

1 Rapid *in vitro* prototyping of O-methyltransferases for pathway 2 applications in *Escherichia coli*

3 Kristina Haslinger*^{1,2}, Thomas Hackl³ and Kristala L.J. Prather*¹

4 ¹ Dept. of Chemical Engineering, Massachusetts Institute of Technology, Cambridge, USA

5 ² Dept. of Chemical and Pharmaceutical Biology, University of Groningen, Groningen, The Netherlands

6 ³ Biomolecular Mechanisms, Max Planck Institute for Medical Research, Heidelberg, Germany

7 *corresponding authors, e-mail: k.haslinger@rug.nl and kljp@mit.edu

8 Abstract

9 O-methyltransferases are ubiquitous enzymes involved in biosynthetic pathways for secondary
10 metabolites such as bacterial antibiotics, human catecholamine neurotransmitters, and plant
11 phenylpropanoids. While thousands of putative O-methyltransferases are found in sequence databases,
12 few examples are functionally characterized. From a pathway engineering perspective, however, it is
13 crucial to know the substrate and product ranges of the respective enzymes to fully exploit their
14 catalytic power.

15 In this study, we developed an *in vitro* prototyping workflow that allowed us to screen ~30 enzymes
16 against five substrates in three days with high reproducibility. We combined *in vitro*
17 transcription/translation of the genes of interest with a microliter-scale enzymatic assay in 96-well
18 plates. The substrate conversion was indirectly measured by quantifying the consumption of the S-
19 adenosyl-L-methionine co-factor by time-resolved fluorescence resonance energy transfer rather than
20 time-consuming product analysis by chromatography. This workflow allowed us to rapidly prototype
21 thus-far uncharacterized O-methyltransferases for future use as biocatalysts.

22 Introduction

23 Methylation of secondary metabolites is a prevalent reaction that alters the bioavailability and reactivity
24 of molecules¹. This effect is important for the native function of secondary metabolites for the producer
25 organism but also for pharmaceutical and nutraceutical applications of natural products. One example is
26 the oxygen-directed methylation (O-methylation) of the lignin precursor caffeic acid towards ferulic
27 acid. This reaction is crucial for regulating the rigidity of lignified cell walls in vascular plants² and has
28 been described to modulate the cytotoxicity and radical scavenging properties of isolated phenolic acids
29 when tested for pharmaceutical applications such as neuroprotection^{3,4}. Similar observations were

30 made for methylated flavonoids (plants)^{5,6}, antimicrobial peptides (bacteria)^{7,8} and dopamine (humans)⁹.
31 O-methylation in nature is carried out by methyltransferases under the utilization of S-adenosyl-L-
32 methionine (SAM) as an electron-deficient methyl donor thereby forming S-adenosyl-L-homocysteine.
33 Some O-methyltransferase (OMT) families additionally require the presence of metal ions such as Mg²⁺.
34 For OMTs acting on small molecules (excluding nucleic acids and proteins), there are several protein
35 families with distinct sequence motifs and with a remarkable breadth in functionality. The functional
36 exploration of these families has been somewhat anecdotal to date and has been very much focused on
37 plant enzymes of the methyltransferase families 2 (PF00891) and 3 (PF01596)¹. This can most likely be
38 attributed to the fact that already in the pre-genomic era, these plant enzymes had been studied with
39 biochemical methods^{10,11}. However, with the rapid expansion of genomes sequenced to date, the
40 methyltransferase protein families are growing by the minute and functional studies are lagging
41 behind¹².

42 In the last decade rapid advances in parallelization of molecular cloning, enzymatic assays, and even
43 fermentation through liquid handling technologies and automation have greatly increased the
44 throughput of functional studies of enzyme libraries^{13,14}. However, the bottlenecks in these screening
45 pipelines remain the failing of molecular cloning steps and the throughput of the reaction readout in the
46 absence of colorimetric or fluorometric assays, which requires time-consuming chromatography
47 methods to analyze the products^{15,16}. To overcome these hurdles in the functional screening of SAM-
48 dependent methyltransferases, we developed a rapid *in vitro* prototyping workflow to express and
49 functionally screen a range of O-methyltransferases against several substrates. To minimize time and
50 effort spent on molecular cloning, we employed a recently developed *in vitro* transcription/translation
51 platform for linear DNA templates with high enzyme yields (myTXTL®)^{17,18}, and combined it with a
52 fluorescence-based read-out^{19,20} to monitor the consumption of the SAM co-factor. For one substrate
53 we translated the newly gained knowledge into the development of a microbial cell factory to produce
54 ferulic acid from simple building blocks.

