Phenylalkylamines, such as the plant compounds ephedrine and pseudoephedrine and the animal neurotransmitters dopamine and adrenaline, compose a large class of natural and synthetic molecules with important physiological functions and pharmacologically valuable bioactivities. The final steps of ephedrine and pseudoephedrine biosynthesis in members of the plant genus Ephedra involve N-methylation of norephedrine and norpseudoephedrine, respectively. Here, using a plant transcriptome screen, we report the isolation and characterization of an N-methyltransferase (NMT) from Ephedra sinica able to catalyze the formation of (pseudo)ephedrine and other naturally occurring phenylalkylamines, including N-methylcathinone and N-methyl(pseudo)ephedrine. Phenylalkylamine N-methyltransferase (PaNMT) shares substantial amino acid sequence identity with enzymes of the NMT family involved in benzylisoquinoline alkaloid (BIA) metabolism in members of the higher plant order Ranunculales, which includes opium poppy (Papaver somniferum). PaNMT accepted a broad range of substrates with phenylalkylamine, tryptamine, β-carboline, tetrahydroisoquinoline, and BIA structural scaffolds, which is in contrast to the specificity for BIA substrates of NMT enzymes within the Ranunculales. PaNMT transcript levels were highest in young shoots of E. sinica, which corresponded to the location of NMT activity yielding (pseudo)ephedrine, N-methylcathinone, and N-methyl(pseudo)ephedrine, and with in planta accumulation of phenylalkylamines. Co-expression of recombinant genes encoding PaNMT and an ω-transaminase (PP2799) from Pseudomonas putida in Escherichia coli enabled the conversion of exogenous (R)-phenylacetylcarbinol (PAC) and (S)-PAC to ephedrine and pseudoephedrine, respectively. Our work further demonstrates the utility of plant biochemical genomics for the isolation of key enzymes that facilitate microbial engineering for the production of medicinally important metabolites.

Phenylalkylamines (PAAs) are a large class of natural and synthetic molecules defined by the occurrence of a phenyl ring linked to an amino group by an alkyl chain of variable length. Functional group substitutions at various positions around the core structural scaffold result in a diverse array of biologically active compounds. In animals, including humans, endogenous molecules of this class such as the catecholamine neurotransmitters dopamine and adrenaline play important roles in normal physiological processes (1). This neurobiology forms the basis for the action of important pharmaceuticals, including synthetic PAA derivatives such as the antidepressant bupropion (e.g. Wellbutrin) (2) and amphetamine-containing attention deficit disorder treatments (e.g. Adderall and Dexedrine) (3). Synthetic PAA derivatives are further used in the treatment of Parkinson’s disease (e.g. selegiline) (4), obesity, anxiety, and nasal congestion. Beyond routine uses of synthetic PAAs in modern medicine, natural sources have been used for millennia in both ritual and medicinal applications. Prominent examples include the cactus-derived hallucinogen mescaline and the various PAAs of Ephedra species and khat (Catha edulis) (5).

PAAs in Ephedra spp. and khat, referred to as substituted α-methyl phenethyllamines or substituted amphetamines, are characterized by a C2 methyl group (the α position relative to the nitrogen) and either a hydroxyl or carbonyl at C1 (Fig. 1). Medicinal use of “ma huang” (a preparation of Ephedra sinica) has been documented in traditional Chinese medicine since at least 200 BCE (6), but isolation of the pharmacologically active metabolites (1R,2S)-ephedrine and (1S,2S)-pseudoephedrine was not reported until 1887 (7). Ephedrine is listed as an essential medicine by the World Health Organization for the prevention of low blood pressure induced by spinal anesthesia, whereas pseudoephedrine is a widely used decongestant. The biosynthesis of Ephedra alkaloids begins with the conversion of L-phenylalanine to trans-cinnamic acid by phenylalanine ammonia lyase, which is the only isolated and partially characterized enzyme in the proposed pathway (Fig. 1) (8, 9). Based on

3 The abbreviations used are: PAA, phenylalkylamine; SAM, S-adenosyl-L-methionine; BIA, benzylisoquinoline alkaloid; CNMT, coaurline N-methyltransferase; CID, collision–induced dissociation; NMT, N-methyltransferase; PaNMT, phenylalkylamine N-methyltransferase; PavNMT, pavine N-methyltransferase; RNMT, reticuline N-methyltransferase; TMNT, tetrahydroprotoberberine N-methyltransferase; PAC, phenylacetylcarbinol; THQ1, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline; THQ2, 6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline; ANOVA, analysis of variance; IPTG, isopropyl 1-thio-β-D-galactopyranoside; CTAB, cetyl trimethylammonium bromide.
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Figure 1. Proposed biosynthesis of phenylalkylamines in Ephedra spp.

The pathway is drawn according to the predictions of radio- and isotopically-labeled tracer experiments, the isolation of putative intermediates, and the detection of enzyme activities in plant extracts. Phenylalanine ammonia lyase (PAL) is the only enzyme for which a corresponding cDNA has been isolated. Dotted arrows for steps between trans-cinnamic acid and (S)-cathinone indicate putative or multiple conversions. Condensation of pyruvate with benzoyl-CoA, or benzaldehyde has been suggested to yield 1-phenylpropane-1,2-dione, but (R,S)-phenylacetylcarbinol and (R,S)-2-hydroxypropiopephedrine have also been implicated as a potential pathway intermediate. Exogenously fed (R,S)-phenylacetylcarbinol can be converted in E. coli to nor(pseudo)ephedrine by an ω-transaminase (PP2799). The isolated PaNMT reported herein catalyzes the final two N-methylations yielding (pseudo)ephedrine and N-methyl(pseudo)ephedrine. N-Methyltransferase activity was detected in plant extracts and shown to yield (1R,2S)-ephedrine from (1R,2S)-norephedrine (12). However, the responsible enzyme was not isolated, and there have been no reports on the molecular characterization of a plant-derived NMT catalyzing the final step in (pseudo)ephedrine biosynthesis. In animals, the well-studied enzyme phenylethanolamine N-methyltransferase catalyzes a similar reaction, converting noradrenaline to adrenaline. The bovine variant of phenylethanolamine N-methyltransferase was shown to N-methylate norephedrine, but not norpseudoephedrine, albeit with a substantially reduced rate compared with its native substrate (13).

