperatures higher than 50°. Whether this feature of the last NMT activity could be responsible for the repetitiously observed caffeine increase (to the extent of theobromine) during manufacturing processes, e.g. tea withering and drying [8], is a matter of speculation.

The isoelectric point (pI), which was determined only for the 2nd NMT activity, was found to be between pH 4.5 and 5.0. At these pHs all of the NMT activity precipitated together with 38 and 23% of the overall protein, respectively. We should mention that this method of pI determination renders only a rough estimate that nonetheless may be very useful for later studies using isoelectric focusing (IEF).

In Fig. 1(a) and (b) the dependence of NMT activities on pH is depicted. The pH optima differed moderately, i.e. pH 7.0–8.0 and pH 8.5 for the 2nd and 3rd NMT activity, respectively. Similar values were obtained for both the 2nd and 3rd NMT activity of coffee berries [9] and tea leaves [10] (pH 8.5) and for the 1st NMT activity of tea [11] (pH 7.5–8.0), indicating that the methyltransferase-mediated steps of caffeine biosynthesis may take place in the cytosol.

Finally, we found for both activities a broad temperature optimum around 35°. Roughly, they were doubled by temperature steps of 10°C from 5° (4°) to 35°. This relatively high temperature optimum found

Table 1. K_m values (μM) for the NMT activities catalysing the final two methylations in caffeine biosynthesis (literature vs these studies)

Author	Plant material	SAM (7-Methylxanthine) (2nd NMT activity)	SAM (Theobromine) (3rd NMT activity)	7-Methylxanthine (2nd NMT activity)	Theobromine (2nd NMT activity)
Suzuki and Takahashi, 1975 [10]	Tea leaves	25	25	1000	1000
Roberts and Waller, 1979 [9]	Coffee berries	10	10	200	200
Baumann *et al.*, 1983 [4]	Coffee tissue culture	5	16	100	200
Mazzafera *et al.*, 1994 [6]	Coffee endosperm	—*	10	496	196
Present studies†	Coffee leaves	203/190/148[c]	67/89/72	390/387/385	573/478/481

* Not determined.
† For details see text and Experimental.
[c] Lineweaver–Burk/Hanes/Eadie-Hofsted.

in vitro corresponds closely to the *in situ* temperature characteristics of *C. arabica*: net photosynthesis was optimum between 24 and 33°, and caffeine formation was accelerated by increasing temperature tested up to 33° [12].

Kinetic parameters

All previous reports on PuA-related NMT activity determinations are based on the use of methyl-radiolabeled SAM of high specific activity in order to measure the radioactive methylxanthine produced in the assay, e.g. ref. [10]. At first glance this approach appears quite rational because of high sensitivity. However, it was ignored that in a two-substrate reaction both substrates should be provided in concentrations higher than the related K_m, if a rapid and sustained product formation is to be expected. This would mean either to add lots of the very costly radioactive SAM, or to dilute it with cold SAM with the risk of losing sensitivity and to introduce into the assay impurities such as the inhibitor SAH or the active sulphonium isomer of SAM [13]. In the present work we established for the first time an assay without radioactivity that should yield for both substrates more accurate K_m values than those obtained with minute SAM concentrations as reported in the literature cited above. A key step was the addition to the assay of adenosine deaminase (ADA) that greatly improved the methyl group transfer from SAM to 7-methylxanthine or theobromine. We had received notice from Dr Felix Keller of our institute that Dr A Richter, from the Institute of Plant Physiology of the University of Vienna, Austria, was successfully using ADA in his work on methylation of inositols. Although ADA turned out to be helpful in our system as well we can only make assumptions regarding its beneficial effect on the methyl group transfer. According to ref [14], ADA may act unspecifically on the inhibitor SAH and remove it by deamination to SIH (*S*-inosylhomocysteine). On the other hand, SAH hydrolase present in the enzyme extract to be tested may lead to an accumulation of adenosine that, by feed back inhibition, subsequently will raise the concentration of SAH. It is suggested that, additionally, ADA may relieve the situation by deaminating adenosine to inosine.