55 Results

56 Design and benchmarking of the prototyping workflow

57 In order to facilitate the fast screening of a library of putative O-methyltransferases against several
58 substrates, we set out to develop a prototyping method that is rapid and parallelizable. Herein, we
59 identified the detection of enzymatic activity and the cloning and expression of the genes of interest as
60 the two major bottlenecks. For the detection of enzymatic activity, we deemed a desirable approach to
61 be independent of the substrates and products and to not require time-consuming chromatography. We

62 turned towards commercially available assays to detect the consumption of the SAM cofactor and
63 decided to use the TR-FRET Bridge-It® S-Adenosyl Methionine (SAM) Fluorescence Assay Kit from
64 Mediomics LLC (St. Louis, Missouri). In this endpoint assay, SAM binds to a DNA-binding protein and
65 induces the association of two fluorescently labeled DNA fragments (donor and acceptor) for Time-
66 Resolved Foerster Resonance Energy Transfer (TR-FRET) to occur. We hypothesized that in this way,
67 several enzymes could be screened against multiple substrates in parallel in the plate reader, with lower
68 TR-FRET readings observed for active enzyme-substrate combinations. We set out to test this detection
69 method with recombinantly expressed and purified MxSafC, an enzyme known to catalyze the
70 methylation of caffeic acid^{25,32}, and compare the TR-FRET read-out with product analysis by HPLC.
71 Compared to the control reaction without enzyme, we saw consumption of caffeic acid and SAM, as
72 measured by HPLC and TR-FRET, respectively, after 3h of incubation (Figure 1a). We observed a good
73 correlation of the biological replicates within and across both detection methods with a slight over-
74 estimation of substrate-consumption with the TR-FRET assay compared to HPLC detection.

75 In order to address the cloning and expression bottleneck, we decided to use an *in vitro*
76 transcription/translation expression platform, myTXTL® from Arbor Bioscience (Ann Arbor, Michigan).
77 This allowed us to express the genes of interest from synthetic, linear DNA fragments and saved us
78 additional time for cloning, sequence verification, transformation, protein expression and cell lysis (~up
79 to 3 weeks of work). The linear DNA fragments were designed to contain a $\sigma 70$ promoter, a T500
80 terminator and flanking overhangs of about 500bp to protect from degradation in the myTXTL® mix.
81 Additionally, GamS protein was added to the reactions to protect the DNA fragments. We first tested
82 the compatibility of the myTXTL® reaction mix with the OMT assay and the TR-FRET detection method
83 with MxSafC expressed from a linear template (Figure 1b). We performed two TXTL reactions at 29°C
84 over night and split them into three OMT reactions each. After stopping the OMT reactions, we analyzed
85 them with the TR-FRET assay (two technical replicates) and HPLC. As negative controls we included two
86 TXTL reactions that did not contain OMT-encoding DNA template (no technical replicates). Looking at
87 the median of the data points, we again observe good correlation of the replicates within and cross the
88 detection methods however, the TR-FRET assay appears to be more sensitive to experimental error than
89 the HPLC detection. The biological replicates of the enzyme expression (TXTL reactions 1 and 2) show
90 only minor deviation, indicating that the experimental error in the expression step of the workflow is
91 minimal.

92 Since we observed good correlation between the two detection methods, yet low overall turnover
93 yields, we proceeded with the established workflow with an extended incubation time for the OMT
94 reaction (24h) in later experiments.

95 **Screening of putative OMTs for methylation of caffeic acid**

96 In order to diversify our knowledge of OMTs in organisms other than plants, the premise of this study
97 was to characterize a range of putative OMTs from various non-plant donor organisms across a relatively
98 wide sequence landscape. Therefore, we first identified distantly related OMTs in the NCBI reference
99 proteome database based on Hidden Markov Models (HMM) constructed from known plant caffeic acid
100 OMTs^{23,24} (search input 1, Extended Data Table 1), and bacterial OMTs previously found to have a broad
101 substrate tolerance towards catechols²⁵⁻²⁸ (search input 2). We found 15,994 unique sequences from all
102 kingdoms of life, from ~190 PFAM families (Supplementary File 1). About 85% of the sequences were
103 annotated as methyltransferases, more specifically 82% as OMTs, and about 10% contained
104 dimerization domains. We filtered the sequences by length and alignment score and constructed a
105 sequence similarity network to group them into clusters by pairwise amino acid sequence similarity.
106 From the thus generated clusters, putative OMTs were chosen for experimental characterization (Table
107 1, Extended Data Figure 1) by taking the following criteria into consideration: the ranking of HMM scores
108 within the clusters, a wide taxonomic spread over the selected enzymes and a balanced selection of
109 enzymes found with the two HMMsearch runs. Multiple enzymes were chosen from the major clusters
110 but also some high scoring putative OMTs were picked from the smallest clusters.