Despite the accumulation of Ephedra alkaloids to ~1–3% of the stem fresh weight, cultivated plants have not remained a viable commercial source for ephedrine or pseudoephedrine because of the slow growth rate of the source plants and the relatively high cost of extraction. Since the early 1930s, commercial production of these compounds has relied on a combined fermentation and chemical strategy whereby exogenous benzaldehyde is initially converted by Saccharomyces cerevisiae to (R)-phenylacetylcarbinol (PAC), followed by extraction, reductive amination, and Welsh rearrangement (5, 14). Since the industrial process already employs a biotransformation step, (pseudo)ephedrine is an ideal target for de novo production using engineered microorganisms with minimal disruption of the existing infrastructure (5). An Escherichia coli “platform strain” that included heterologous expression of an engineered pyruvate decarboxylase (15) enabled conversion of benzaldehyde to (R)-PAC in a reaction equivalent to that used in the yeast-based (pseudo)ephedrine production process (16). From (R/S)-PAC, the two remaining chemical steps to (pseudo)ephedrine (i.e., transamination and N-methylation) can also be enzymatically catalyzed. Several bacterial ω-transaminases have been characterized that are capable of yielding one or more of the four possible nor(pseudo)ephedrine stereoisomers (17–20), including the Pseudomonas putida enzyme PP2799 (Fig. 1). As part of our overarching goal of elucidating alkaloid biosynthesis in E. sinica, we targeted the N-methyltransferase step, with the additional aim of providing a new gene potentially useful for fermentation-based production of (pseudo)ephedrine pharmaceuticals. Herein, we report the isolation and characterization of a phenylalkylamine NMT (PaNMT) from E. sinica capable of producing (pseudo)ephedrine both in vitro and in engineered E. coli. Positive correlation of PaNMT transcript levels with NMT activity in E. sinica plant organs supports a physiological role for the corresponding enzyme in the formation of (pseudo)ephedrine and related alkaloids. Unexpectedly, the in vitro substrate preference of PaNMT suggests the possibility of a bifurcation in the pathway leading to Ephedra alkaloids, with (S)-N-methylcathinone serving as a potential biosynthetic intermediate. Interestingly, PaNMT exhibits a broad substrate range that includes various phenylalkylamines, along with several tetrahydroisoquinolines and benzylisoquinolines. Our discovery completes the set of enzymes required for the potential commercial production of (pseudo)ephedrine, and other phenylalkylamines, in engineered microorganisms.
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Results

Selection and screening of NMT candidates

Combined tBLASTn and HMMer searches of an E. sinica stem transcriptome (11) using six canonical plant NMT proteins as queries yielded 27 candidate enzymes potentially able to catalyze the N-methylation of phenylalkylamine intermediates in the biosynthesis of (pseudo)ephedrine (Table S1; Fig. S1). Full-length open reading frames corresponding to 18 genes encoding candidate NMT enzymes were amplified by RT-PCR using total RNA from E. sinica stems (Table S1), 16 of which yielded sufficient His6-tagged recombinant protein in E. coli to allow for partial purification using a cobalt–affinity resin (Fig. S2). Candidate proteins (supporting Data Set 1) were tested in vitro for SAM-dependent N-methylation activity using racemic (±)-norephedrine and (±)-ephrine as substrates (Fig. S3). Only candidate A (Singlet3659) catalyzed the formation of ephedrine and N-methylephedrine from (±)-norephedrine and (±)-ephrine, respectively, and the enzyme was named phenylalkylamine N-methyltransferase (PaNMT).

Characterization of PaNMT

The amino acid sequence of PaNMT shared considerable (43–50%) identity with NMTs involved in benzyloisoquinoline alkaloid (BLA) biosynthesis (Fig. S4). In addition to the SAM-binding residues, which are entirely conserved, PaNMT also showed substantial conservation of residues predicted to form part of the homodimer interface and the alkaloid-binding domain in the TfpavNMT X-ray crystallographic structure (21). No canonical subcellular targeting signals were detected in the polypeptide sequence using TargetP (22). The purified His6-tagged PaNMT protein displayed an empirical molecular mass of 41 kDa (Fig. S5), which was in agreement with the predicted size of the translation product.

In addition to (±)-norephedrine and (±)-ephrine, PaNMT catalyzed the N-methylation of (±)-cathinone and (1R,2R)-(−)-norpseudoephedrine (Fig. 2; Fig. S12). Although the isomer (1S,2S)-(−)-norpseudoephedrine was not commercially available to test as a substrate, PaNMT had the capacity to remove precipitated protein prior to analysis of the diluted supernatant by LC-MS. PaNMT exhibited an apparent turnover number (1 s−1) for (−)-cathinone and (1R,2R)-(−)-norpseudoephedrine, respectively.

PaNMT was tested for potential NMT activity on 45 nitrogenous compounds exhibiting diverse scaffold structures and functional group substitutions (Fig. S8). Reaction products consistent with N-methylation activity (i.e. showing an increase of 14 atomic mass units with respect to the corresponding substrate) were detected for 23 different compounds (Fig. S9), which belonged to several distinct structural categories, including phenylalkylamines, tryptamine alkaloids, tetrahydroiso-
quinoine alkaloids, β-carboline, and BIAs (Fig. 4). PaNMT catalyzed the N-methylation of compounds containing primary, secondary, and tertiary amine moieties. Authentic standards for most reaction products were not available, and turnover rates were relatively low (i.e., less than 5%), which precluded an accurate quantitative determination of relative substrate acceptance.