The reactions were found to run linearly during the first 30 min at 25°. The apparent K_m values determined by graphical plots are listed in Table 1 and compared to those from the literature. It turns out that our K_m values of SAM are considerably higher than those reported previously and obtained under non-saturated conditions. They behave in an inverse manner to the K_m values of the methylxanthines, i.e. the 2nd NMT reaction is characterized by a K_m value relatively higher for SAM, and comparably low for the methylxanthine, and vice versa for the 3rd NMT reaction. Under standard assay conditions much more product is formed by the 2nd than by 3rd NMT

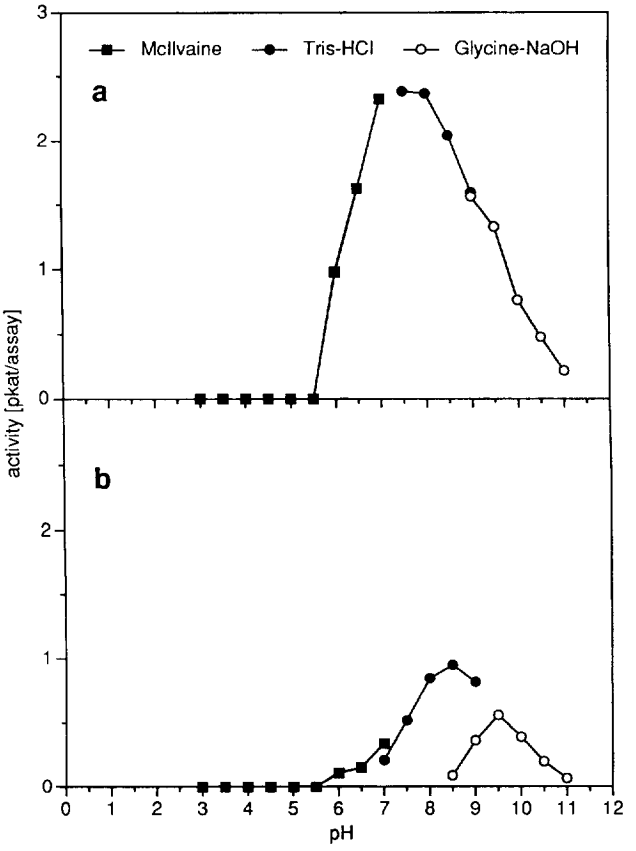


Fig. 1. Second (a) and 3rd (b) NMT activity in dependence of the pH. Standard assays were run for 30 min at 21° in three different buffers covering the pH range of 3–11.

activity and the related v_{max} values were 8.8 and 3.2 pmol s^{-1}. We are aware of the fact that the presented values could be refined using a two-substrate rate equation. In our view, however, they should be assessed later when the enzyme(s) will be purified to homogeneity.

NMT activities during leaf development

In preliminary experiments, NMT activities were measured in the crude enzyme extracts, then in enzyme preparations concentrated by ammonium sulphate precipitation of leaflets up to *ca* 400 mg fr. wt. Later, the experiment was repeated but extended to fully expanded leaves (*ca* 1900 mg fr. wt). In Fig. 2(a) and (b) these later results are presented. They are essentially identical to those of the preliminary studies. Even though the leaflets originated from different plants, the activities per g fr. wt (Fig. 2(a)) exhibit a rather consistent curve with a very early maximum during the first stages of emergence of the leaflets (*ca* 7–20 mg fr. wt; day 2 to day 7), and with the 2nd NMT activity mostly considerably higher than the 3rd one. It is interesting to note that the NMT peaks coincide well with the maximum concentration of the resulting products, theobromine and caffeine (see inset Fig. 2(a) and ref. [15]). Thus, the biosynthetic potential for the formation of chemical defence compounds, i.e. the related actual enzyme activity per amount tissue, is strongly correlated with leaf emergence. After completion of emergence (*ca* 25–40 mg fr. wt: day 9 to day 11), that is when the leaflets of the pair start to separate from each other and enter the phase of rapid expansion, both the relative enzyme activities and the purine alkaloid concentrations decrease concomitantly. One could assume that this sharp decline basically results from a simple dilution-by-expansion process, which in view of chemical defence appears quite conceivable, since the nutritive value per unit tissue is proportionally diminished by expansion as well. Indeed, after the early rapid induction the total enzyme activity (Fig. 2(b)) either declines slowly (2nd NMT), or remains more or less constant for the most part of leaf expansion (3rd NMT). This holds also for theobromine and even for caffeine showing an increase on an absolute basis (Fig. 2(b), inset). When leaf expansion is terminated, the activity is no longer (2nd NMT) or hardly (3rd NMT) detectable. The time course of the specific activities (the peaks at 25 and 12 nkat per g protein for the 2nd and 3rd NMT respectively; not shown) during leaf development is similar to that of the relative activities in Fig. 2(a), demonstrating again that chemical defence mediated by the caffeine methyltransferases is, also in terms of soluble protein, prevailing during the critical early phase of leaf development.