111 The first large-scale screen of the selected putative OMTs was performed with caffeic acid as the
112 substrate. We analyzed the enzymatic reactions by HPLC and TR-FRET (Figure 2a,b) and repeated the
113 experiment on a different day with a slightly different sample-handling workflow that allowed the
114 consistent use of multichannel pipettes throughout the experiment (Figure 2c,d). The data points in the
115 plots are ordered by increasing substrate turnover based on panel C. The HPLC analysis shows a good
116 correlation of the independent experiments with each other, both in relative terms (ranking of the
117 tested enzyme by performance) and in absolute terms. This again indicates that the expression levels in
118 the TXTL reactions is highly reproducible and that the technical error in the OMT reaction is low.
119 However, for the TR-FRET analysis of the first experiment (Figure 2b), it is evident that the technical
120 error by manual sample dilution and setup of the TR-FRET detection assay is very high and therefore, the
121 technical replicates deviate strongly. The overall noise of the experiment is very high, which becomes
122 most apparent in the wells that appear to have higher SAM concentrations than the negative controls

123 (here shown at the bottom of the plot). These experimental errors were overcome with a slightly
124 different sample handling procedure in the second experiment (Figure 2d), which shows dramatically
125 decreased noise in the data and a clear distinction between true- and false-positives. However, even in
126 the first experiment with high background noise the best-performing enzymes can be clearly
127 distinguished from the other ones. In the intermediate range, it is difficult to make a distinct cut-off.
128 However, depending on the goal of this screening step, the cut-off can be set at a lower or higher level
129 of SAM consumption at the risk of including false-positives or excluding false-negatives, respectively. In
130 this case, we decided to make a very conservative cut-off and to even carry some true-negatives
131 forward to the characterization in *E. coli* (*vide infra*). Overall, we observe a clear correlation between the
132 HPLC and TR-FRET read-out and were therefore encouraged to screen the enzymes against four other
133 potential substrates: 1,2-dihydroxybenzene (catechol), ferulic acid, quercetin and dopamine.

134 **Screening of putative OMTs against other substrates**

135 Next, we sought to use our *in vitro* expression and testing workflow to screen our panel of putative
136 OMTs against other substrates. We selected catechol and dopamine - two known substrates for MxSafC
137 and plant caffeic acid OMTs-, quercetin - a flavonoid also often converted by plant caffeic acid OMTs -,
138 and ferulic acid - the precursor for a non-natural double-methylated product. We ran all reactions in
139 parallel by diluting the TXTL reactions after overnight expression and aliquoting them into microtiter
140 plates with the OMT reaction mixes. After 24h we stopped the OMT reactions and assessed the SAM
141 levels with the TR-FRET assay. We observed increased SAM consumption by 11 OMTs in the presence of
142 catechol (Figure 3a) and by 15 OMTs in the presence of dopamine (Figure 3b), whereas in the presence
143 of ferulic acid and quercetin, only low levels of SAM conversion were observed that are difficult to
144 separate from the background noise of the assay (Figure 3c and d). Furthermore, we did not have any
145 true positive controls for these substrates in the panel of enzymes. Therefore, we are inclined to
146 interpret the results as negative for these substrates. Also, in the presence of dopamine, the separation
147 of positives and negatives is less clear-cut than with catechol. However, since the background noise
148 appears to be rather small, we'd suggest a more inclusive cut-off for further analysis.