To compare with PaNMT, three previously characterized BIA NMTs (23, 24) were heterologously expressed in *E. coli*, affinity-purified (Fig. S5), and subjected to the same determination of substrate range (Fig. S10). As anticipated, the enzymes exhibited preference for their respective “primary” BIA substrate: GfCNMT (*Glaucium flavum* coclaurine N-methyltransferase) was largely specific for (S)-coclaurine; GfTNMT (*G. flavum* tetrahydroprotoberberine N-methyltransferase) was most active on the protoberberine alkaloid (R,S)-stylopine; and PsRNMT (*Papaver somniferum* reticuline N-methyltransferase) preferred (S)-reticuline. With the exception of a tetrahydroiso-

**Physiological relevance of PaNMT in *E. sinica***

To assess the physiological role PaNMT in (pseudo)ephedrine biosynthesis, relative transcript abundance, NMT activity, and
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Figure 5. Relative abundance of PaNMT transcripts, phenylalkylamine N-methyltransferase activity, and phenylalkylamine content in shoots and roots of E. sinica. A, schematic showing the sampling location of various shoot and root tissues. Segment 1 contained partially expanded internodes proximal to the shoot tip, and each successive segment contained a single expanded internode. Roots (R) were collected as a single tissue type. B, relative PaNMT transcript abundance in internodes 1–8 and in roots. Letters above bars for stem internodes denote statistical significance as determined using a one-way ANOVA and Holm-Sidak test for multiple pairwise comparisons (p < 0.02). Root data were not included in the statistical analysis of the stem internode data but were significantly different (p < 0.001) compared with all stem samples. C, N-methyltransferase activity using various phenylalkylamine substrates in protein extracts of combined shoot samples corresponding to internodes 1–3, internodes 4–6, and internodes 7–8. No activity was detected in roots. The statistical significance of the data are provided in Figures S13 and S14. D, accumulation of major phenylalkylamines detected at 10–1000-fold lower levels compared to their production in engineered microorganisms. A demonstration of this potential was pursued by transforming two E. coli strains with expression vectors containing inducible genes encoding (i) a ω-transaminase (PP2799) from P. putida and (ii) PaNMT from E. sinica. A co-culture of the strains producing recombinant FLAG-tagged PP2799 and His6-tagged PaNMT (Fig. S15) was able to convert exogenous (R)-PAC or (S)-PAC (along with SAM) to (pseudo)ephedrine (Fig. 6; Figs. S16 and S17). In co-cultures fed (S)-PAC, norterpseudoephedrine was detected in the medium at 24 h, and levels remained stable for the duration of the time course to 96 h. Pseudoephedrine was also detected at 24 h, and its concentration increased over the period of the time course. Similarly, in co-cultures fed (R)-PAC, norephedrine was detected at a stable level in the culture medium from 24 to 96 h, whereas ephedrine was detected at 24 h and increased in concentration at each sampling point. When the transaminase step was bypassed by supplementing cultures directly with norephedrine or norterpseudoephedrine, the time course of ephedrine and pseudoephedrine accumulation was similar to the previous

Production of (pseudo)ephedrine in E. coli

An NMT able to N-methylate phenylalkylamines permits the production of (pseudo)ephedrine in engineered microorganisms. A demonstration of this potential was pursued by transforming two E. coli strains with expression vectors containing inducible genes encoding (i) a ω-transaminase (PP2799) from P. putida and (ii) PaNMT from E. sinica. A co-culture of the strains producing recombinant FLAG-tagged PP2799 and His6-tagged PaNMT (Fig. S15) was able to convert exogenous (R)-PAC or (S)-PAC (along with SAM) to (pseudo)ephedrine (Fig. 6; Figs. S16 and S17). In co-cultures fed (S)-PAC, norterpseudoephedrine was detected in the medium at 24 h, and levels remained stable for the duration of the time course to 96 h. Pseudoephedrine was also detected at 24 h, and its concentration increased over the period of the time course. Similarly, in co-cultures fed (R)-PAC, norephedrine was detected at a stable level in the culture medium from 24 to 96 h, whereas ephedrine was detected at 24 h and increased in concentration at each sampling point. When the transaminase step was bypassed by supplementing cultures directly with norephedrine or norterpseudoephedrine, the time course of ephedrine and pseudoephedrine accumulation was similar to the previous
results, except that the final product concentrations were 10-fold higher. Without exogenous SAM, phenylalkaline products were detected at 5–20-fold lower levels. No products were detected in co-cultures containing E. coli transformed with empty vectors (Fig. S16).

Discussion

We have isolated and characterized a novel enzyme from E. sinica able to N-methylate various phenylalkalines involved in the biosynthesis of (pseudo)ephedrine (Fig. 2). PaNMT exhibited relatively low affinity for (±)-norephedrine and (1R,2R)-(−)-norpseudoephedrine (apparent $K_m$ of 1.2 and 1.7 mM, respectively) (Table 1). However, PaNMT also accepted (±)-cathinone, an upstream pathway intermediate in Ephedra spp. not previously proposed as a target for N-methylation. The affinity of PaNMT for (±)-cathinone (apparent $K_m$ of 630 μM) was the greatest of all substrates examined (Table 1). Furthermore, the $V_{max}$ of PaNMT with (±)-cathinone was 6.8- and 7.9-fold greater than the $V_{max}$ observed for (±)-norephedrine and (1R,2R)-(−)-norpseudoephedrine, respectively, whereas the catalytic efficiency with cathinone similarly outpaced other substrates (e.g. 21-fold greater than with (1R,2R)-(−)-norpseudoephedrine). These results raised the possibility that N-methylation occurs earlier in the pathway than previously proposed (12, 26). In contrast, the most closely related and functionally characterized enzymes to PaNMT showed higher affinities for their respective substrates. For example, recombinant CjCNMT, PsTNMT, PsRNMT, and TiPavNMT were reported to display $K_m$ values between 0.6 and 160 μM (24, 27–29). In contrast, enzymes similar to PaNMT in terms of substrate profile exhibit comparably low substrate affinities. Few enzymes are known to accept Ephedra alkaloids, although those that do include bovine phenylethanolamine NMT ($K_m$ 1.3 mM for (1R,2S)-norephedrine) and mammal indolethylamine NMTs, which accept both norephedrine and ephedrine, albeit at a much reduced rate compared with the native substrate (30, 31). To the best of our knowledge, no other enzymes accept (nor)pseudoephedrine substrates. Significantly, mammal enzymes capable of N-methylating PAA alkaloids exhibit very broad substrate ranges, a feature reflected by PaNMT (Fig. 4). This commonality suggests that relatively low substrate affinity might be an inherent feature of NMTs accepting a broad range of alkylamine substrates.