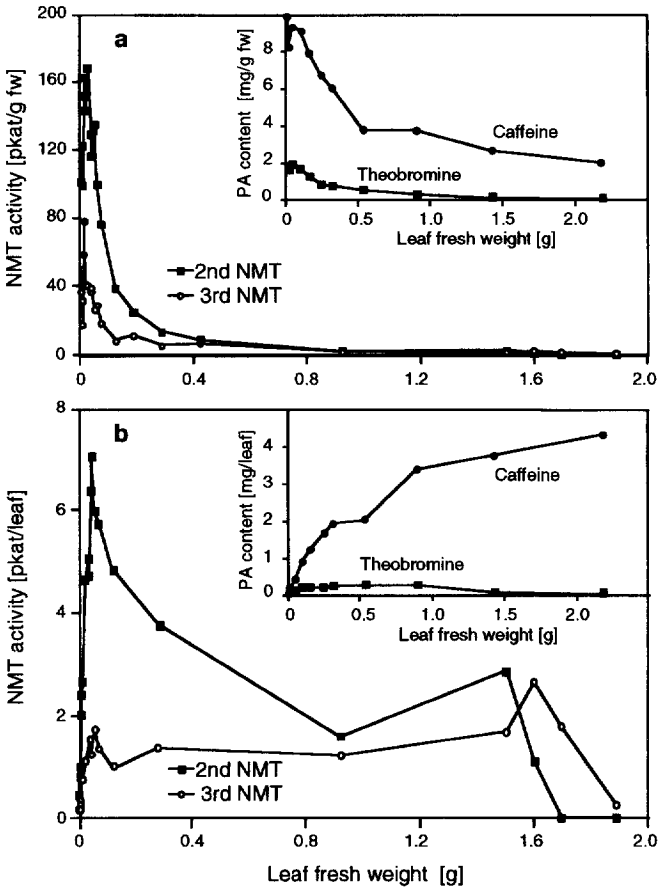


Fig. 2. Time course of NMT activities during leaf development in *C. arabica*: leaves (5–70, depending on leaf size) of each stage were pooled and extracted as described in the Experimental. The youngest stage harvested had a fr. wt of *ca* 5 mg, the oldest had one of 1900 mg. The latter corresponded to the fully expanded, but still glossy and soft leaf, 30–35 days old. (a) Activity per g leaf fr. wt; inset: purine alkaloid contents related to fr. wt. (b) Total activity (pkat) per leaf; inset: absolute amounts of the purine alkaloids theobromine and caffeine per leaf. The data presented in the insets are from a separate experiment with 11 leaf classes (see Experimental) and not from the leaf series that was used for the determination of the enzyme activities.

The velocity of caffeine biosynthesis, as it can be seen from the slope of the formation curve in the inset of Fig. 2(b), was measured in an earlier study by infiltrating leaves with ring-labelled theobromine and found to be, per unit leaf tissue, 1000-fold higher in emerging than in expanding leaves [15, 12 (Table 1, erroneously the dry weights listed are too small by a factor of ten!)].

Finally we should comment on the consistent and considerable difference between the 2nd and 3rd NMT activity during the most part of leaf development (Fig. 2(a) and (b)). Firstly, it may indicate that the methylation steps are mediated by one and the same enzyme, and if so, that a separate modulation of the one or the other activity rarely occurs. Secondly, since the 2nd NMT activity yielding theobromine is much higher than the 3rd one catalysing the formation of caffeine, one would expect a massive accumulation of theobromine, and not the slight and only transient increase shown in Fig. 2(b) (inset). Clearly, the *in vitro* results obtained under saturation conditions cannot be directly related to the *in vivo* situation. However, the 'brief presence' of theobromine is so typical of rapidly growing coffee organs such as leaflets, young fruits and seeds (mature organs are virtually theobromine-free), that it calls for an explanation even though it may be speculative. One reason could be the competition for SAM. As listed in Table 1, the affinity of the 3rd NMT for SAM is higher by a factor of 2–3. Alternatively, a limited supply of purine precursors for the second or even the first methylation may impair the accumulation of theobromine. In any case, the emerging leaflets contain not only the highest purine alkaloid concentrations but also the potentially highest enzyme activities on a relative and absolute basis. Moreover, the purine alkaloid-complexing phenols, predominantly the chlorogenic acid 5-CQA, are accumulated in a similar manner [16]. Hence, the freshly formed foliage is extremely well protected against all kinds of pathogens and predators, this being quite in accordance with the concept of chemical defence [17].