149 Only considering the enzymes with highest SAM conversion, we see overlap in substrate acceptance for
150 StyLOMT, RetFOMT, StrAOMT, OmnOMT and MesMOMT. While the former enzymes show increased
151 SAM conversion in the presence of all three substrates, MesMOMT is not stimulated by dopamine.
152 Several enzymes appear to display stronger substrate selectivity: KibPOMT and StiAOMT are selective
153 for caffeic acid, HymGOMT, LegHOMT, MedSOMT, SapPOMT, SarHOMT and SeISOMT are selective for

154 catechol, and SalOMT is selective for dopamine. A sequence comparison of the tested OMTs shows that
155 enzymes with similar activities also share higher sequence similarity with each other (Figure 4). Looking
156 at the active site residues predicted based on multiple sequence alignments, we see that some of the
157 tested OMTs display activity although they carry changes in the putative catalytic triad (Table 1). In the
158 group of enzymes from HMMsearch 1 (plant input sequences) the catalytic triad should be H-E-E (in
159 *Medicago sativa* COMT1_MEDSA residues H269, E297 and E329³⁹) and is highly conserved with a few
160 exceptions: CreAOMT, GloKOMT, GloOMT, LegHOMT, with all enzymes being active. In the group of
161 enzymes from HMMsearch 2 (bacterial input sequences) the catalytic triad should be K-N-D (in MxSafC
162 residues K145, N69, D212⁴⁰) and is even more conserved. Only three sequences AciOMT, DesAOMT and
163 StiAOMT show significant changes in these amino acids, with only AciOMT being inactive. This suggests
164 that there must be other changes in the active site architecture of these OMTs that compensate for
165 these amino acid substitutions. With a stricter pre-selection based on sequence similarity and active site
166 conservation, we might have missed these interesting OMTs, whereas our pre-screening approach
167 enabled us to explore a wider sequence space.

168 Application of pre-screened OMTs in a pathway towards (iso-)ferulic acid

169 Lastly, we sought to use the pre-screened OMTs in an *E. coli* microbial cell factory. We chose to expand
170 our previously constructed and optimized^{31,41} pathway from tyrosine to caffeic acid^{31,41} by one enzymatic
171 step in order to generate the pharmaceutically relevant phenolic acid, ferulic acid and its regio-isomer
172 4-methoxy-3-hydroxy-cinnamic acid (iso-ferulic acid). In order to select enzymes for testing in the
173 recombinant pathway, we first explored the data from the pre-screening assay in the context of enzyme
174 expression, protein sequence and the donor organism. We found that the presence or absence of a
175 band of the appropriate size in the SDS PAGE did not correlate with observed enzymatic activity
176 (Extended Data Figure 2). For instance, in the lane of one of the best performing enzyme, StyLOMT, we
177 did not see a distinct band on SDS PAGE. Whereas for some inactive enzymes such as, HalOMT, we saw a
178 distinct band on SDS PAGE. This indicates that some enzymes are expressed at a low level yet active,
179 whereas others are either not correctly expressed and folded, or were simply not challenged with the
180 right substrate in this study. When mapping the pre-screening results onto the sequence similarity
181 network we notice that the enzymes active on caffeic acid (Extended Data Figure 1, filled symbol) are
182 distributed across the network with most of them being part of the main cluster. All active OMTs except
183 MesMOMT show highest sequence similarity with the bacterial seed sequences (yellow box). This
184 indicates that the bacterial input sequences provided a better search template for identifying new
185 caffeic acid OMTs than the plant input sequences. Since some of the putative OMTs found with the plant

186 input sequences display activity against catechol and dopamine, we can exclude that the lack of activity
187 on caffeic acid is caused by a general problem with our *in silico* selection, *in vitro* expression and pre-
188 screening approach. However, for the putative OMTs that did not display activity on any of the tested
189 substrates, we cannot rule out protein expression and folding problems. Two of the active enzymes,
190 RetFOMT and StyLOMT, are from eukaryotic donors and the rest from bacterial donors. This indicates
191 that the pre-screening method is also applicable to eukaryotic enzymes.