The affinity ($K_m$) of an enzyme has been proposed to proportionally reflect the physiological concentration of the substrate (32). Although the cellular concentration of (pseudo)ephedrine pathway intermediates is not known, levels can be approximated based on their accumulation in stem internodes (Fig. 5; Fig. S13). Conservatively, and assuming that plant cells are similar in density to water, norephedrine and norpseudoephedrine would occur at a mean concentrations of ~0.4 and 3 mM, respectively. Knowing that (pseudo)ephedrine pathway intermediates are reported to accumulate at substantially higher levels in mature E. sinica stems (12), and given the typical confinement of specialized metabolic pathways to specific cell types or subcellular compartments, the concentration of PaNMT substrates could be high enough to ensure that the low substrate affinities of the enzyme do not impede pathway flux. Relative to the phenylalkaline substrates, PaNMT showed a much higher affinity for SAM (140 μM; Table 1), which was comparable with $K_m$ values reported for recombinant PsRNMT (160 μM) (24) and CjCNMT (390 μM) (27). Other BIA NMTs had substantially better affinity for SAM, with $K_m$ values between 43 (TfCNMT) and 1.2 μM (ScTNMT) (28, 29, 33, 34). SAM concentration in plant cells is reported to be in the range of 15 μM, suggesting that PaNMT activity might be regulated by co-substrate availability (35, 36).

Compared with most other enzymes, PaNMT displayed low apparent turnover numbers ($k_{cat,app}$) with all tested substrates (Table 1). However, similarly modest values have been obtained for some BIA NMTs, including TTPavNMT with respect to (±)-pavine (4.7 × 10$^{-5}$ s$^{-1}$) and (R,S)-tetrahydropapaverine (7.9 × 10$^{-4}$ s$^{-1}$), and PbTNMT with (R,S)-stylopine (0.001 s$^{-1}$) (21, 29). As a group, recombinant BIA NMTs are relatively inefficient enzymes as evidenced by the highest turnover number of only 0.3 s$^{-1}$ (EcTNMT) (29). The biochemical and evolutionary significance of these observations are not known; however,
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enzymes of specialized metabolism often exhibit remarkable inefficiency. Slow turnover by PaNMT necessitated relatively long incubation; however, determination of the linear range for product formation indicated that measurements over this time period accurately reflected apparent initial rates (Fig. S6). Nevertheless, all Michaelis-Menten kinetic parameters are qualified as “apparent” to account for the possibility that enzyme instability, product inhibition (e.g. by S-adenosylhomocysteine), or other considerations impacted the accuracy of our measurements. A potential factor contributing to the kinetic parameters of PaNMT is the racemic nature of most tested substrates (Figs. S8 and S20). The measured $K_m$ value would be artificially high if PaNMT accepted only one enantiomer (i.e. the one naturally occurring in the plant). Furthermore, the alternative enantiomer could function as an inhibitor. In support of this, bovine phenylethanolamine NMT accepts only (1R,2S)-norephedrine, whereas all other diastereomers act as inhibitors (13). However, the similarity of PaNMT enzyme kinetics with racemic (±)-ephedrine compared with naturally-occurring (1S,2S)-(−)-pseudoephedrine and with racemic (±)-norephedrine compared with unnatural (1R,2R)-(−)-norpseudoephedrine suggests that the enzyme is not stereospecific and does not experience inhibition by unnatural stereoisomers (Table 1; Fig. 3).

The PaNMT pH optimum of 9.0 is in agreement with the more alkaline pH optima determined for recombinant BIA NMTs, which range from 7.0 to 9.0 (24, 27, 28). Given that enzymes likely evolve for efficient catalysis under conditions in their subcellular environment (37), a high pH optimum might suggest that PaNMT functions in a relatively alkaline environment, such as can be found in peroxisomes, the mitochondrial matrix, or plastidial stroma (38). Nevertheless, the lack of recognizable targeting signals supports a predicted localization of PaNMT to the cytoplasm, as shown previously for a BIA NMT with similar sequence features (39). An alternative explanation for the high pH optimum of PaNMT is the predicted high $pK_a$ of its phenylalkylamine substrates (9.4 to 9.6) compared with BIA substrates (5.3 to 8.6) (40). Accordingly, the observed pH optimum might reflect a balance between substrate deprotonation facilitating methyl group transfer and alkaline-induced protein denaturation.

Unlike BIA NMTs, which accept a limited range of substrates (Fig. S10), PaNMT accepted a broad array of substrates containing one or more aromatic rings, but with substantially less turnover (Fig. 4). Most substrates with an amino group separated from a phenyl moiety by two carbons (i.e. phenethylamines) were accepted, whereas those separated by a single carbon were not. Within this group, PaNMT activity was relatively insensitive to functional groups on the phenyl ring and accepted substrates with hydroxyl, methoxyl, or methylenedioxy substitution. Similarly, carbonyl, hydroxyl, or methyl group substitutions along the ethyl moiety, and stereochemistry at these carbon centers, did not noticeably impact enzyme function. However, catecholamines (i.e. dopamine and noradrenaline) were not accepted, and a tetrahydroisoquinoline alkaloid with the equivalent aromatic substitution pattern (THQ1) was turned over much less than the O-methylated equivalent (THQ2). The absence of detectable activity on amino acids indicates that PaNMT does not tolerate a large polar carboxylic acid moiety. Apart from phenethylamines, PaNMT was active on tetrahydroisoquinoline, β-carboline, and benzylisoquinoline alkaloids, as well as tryptamine and proranol, which contain indole and naphthalene ring structures, respectively. Molecules containing imidazole, tropane, and purine rings were not accepted. Specifically, PaNMT did not accept xanthosine or theobromine, which are substrates for NMTs in caffeine biosynthesis (41, 42). Irrespective of substrate structural group, PaNMT accepted primary, secondary, and tertiary amines, which is similar to the functional range of BIA NMTs (24, 27, 28). Although most of these compounds are not known to occur in Ephedra spp., this broad substrate range is consistent with a previous hypothesis that the NMT involved in (pseudo)ephedrine biosynthesis was a promiscuous enzyme with alternative physiological roles (10). Because authentic standards for most of the putative products were unavailable, and quantities sufficient for NMR analysis were not produced, the formation of N-methylated products reported here requires further confirmation; however, this caveat applies to the reported substrate range of most other enzymes discussed herein (21, 23, 24, 27–31, 33, 34, 43). Our results indicate that PaNMT has one of the broadest reported substrate ranges of any NMT characterized at the molecular level and, as such, represents an ideal starting point for engineering of many desired catalytic activities.