EXPERIMENTAL

Chemicals. SAM and SAH were purchased from Sigma. Caffeine, theobromine and 7-methylxanthine were obtained from Fluka (Buchs, Switzerland). Adenosine deaminase (ADA; 2 mg ml^{-1} 3.2 M $(NH_4)_2SO_4$) was from Boehringer (Mannheim, Germany).

Buffers. Extraction buffer (EB): 0.2 M Na-Pi (pH 7.5) containing 50 mM $NaCl_2$, 5 mM DTT, 2 mM Na-EDTA, 0.6% Na-ascorbate, 20% (v/v) ethylene glycol. Storage buffer (SB): same as the extraction buffer but with 0.1 M Na-Pi and 40% ethylene glycol. Desalting buffer (DB): 50 mM Tris-HCl (pH 8.0) containing 0.06% Na-ascorbate. For determination of pH stability and pH optimum, 50 mM buffers with 0.06% Na-ascorbate were used (pH): McIlvaine (5–7), Tris-HCl (7–9), and glycine-NaOH (8.5–11).

Plant material. Coffee plants (*C. arabica* L. var. *catuai*) were raised in the greenhouse from seeds originating from parent trees kept in the environmental chamber at short-day (8 hr; 24°), flower-inducing conditions [18, 19]. For the leaf development-related NMT studies, young leaves of various size (6–175 mm long, i.e. 5–1900 mg fr. wt) were randomly harvested from several plants and grouped into 21 classes that cover the growth period of 30–35 days from early emergence to full expansion. Due to the large fr. wt, differences between 70 (youngest stage) and 5 (oldest stage) leaves were harvested for each class. For the determinant of the PuA content, a second series of leaves (7–2000 mg, $n = 14–5$) was plucked and analysed.

Extraction of PuA. The leaves were extracted in 0.1 N HCl (2.5 per 100 mg fr. wt) at 40° for 30 min by sonication. The extract was purified using Extrelut (Kieselgur, Merck) as described in ref. [20]. For HPLC (see below) the PuAs were dissolved in 8% MeOH.

Extraction of NMT activities. Unless stated otherwise, all procedures were carried out at 0–4°. About 100 youngest leaf pairs (*ca* 10–15 mm long) of 2-year-old coffee plants were harvested and corresponded approximately to a fr. wt of 4 to 5 g. They were then homogenized in a prechilled mortar in the presence of a few ml of EB and 0.6 g PVPP previously equilibrated with EB. The slurry was, by rinsing with EB, poured into a tube and centrifuged at 20 000 *g* for 10 min. The supernatant (*ca* 40–50 ml) was 40% satd with $(NH_4)_2SO_4$. After centrifugation, the sediment was discarded, the supernatant adjusted to 60% $(NH_4)_2SO_4$ and again centrifuged. The pellet was dissolved in SB (final vol. *ca* 4–5 ml) and portioned into frs of 0.6 ml. These were quickly frozen and stored in liquid N_2 without loss of activity for at least 5 months. Alternatively, *ca* 50 leaflet pairs corresponding to a fr. wt of 2.17 g were extracted as described above. However, the supernatant (18 ml) was at once adjusted to 75% $(NH_4)_2SO_4$, the pellet redissolved in EB to give 4 ml and to be immediately used for the characterization of NMT activities. To determine the NMT activity during leaf development, the leaves of each class were pooled and homogenized with a stainless steel pestle in a polycarbonate centrifuge tube (15 or 40 ml) in the presence of white quartz sand (−50+70 mesh, Sigma) and little EB containing 20 mM instead of 5 mM DTT. The resulting slurry was centrifuged at 12 000 *g* for 3 min. The pellet was extracted a second time and the supernatants were combined and adjusted to 70% $(NH_4)_2SO_4$. After centrifugation at 38 000 *g* for 5 min the pellet was redissolved in EB in a vol. that was 1.5× the fr. wt (v/w) of the original leaf material. An aliquot (300 μl) was desalted over Sephadex G-25 fine (5 ml) as described below, and then directly assayed by the slightly modified standard procedure.