192 Based on the prescreening results, we chose the ten top-performing enzymes including the previously
193 characterized StrAOMT²⁸ and MxSafC^{25,32} and two enzymes that were inactive in the pre-screen: HalOMT
194 (archaeal donor, visibly expressed) and SalOMT (archaeal donor, not visibly expressed). We cloned the
195 respective genes into the vector pRSFduet::FjTAL, which already contained the tyrosine ammonia lyase
196 gene from *Flavobacterium johnsoniae* (FjTAL, first pathway step) in a separate expression cassette, for
197 expression under the T7 promoter (Extended Data Table 2). We co-transformed each new plasmid with
198 two other plasmids encoding for the Cytochrome P450 monooxygenase CYP199A2 F185L Δ 7 and its
199 redox partners (second pathway step) from our previous study³¹ into *E. coli* K12 MG1655DE3. In the
200 resulting strains (s01-s12, Extended Data Table 3) L-tyrosine will be converted to *p*-coumaric acid by
201 FjTAL, to caffeic acid by CYP199A2 F185L Δ 7 and to (iso-)ferulic acid by the OMTs. As a negative
202 control, we used a strain with the pRSF::FjTAL plasmid lacking an OMT gene (s00). In initial fermentation
203 experiments with the modified M9 minimal media composition that we had previously used³¹, we did
204 not observe significant product formation from glucose or fed L-tyrosine (data not shown) and therefore
205 decided to first optimize the conditions for the OMT catalyzed step with a subset of the strains and in
206 smaller scale reactions with fed caffeic acid. We observed that the addition of Mg²⁺ (obligate co-factor
207 for OMTs) by itself only led to slightly higher caffeic acid conversion, whereas feeding of L-methionine as
208 a precursor for SAM improved the turnover by 2.2- to 3.6-fold (Figure 5a). This finding is consistent with
209 previous observations for vanillin biosynthesis in *E. coli*⁴² and indicates that SAM supply is limited and
210 needs to be increased for OMT containing pathways to be efficient. With this knowledge, we tested all
211 strains in 15 mL fermentations with glucose as a carbon source, L-tyrosine as a pathway precursor and
212 Mg²⁺ and L-methionine as additives for the OMT reaction. We observed product formation for all strains
213 expressing OMTs that had tested active in the pre-screening step (Figure 5b). In most strains more than
214 half of the caffeic acid formed was converted to the methylated products and four strains even achieved
215 full conversion: s06 expressing PhoAOMT, s08 expressing StyLOMT, s09 expressing RetFOMT and s11
216 expressing StrAOMT. In terms of titers, s11 displays the most desirable outcome with low titers for
217 pathway intermediates and side products, and a high product titer of 0.49 mM +/- 0.06 mM (Figure 5c).

218 Interestingly, all OMTs displayed a strong regioselectivity for the meta-position over the para-position *in*
219 *vivo*, although some showed a preference for the para-position in the *in vitro* screening step (Extended
220 Data Table 4). This indicates that *in vitro* data cannot necessarily be directly translated into whole-cell
221 applications. Nevertheless, our pre-screening step decreased the experimental load for cloning,
222 fermentation and product analysis by HPLC by at least two-thirds. The best performing OMT, StrAOMT,
223 had previously been observed to act on caffeic acid, however with low catalytic efficiency²⁸. To our
224 surprise it was one of the top-performers in the pre-screening and the pathway application in this study.
225 To the best of our knowledge, StrAOMT has not been used in the context of a pathway before.

226 Discussion

227 Closing the gap between computational annotations and the biotechnological exploitation of natural
228 enzymes as industrial biocatalysts requires extensive functional screening of enzyme libraries.
229 Alternatively, scarce sampling of enzyme families and deep functional analysis (“thick data”) can be
230 utilized to improve annotation pipelines and thus the interpretation of big data. In this study, we
231 developed a rapid prototyping platform for SAM-dependent methyltransferases, an enzyme superfamily
232 that has great potential for functionalization of natural product-inspired pharmaceuticals. The chosen
233 approach follows in the footsteps of a range of studies utilizing *in vitro* transcription/translation systems
234 for prototyping of antibodies⁴³, protein expression enhancing factors⁴⁴, transcription regulatory
235 elements⁴⁵, GPCRs⁴⁶, quorum-sensing systems⁴⁷ and entire biosynthetic pathways⁴⁸, which highlights the
236 generalizability of this expression system⁴⁹. In our study we observed that the enzymatic activity is highly
237 reproducible across biological replicates of the *in vitro* transcription/translation system, which is in good
238 agreement with the previous studies. In addition to the time and effort saved on molecular cloning,
239 transformation and protein expression in *E. coli* (1-3 weeks), another advantage is that no additional
240 lysis and clearing steps are required before the enzymatic reaction is performed. This is particularly
241 advantageous for enzymes requiring cofactors that cannot cross the cell membrane, such as SAM. In our
242 workflow, we combined the *in vitro* expression system with a microliter-scale enzymatic assay coupled
243 to a TR-FRET read-out. While this read-out is sensitive to experimental error due to the small volumes
244 and the required dilution steps, we were able to generate robust results by using master mixes and
245 multichannel pipettes for all steps. The TR-FRET-based detection of the SAM cofactor - rather than a
246 specific substrate or product - allows for the screening of a library of substrates. The format can
247 furthermore be used to swiftly optimize reaction conditions, such as buffers, salts and substrate
248 concentrations and thus generate “thick data”. The entire workflow should be amenable to automation
249 by using liquid handling robots and is therefore scalable to also screen large enzyme libraries.