In agreement with earlier reports on the distribution of secondary metabolites in Ephedra spp., phenylalkylamines were detected almost exclusively in shoots (12, 44). The variety used in our work contained similar levels of (1R)- and (1S)-alkaloids and corresponds to the “mixed” chemotype described previously (12). PaNMT’s apparent lack of stereospecificity in vitro is consistent with the chemotype of the variety from which it was isolated (Table 1). NMT activity on five phenylalkylamine substrates was detected in stems, but not in roots (Fig. 5C), as reported previously for norephedrine (12). The detection of PaNMT transcripts primarily in stem samples (Fig. 5B) reasonably correlates with the occurrence of NMT activity and the occurrence of phenylalkylamine substrates and reaction products. Furthermore, PaNMT transcript abundance and NMT activity were correlated across stem internode samples, where both showed a decreasing trend in successively older tissues.

The relative substrate preferences for recombinant PaNMT were similar, but not identical, to NMT activity in E. sinica stem protein extracts (Table 1; Fig. 5C). Native NMT activity was highest with (±)-norephedrine substrate, although (±)-cathinone was also readily accepted. In comparison, recombinant PaNMT exhibited a preference for (±)-cathinone over other Ephedra alkaloids. The plant extracts tested were enriched for NMT activity, but purification was not performed, and thus we cannot rule out the existence of a second, as yet uncharacterized NMT, which may contribute to observed activity. It is also possible that raising recombinant PaNMT in a prokaryotic host precludes important post-translational modifications acquired in planta or that epitope tagging of recombinant PaNMT affects its folding. In either case, discrepancies could arise between native and recombinant NMTs. Similar differences in
substrate preference between native and recombinant enzymes were reported for other plant NMTs of specialized metabolism (27, 43). Regardless, the preference of recombinant PaNMT for (+)-cathinone in vitro (Table 1) and acceptance of this substrate in planta suggest a previously undetected bifurcation of the biosynthetic route to (pseudo)ephedrine, with (S)-N-methylcathinone (methcathinone) occurring as intermediate. The physiological relevance of this observation is supported by the following: 1) detection of substantial (+)-cathinone NMT activity, and 2) occurrence of methcathinone, in E. sinica stem extracts (Fig. 5C; Figs. S13 and S14). Methcathinone might subsequently undergo reduction, as occurs with cathinone, yielding the (pseudo)ephedrine diastereomers (12, 26).

E. sinica PaNMT is the first reported member of the BIA NMT clade outside the plant order Ranunculales shown to accept alkaloid substrates, and as such, its discovery highlights a number of intriguing questions. The partial functional overlap between PaNMT and BIA NMTs will broaden the scope of structure–function investigations for this important group of enzymes. The superimposability of PAA over the isoquinoline lap between PaNMT and BIA NMTs will broaden the scope of NMT clade outside the plant order Ranunculales shown to

Experimental procedures

Chemicals

(R)-PAC, (S)-PAC, and (1R,2R)-(−)-norpseudoephedrine were purchased from Toronto Research Chemicals. (R)/(S)-(±)-Cathinone hydrochloride was a gift from Efrem Levinsohn (Newe Ya’ar Research Center, Israel). (1R,2S/1S,2R)-(±)-Norephedrine hydrochloride, (1R,2S/1S,2R)-(−)-epheedrine hydrochloride, and (+)-methyleneephedrine hydrochloride were obtained from Sigma. (1S,2S)/(−)-Pseudoephedrine was isolated from an off-the-shelf decongestant tablet by acid/base organic extraction. (1R,2S/1S,2R)-(−)-Methylcathinone hydrochloride was produced by oxidation of (1R,2S/1S,2R)-(−)-epheedrine hydrochloride (49). Except for (R)-PAC and (S)-PAC and (1R,2R)-(−)-norpseudoephedrine, the identity and purity of these compounds were verified by LC-MS, 1H NMR (Table S2; supporting Data Set S2) and, in some cases, CD spectroscopy (Fig. S20). S-(5′-Adenosyl)-L-methionine chloride dihydorochloride, taurine, benzylamine, 1-phenethylamine, tyramine, (±)-octopamine hydrochloride, (±)-norephedrine, dopamine hydrochloride, (±)-norepinephrine bitartrate, nicotinamide, p-dimethylaminobenzaldehyde, anthranilic acid, histamine dihydrochloride, L-phenylalanine, 3,4-dihydroxy-L-phenylalanine, L-tyrosine, L-tryptophan, tryptamine, tropinone, adrine hemisulfate, theobromine, xanthosine, 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (THQ1),6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline (THQ2), (±)-amphetamine, phentermine, (±)-methyleneoxypamphetamine, and mescacline hydrochloride were purchased from Sigma. Propranolol was isolated from a Teva-Propranolol tablet by acid/base organic extraction. Harmine and harmaline were isolated from seeds of Peganum harmala (50). Mitragynine was isolated from dried leaves of Mitragyna speciosa (51). (S)-Norlaudanosoline, (S)-coclaunine, (S)-reticuline, papaverine, (S)-styloline, and noscapine were obtained as reported previously (28). All other reagents were obtained from Sigma or BioShop Canada.