NMT standard assay. The enzyme extract was desalted on Sephadex G-25 fine (Pharmacia) by centrifugation [21]: the column (5 ml, in a plastic syringe barrel), previously equilibrated by three consecutive runs (1400 *g*: 2 min) each with 1 ml of DB, was charged with 300–750 μl extract, mounted onto a Eppendorf tube and spun under the same conditions, this yielding an eluate (enzyme extract) not diluted by the desalting procedure. The standard reaction mixture (110 μl) consisted of 55 μl (5 mM in H_2O) methyl-group acceptor, 5 μl (2 unit) adenosine deaminase (ADA), 40 μl desalted enzyme soln, and 10 μl SAM (16.65 mM in 0.01 N H_2SO_4). Due to the enzyme soln (DB) the pH of the assay was 8.0. The final concns were as follows: DB 18 mM, methylxanthine substrate 2.5 mM, SAM 1.5 mM. Incubation was carried out at 25° for 15–30 min as indicated separately in the various studies. The reaction was stopped by the addition of 10 μl 1.2 M $HClO_4$. The NMT activities during leaf development were measured by the same assay for 20 min at 25° but with the vols multiplied by a factor of 1.5. After a short centrifugation (microfuge) the methylated product, theobromine or caffeine, was determined directly in the supernatant by HPLC on a Nucleosil 100-5 ODS column (5 μm; 4×125 mm; precolumn 4×8 mm; ChromCart, Macherey-Nagel, Oensingen, Switzerland). Parameters were controlled by a Hewlett-Packard liquid chromatograph equipped with a diode array detector set at 272 nm. Chromatography was carried out using a MeOH (%) gradient in H_2O: 0–3 min (8–15), 3–7 min (15–25), 7–8 min (25). The R_ts (min) were 1.3, 3.4, 5.2, 8.9 and 9.3 for SAM, 7-methylxanthine, theobromine, caffeine and methylthioadenosine, respectively. The flow rate was 1.0 ml min^{-1} and the injection vol. 60, or 120 μl (leaf developmental studies). Since by this system caffeine was not completely separated from methylthioadenosine, caffeine formation was monitored using the following gradient: 0–4 min with 0–7.5% MeOH and 0–2.5% AcN, 4–20 min with 7.5% MeOH and 2.5% AcN. The R_ts (min) were 1.5, 4.9, 6.9, 15.1, and 18.2 for SAM, 7-methylxanthine, theobromine, caffeine, and methylthioadenosine, respectively. The flow rate was 1.1 ml min^{-1}. Peak identification was achieved by comparing UV spectrum (library estab-

lished under separating conditions) and retention time of authentic standards. The enzyme activity was calculated from the peak area (UV absorption) of the methylated product formed and expressed as pkat g^{-1} fr. wt or nkat g^{-1} protein.

Characterization of NMT activities. The pH stability was tested between 5 and 9 (see "Buffers"), with increments of 0.5 between 6 and 8. The enzyme sample (350 μl, in EB) was applied onto Sephadex G-25 fine (see NMT standard assay) previously equilibrated with the buffer of the corresponding pH. After centrifugation one half of the probe was converted into DB by centrifugation and directly assayed at standard conditions (at pH 8), whereas the other half was stored for 90 min at 4°. The latter probe was brought to pH 8.0 by centrifugation through Sephadex G-25 fine and assayed under standard conditions. The activity (stability) was related to the sample which was (after conversion into DB) immediately assayed at pH 8.0. The temperature stability was assessed at 35° in desalted probes that were pre-incubated between 5 and 50° for 30 min. The pI was determined by isoelectric precipitation. Portions (2–5 ml) of crude enzyme extract were acidified (with 1 M HAc) or alkalinized (with 1 M Tris base) to give a range of pH 4–8 with an increment of 0.5. After 15 min at 4° the probes were centrifuged, and the pellets dissolved, desalted and assayed by the standard procedure.

To locate the pH optimum, standard assays were run between pH 3 and 11 with 0.5 increments (see "buffers"). The required pH in the assay was achieved by passing (desalting) the enzyme probe through the Sephadex column with the appropriate pH. The assays were stopped after 30 min at 21°. The temperature optimum was determined at pH 8.0. Assays were run between 5° and 50° (with an increment of 5°) for 20 min. To establish the kinetic parameters (K_m, v_{max}), the concentration in the assay of the related methylxanthine (7-methylxanthine or theobromine) was kept at 2.5 mM, i.e. close to its saturation, and that of SAM varied between 75 and 1514 μM (K_m for SAM), or alternatively SAM was at 1.51 mM and the methylxanthine ranged from 0.33 to 2.5 mM (K_m for the methylxanthine). The assays were incubated for 30 min (2nd NMT) and 25 min (3rd NMT) at 25°. The K_m values were calculated using the Lineweaver–Burk, Eadie–Hofsted, and Hanes plots. Protein was determined by the method of Bradford [22] (Bio-Rad), modified for the use in 96-well microtiter plates. The absorption at 620 nm was measured in a microplate reader (Anthos 2001) with albumin as standard.

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