250 The workflow allowed us to rapidly screen ~30 enzymes against 5 substrates. In particular for caffeic
251 acid as a substrate, we identified several distant homologues with remarkable activity, two of which
252 don't even carry the conserved active site residues. With a more conservative *in silico* approach of
253 selecting enzymes of interest, we might not even have considered these as suitable enzymes. However,
254 our approach allowed us to cast a wide net, explore the activity of these distantly related enzymes and
255 use them in an *E. coli* cell factory for ferulic acid, a methylated phenolic acid of pharmaceutical interest.
256 Zooming in on these unexpected hits and their close relatives with structural and functional studies will
257 allow us to better understand the underlying mechanisms of substrate selectivity and regioselectivity in
258 OMTs.

259 Lastly, we tested a subset of the pre-screened OMTs in the context of a recombinant biosynthetic
260 pathway in *E. coli*. We observed that all enzymes seen to be active in the pre-screening step were also
261 catalytically active in the pathway, whereas enzymes found to be inactive in the screen, remained
262 inactive in the pathway. The trends in substrate conversion levels and regio-selectivity, however, were
263 not necessarily correlated between the *in vitro* and *in vivo* experiments. This is a hurdle well known to
264 metabolic engineers and is inherent to *in vitro* characterization of enzymes. However, the high cost of *in*
265 *vivo* screening in terms of time and consumables justifies the need for *in vitro* prototyping.

266 **Methods**

267 **Selection of enzymes of interest**

268 Two different multiple sequence alignments of known OMTs were generated with the Clustal Omega EBI
269 webtool²² (Extended Data Table 1) and used as input for HMMsearch (EBI webtool version 2.23.0²¹; data
270 base of reference proteomes of all taxa excluding green plants (taxid: 33090), significance E-value cut-
271 off 0.01 for the entire sequence and 0.03 for hits). The significant results were combined into one data
272 set (Supplementary File 1) and used as an input for calculating a sequence similarity network with a
273 webtool of the Enzyme Function Initiative (EFI-EST²⁹; node selection cut-off: protein length between 180
274 and 400 amino acids, edge selection cut-off: alignment score >30). The finalized network was visualized
275 in Cytoscape 3.8.0³⁰ with the yFiles organic layout. For the representation in Extended Data Figure 1, the
276 nodes were further filtered to exclude all nodes with an HMM score below 70 and all edges with
277 sequence identity below 50%. From the thus generated clusters enzymes were chosen for experimental
278 characterization (Table 1).

279 Design and synthesis of DNA templates for TXTL reactions

280 The selected genes were codon-optimized for expression in *E. coli* with the Integrated DNA Technologies
281 (IDT) optimization algorithm and manually modified to exclude recognition sites for BsaI, NcoI, XhoI and
282 where possible NdeI restriction enzymes. The 5' end of all DNA fragments was designed with an
283 overhang of 500bp, the p70a promoter sequence and an NcoI recognition site to facilitate cloning into
284 the pET21b(+) (Novagen) and pBEST (Arbor Bioscience) expression vectors. The 3' end was designed to
285 include an XhoI recognition site, the T500 terminator and a 500bp overhang. The synthetic DNA was
286 obtained from Arbor Bioscience (Ann Arbor, MI, USA) with an additional purification step to allow for
287 direct use in the myTXTL[®] reaction for linear templates.