Selection of NMT candidates

E. sinica transcriptome assemblies were generated as described previously (11). The transcriptome library was searched for contigs representing candidate NMTs using tBLASTn and HMMPer (52) based on six previously reported plant NMT sequences as queries (Table S1). Selected contigs were manually inspected to verify the presence of a complete ORF. Redundant and incomplete contigs were discarded. Candidate NMT amino acid sequences were aligned using the MUSCLE algorithm implemented in MEGA7 with default parameters (53, 54). Evolutionary history was inferred using the maximum

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Likelihood method based on the JTT matrix-based model, assuming a discrete Gamma distribution to model evolutionary rate differences among sites (five categories (+G, parameter = 9.2796), followed by a bootstrap test of phylogeny. The tree with the highest log likelihood was drawn with branch lengths proportional to the number of substitutions per site, and nodes were labeled with the percentage of 500 bootstrapped trees in which the associated taxa clustered together. The analysis involved 35 sequences whereby positions with less than 50% site coverage were eliminated, resulting in a final data set with 423 positions. Abbreviations and accession numbers for query sequences and phylogenetic reference points were as follows: Arabidopsis thaliana protein arginine methyltransferase, AtPRMT (NP_199713); Atropa belladona putrescine N-methyltransferase, AbPMT (BA8A8264); Solanum lycopersicum phosphoethanolamine N-methyltransferase, SIEPANMT (AAG5989); A. thaliana histone-lysine N-methyltransferase, ATSuVH (NP_196113); Coffea arabica 3,7-dimethyllanthine N-methyltransferase caffeine synthase, CaCS (BAC75663); Papaver somniferum (S)-tetrahydroprotoberberine N-methyltransferase, PsTNMT (AAY79177); Coptis japonica coaularine N-methyltransferase, CJCNMT (BAB71802); P. somniferum reticuline N-methyltransferase, PsRNMT (KKX369612).

Materials

E. sinica seeds acquired from open pollinated wild populations originating in northern China (Horizon Herbs) were sown in a 50:50 blend of industrial quartz (Unimim) and soil-less greenhouse mix containing perlite (Supreme Perlite), vermiculite (Perlite Canada), and sphagnum peat moss (PRO-MOSS Hort) in a volumetric ratio of 20:20:60. Water-soluble fertilizer (ArcticExpress) or 4-h (SG13009) incubation, cells were harvested, flash-frozen on liquid nitrogen, and ground to a fine powder using a mortar and pestle. Total RNA was extracted from this material using the CTAB method (55) and resuspended in diethyl pyrocarbonate-treated ultrapure water. cDNA synthesis was performed on 1.5 μg of total RNA using Moloney murine leukemia virus reverse transcriptase (Invitrogen). Full-length coding regions were amplified from cDNA using Q5 high-fidelity DNA polymerase (New England Biolabs) and specific oligonucleotide primer pairs (Table S3). Amplicons were cloned into the pET47b expression vector. NMT candidates A–D and G were also synthesized as codon-optimized genes (Genscript) for expression in E. coli and cloned into the pQE30 expression vector.

Single colonies of E. coli strain ArcticExpress (Agilent) individually transformed with the pET47b expression constructs were used to inoculate 50 ml of lysogeny broth (LB) medium supplemented with 50 μg/ml kanamycin and 20 μg/ml gentamycin. Similarly, colonies of E. coli strain SG13009 (Qiangen) transformed with each of the pQE30 expression constructs were used to inoculate 50 ml of LB medium supplemented with 50 μg/ml kanamycin and 100 μg/ml ampicillin. Seed cultures were grown for 16 h at 30 °C with shaking at 250 rpm and used to inoculate 1 liter of LB medium without antibiotics. Cultures were grown at 30 °C to A600 of ~0.6, cooled to 16 °C (ArcticExpress) or room temperature (SG13009), and supplemented with IPTG to a final concentration of 1 mM. Following a 24-h incubation (ArcticExpress) or 4-h (SG13009) incubation, cells were harvested by centrifugation at 3000 × g for 20 min and stored at −80 °C.

Cell pellets were resuspended in protein extraction buffer (50 mM Tris-HCl, pH 8.5, 10% (v/v) glycerol) and lysed by sonication. Crude lysates were centrifuged at 4 °C and 10,000 × g to remove cellular debris, and the resulting supernatant was applied to TALON cobalt-affinity purification resin (Clontech) and incubated on ice for 1 h. The resin was washed with two 10-mL volumes of protein extraction buffer, followed by two additional buffer volumes supplemented successively with 5 and 10 mM imidazole. Purified proteins were eluted stepwise using 1 mL of buffer supplemented with 100 mM imidazole. Eluted protein fractions were passed through PD-10 columns (GE Healthcare) to remove imidazole. Protein concentration was determined by Bradford assay using bovine serum albumin (BSA) as the standard. Protein fractions were assessed by SDS-PAGE and anti-His immunoblot analysis. Recombinant G. flavum CNMT and TNMT and P. somniferum RNMT were expressed and purified as described previously (23, 24).

In vitro screening of NMT candidates

Initial screens were conducted in assay buffer (50 mM Tris-HCl, pH 8.5, 10% (v/v) glycerol, and 5 mM DTT) containing 1 mM alkaloid substrate (i.e. cathinone, norephedrine, norpseudoephedrine, ephedrine, or pseudoephedrine), 1 mM SAM, and 20 μg of purified protein in a reaction volume of 50 μL incubated at 37 °C for 24 h. Negative control reactions were performed without the addition of SAM and by using protein dena-
tured by boiling for 20 min. Assays were quenched with 500 μl of 1 M ammonium hydroxide, pH 10, followed by extraction with 500 μl of ethyl acetate. The organic phase was transferred to a clean tube and dried at room temperature overnight, and the residue was dissolved in ultrapure H₂O containing 0.1% (v/v) formic acid (solvent A) and analyzed by LC-MS. Screening assays were conducted in triplicate and repeated independently at least three times for each candidate.

**In vitro characterization of PaNMT**

To determine pH optimum, assays were performed in 100 mM MES (pH 5.5–6.5), MOPS (pH 6.5–7.5), Tris-HCl (pH 7.5–9.0), or Na₂CO₃/NaHCO₃ (pH 9.0–10.5) containing 1 mM (±)-norephedrine, 1 mM SAM, and 25 μg of purified protein in a final volume of 50 μl. Assays were incubated at 37 °C for 3 h, which was within the linear range for product formation (Fig. S6), and quenched with 200 μl of methanol. To determine temperature optimum, assays were performed in 100 mM Tris-HCl, pH 9.0, and between 4 and 65 °C, as indicated. Assays were equilibrated at each temperature for 10 min prior to the addition of purified protein. Kinetic parameters were measured at 37 °C in 100 mM Tris-HCl, pH 9.0. Phenylalkylamine substrates (i.e. cathinone, norephedrine, norpseudoephedrine, ephedrine, and pseudoephedrine) were used at concentrations between 15 μM and 10 mM, with SAM constant at 1 mM. SAM was varied from 4 to 1000 μM at a fixed concentration of 1 mM norephedrine. Products were quantified by LC-MS analysis employing five-point standard curves (6 nM to 4 μM or 160 nM to 500 μM) prepared from authentic standards. Saturation curves and kinetic constants were calculated according to the Michaelis-Menten model using Prism 5 software (GraphPad). Substrate range was determined at 37 °C in 100 mM Tris-HCl, pH 9.0, using 1 mM of potential substrates. Substrate range assays were incubated for 3 h and, in addition, for 16 h to ensure no trace activities were overlooked. Turnover was detected by the formation of an analyte with m/z 14 atomic mass units higher than the corresponding substrate. Negative control reactions were performed using purified protein denatured by boiling for 20 min. Methanol-quenched reactions were centrifuged at 21,000 × g and 4 °C for 30 min to remove insoluble protein, and the diluted soluble portion of the assay (supernatant) was directly analyzed by LC-MS.

**Production of (pseudo)ephedrine in E. coli**

Single colonies of *E. coli* strain SG13009 transformed with pQE30-PaNMT or pQE30 were used to inoculate 3 ml of LB medium supplemented with IPTG to a final concentration of 1 mM and incubated for an additional 16 h. Single colonies of *E. coli* strain BL21 transformed with pACE2–PP2799 or pACE2 were similarly cultured in LB medium containing only 20 μg/ml tetracycline. Cultures (i.e. SG13009 harboring pQE30–PaNMT and BL21 harboring pACE2–PP2799; SG13009 harboring pQE30 and BL21 harboring pACE2) were combined at a 1:1 ratio, and 2-ml aliquots were centrifuged at 3000 × g for 5 min to remove the culture medium. Cell pellets were resuspended in 500 μl of LB medium supplemented with 1 mM IPTG and 500 μM of either (S)-PAC, (R)-PAC, norephedrine, or norpseudoephedrine and incubated at 37 °C for 96 h. After the first 48 h, each culture was supplemented with 1 mM SAM, and the pH was adjusted to 8.5 using 100 mM Tris-HCl. Product formation was measured in culture supernatant collected at various incubation time points between 0 and 96 h. Supernatants were diluted 1:1 with ultrapure methanol, centrifuged at 21,000 × g and 4 °C for 30 min, and analyzed by LC-MS. Soluble cellular protein was assessed by SDS-PAGE and immunoblot analysis to confirm production of recombinant proteins.

**RNA extractions**

Total RNA was extracted from plant tissues using a modified CTAB method (56). Quality and quantity were assessed by agarose gel electrophoresis and Nanodrop spectrophotometry. cDNA synthesis was performed on 2 μg of total RNA using the 5× All-in-One RT Master Mix kit, which contains both oligo(dT)s and random primers (Applied Biological Materials).

**Gene expression analysis**

 Primer pairs for quantitative RT-PCR were designed using the PrimerQuest (IDT) tool and verified by examining melt curves and agarose gel electrophoresis of amplicons. Five plant housekeeping gene orthologs (*EF-1α, ACT2, LBC, PP2A, and TIF*) were identified in the *E. sinica* transcriptome and tested as potential reference genes (57). The geometric mean of C₅ values determined for the three genes showing the most stable expression (*ACT2, PP2A, and TIF*) was used as the reference to calculate relative transcript abundance of PaNMT in plant tissues using the 2⁻ΔΔC₅ method. PCR amplification efficiency, calculated from raw fluorescence data using LinRegPCR (58), was between 90 and 110% for all primer pairs. Results were normalized to the sample showing the lowest expression level. Real-time PCR and melt curve analyses were performed using SYBR Green detection in a QuantStudio 3 system (ThermoFisher Scientific). Each 20-μl reaction contained 2 μl of cDNA, 6 pmol of each primer (Table S3), and 10 μl of 2× Power Up SYBR Green Master Mix as recommended by the manufacturer (Applied Biosystems). Cycling was performed as follows: 2 min at 95 °C, followed by 45 cycles of 5 s at 95 °C and 20 s at 60 °C. Melt curve analysis was performed by increasing the block temperature from 60 to 95 °C at a ramp rate of 0.15 °C/s.

**Alkaloid extraction**

Phenylalkylamines were extracted from plant material by adding 750 μl of ultrapure water to 100 mg of a ground tissue, followed by vortexing and sonication at room temperature for 2 min (12). Suspensions were centrifuged at 21,000 × g for 5 min at room temperature to pellet debris, and the supernatant was transferred to a clean microtube, and 7.5 μl of 10 M NaOH was added to recover phenylalkylamines in uncharged form. The basified aqueous solution was extracted three times with 900 μl of diethyl ether, after which the organic fractions were pooled and
evaporated to dryness under reduced pressure. Residual solids were dissolved in ultrapure methanol and analyzed by LC-MS.

**Soluble protein extraction**

Soluble protein was extracted from pooled plant materials by adding 10 ml of ice-cold protein extraction buffer (100 mM Tris-HCl, pH 8.5, 10% (v/v) glycerol, 1% (w/v) PVP-40, 5 mM DTT, 1 X Plant Protease Inhibitor Mixture (Bioshop Canada)) to 1-2 g of ground plant tissue, followed by vortexing and sonicication in an ice-cold water bath for 5 min. The suspension was centrifuged at 21,000 × g for 20 min at 4 °C to pellet debris, and the supernatant was transferred to a clean tube. Oven-dried and finely ground ammonium sulfate was added to a final concentration of 30% saturation, and the sample was incubated on ice for 1 h. The sample was centrifuged at 16,000 × g for 20 min at 4 °C and the precipitated pellet discarded. Saturated ammonium sulfate solution was added to the supernatant to a final concentration of 60% saturation, and the sample was incubated for 1 h at 4 °C. The sample was centrifuged at 16,000 × g for 20 min at 4 °C, and the precipitated pellet (30-60% fraction) was discarded. Saturated ammonium sulfate solution was added to the supernatant to a final concentration of 85% saturation, and the sample was incubated for 2 h at 4 °C. The sample was centrifuged at 16,000 × g for 20 min at 4 °C, and the supernatant was discarded. The remaining pellet (60-85% fraction) was resuspended in 2 ml of ice-cold protein extraction buffer and desalted using a PD-10 column (GE Healthcare). Protein concentration was determined by Bradford assay with BSA as the standard.

**Plant activity profiling assays**

Enzyme assays were performed in plant protein extraction buffer containing 1 mM phenylalkylamine substrates (i.e. cathinone, norephedrine, norpseudoephedrine, ephedrine, or pseudoephedrine), 1 mM SAM, and 12 μg of plant protein (60-85% ammonium sulfate fraction) in a 50-μl reaction at 37 °C for 1.5 h. As reported previously, NMT activity was restricted to the 60-85% fraction so the 30-60% fractions were not analyzed (10). Negative control reactions were performed using protein extracts denatured by boiling for 20 min. Assays were quenched with 200 μl of methanol and analyzed by LC-MS.

**LC-MS analysis**

LC-MS analysis was performed using a 1200 HPLC coupled to a 6410 triple quadrupole mass spectrometer (Agilent). Initial screening assays were analyzed by injection onto a 150 × 2-mm Prodigy Phenyl-3 column (particle size 5 μm, pore size 100 Å) at room temperature (21 °C). Analyses were eluted in a gradient of solvent A (0.1% (v/v) formic acid in ultrapure H2O) and solvent B (0.1% (v/v) formic acid in ultrapure methanol) at a flow rate of 200 μl/min. The gradient was initiated at 5% B, ramped linearly to 70% B by 20 min, and increased to 90% B by 21 min. From 22 to 23 min, the mobile phase composition was returned to and remained at 5% B, for a 5-min re-equilibration period. All other samples were analyzed by injection onto a 250 × 2 mm Luna Phenyl-Hexyl HPLC column (particle size 5 μm, pore size 100 Å) at 45 °C. Analyses were eluted in a gradient of solvent A (0.1% (v/v) formic acid and 5% (v/v) ultrapure methanol in ultrapure H2O) and solvent B (0.1% (v/v) formic acid in ultrapure methanol) at a flow rate of 300 μl/min. The gradient was initiated at 0% B, ramped linearly to 35% B by 12 min, increased to 85% B by 14 min, remained at 85% B until 18 min, and returned to 0% B by 21 min for a 6-min re-equilibration period.

Mass spectrometry was performed using electrospray ionization operating in positive mode under the following conditions: capillary voltage 4000 V, fragmentor voltage 100 V, source temperature 350 °C, nebulize pressure 50 p.s.i., gas flow 10 liters/min. For initial screening assays, samples were analyzed in selected ion mode where quadrupoles 1 and 2 were set to RF (radio frequency) only, whereas quadrupole 3 was filtered for m/z corresponding to the substrate and reaction product (+14 m/z). For substrate range experiments, samples were analyzes in full scan mode, where quadrupoles 1 and 2 were set to RF only, whereas quadrupole 3 was scanned from m/z 100 to 700. All other samples were analyzed in selected reaction monitoring mode where quadrupole 1 was filtered for select precursor ions; quadrupole 2 applied collision energy of 10 or 20 eV, and quadrupole 3 was filtered for select product ions. Transitions (precursor and product ion pairs) were selected empirically on the basis of collision-induced dissociation experiments with authentic standards.

Analyte identities were confirmed by comparison of retention time and CID spectra for experimental samples and authentic standards. For CID experiments, precursor ions ([M + H]+ or [M + H – H2O]+) were selected in quadrupole 1; collision energy of 10, 15, or 20 eV was applied in quadrupole 2, and quadrupole 3 was scanned for product ions from 40 m/z to +20 m/z relative to the precursor. When possible, alkaloids were quantified by comparison with five-point standard curves (6 nM to 4 μM or 160 nM to 500 μM) prepared from authentic standards. N-Methylpseudoephedrine, for which an authentic standard was not available, was quantified using N-methylephedrine as an equivalent.

**NMR and CD spectroscopy**

1H NMR spectra were recorded on an AVIII-400 spectrometer (400 MHz) and analyzed using TopSpin software (Bruker). Mass spectrometry was performed on 2–5 mg of each analyte in 300 μl of either DClO4 or D2O, as indicated. CD spectra were recorded using a J-715 spectropolarimeter (Jasco) on 10 mg of each analyte dissolved in 3 ml of ultrapure methanol or H2O contained in a 1-cm path length quartz cuvette. The instrument scanned from 400 to 200 nm at a speed of 20 nm/min, and three spectra were averaged to reduce noise. The instrument was calibrated using (R)-(−)-10-camphorsulfonic acid.

**Statistical analysis**

Statistical significance was determined using a one-way ANOVA and Holm-Sidak test for multiple pairwise comparisons (59).

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