288 Construction of plasmids

289 All molecular cloning and plasmid propagation steps were performed in chemically competent
290 *Escherichia coli* *E. cloni*[®] 10G (F- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) *endA1 recA1* Φ 80*dlacZ* Δ M15
291 Δ *lacX74 araD139 Δ (*ara, leu*)7697*galU galK rpsL nupG* λ -*tonA*) produced by Lucigen (Middleton, WI,
292 USA). Genes encoding for OMTs selected for *in vivo* testing were cloned directly from the synthetic DNA
293 fragments by restriction and ligation (NcoI/XhoI) into pET21b(+) for expression under the T7 promoter
294 (Extended Data Table 2). From there the genes were amplified by polymerase chain reaction (PCR) with
295 gene specific 5'primers and the T7 terminator primer to generate an NdeI recognition site at the 5'
296 prime end. The PCR products were inserted by restriction and ligation (NdeI/XhoI) into the second
297 multiple cloning site of the plasmid c71 (pRSF::FJTAL) for expression under the T7 promoter (Extended
298 Data Table 2). All constructs were verified by sequencing by ETON Bioscience (Charlestown, MA, USA).
299 Plasmids c71, c84 and c86 were constructed in a previous study³¹.*

300 *In vitro* transcription/translation

301 *In vitro* transcription/translation was performed with the myTXTL[®] kit from Arbor Bioscience according
302 to the manufacturer's instructions. In brief, the synthesized DNA fragments were dissolved in nuclease-
303 free water to a final concentration of 109.1 nM and stored at -70°C between experiments. All assay
304 components were thawed on ice (myTXTL[®] lysate, GamS protein and DNA templates) and mixed by
305 carefully pipetting up and down. To minimize pipetting errors, a master mix of myTXTL[®] lysate (9 μ L per
306 reaction) and GamS protein (0.8 μ L per reaction) was prepared on ice and aliquoted into 1.5 mL
307 microcentrifuge tubes. 2.2 μ L of DNA template were added to each tube and mixed by carefully
308 pipetting up and down (final concentration 20 nM). The reactions were incubated on ice for 5 min and
309 then transferred to a water bath at 29°C for 16 h. As negative controls ("no OMT"), one reaction was

310 performed with a DNA template not encoding for an OMT enzyme and one reaction with nuclease-free
311 water without DNA.

312 SDS PAGE

313 To visualize protein expression 1 μ L of the TXTL reactions was mixed with 2 μ L of water and 3 μ L of 2x
314 Laemmli loading dye (Bio-Rad, Hercules, CA, USA) and incubated at 90°C for 3 min. The denatured
315 samples were loaded onto AnyKD™ Mini-PROTEAN® TGX™ precast protein gels (Bio-Rad, Hercules, CA,
316 USA) and separated for 40 min at 40 mA. Protein bands were visualized by staining with InstantBlue®
317 protein stain and imaging with the Bio-Rad ChemiDoc™ imager.

318 Expression and purification of MxSafC

319 Plasmid c157 was transformed into chemically competent *E. coli* BL21 DE3 ($F^- ompT gal dcm lon hsdS_B(r_B^-$
320 $m_B^-)$ λ (DE3 [*lacI lacUV5-T7p07 ind1 sam7 nin5*]) [*malB'*]_{K12}(λ^S)) and maintained on selective LB agar
321 containing 100 μ g/mL carbenicillin. A starter culture was inoculated from a single colony (5 mL, LB with
322 carbenicillin) and incubated overnight at 37°C, 250 rpm. The main culture was inoculated from the
323 starter culture (1:100) and incubated at 37°C, 250 rpm until an optical density OD₆₀₀ of 0.7 was reached.
324 Expression was induced with Isopropyl β -D-1-thiogalactopyranoside (IPTG, 1 mM final) and the
325 temperature was lowered to 30°C (250 rpm, overnight). All following steps were performed at 4°C with
326 chilled buffers. The cells were harvested by centrifugation (10 min, 3,000 rpm) and resuspended in
327 20 mL lysis buffer (buffer A including one EDTA-free protease inhibitor tablet (Roche) and 10 mg/mL
328 lysozyme; buffer A: 50 mM Tris/HCl pH 7.4, 500 mM NaCl, 10 mM imidazole). The cell suspension was
329 incubated on ice for 20 min, lysed by sonication (20% duty cycle, 10 cycles of 15 s ON/15 s OFF) and
330 cleared by centrifugation for 20 min at 40,000 x g. The supernatant was loaded onto the affinity matrix
331 equilibrated with buffer A by gravity flow (Qiagen, Ni-NTA agarose slurry, 0.25 mL column volume). The
332 column was washed with 20 column volumes of buffer A and eluted stepwise with one column volume
333 of buffers B1 to B6 (buffers B1-B6: 50 mM Tris/HCl pH 7.4, 500 mM NaCl, 50 mM imidazole/ 100 mM
334 imidazole/ 150 mM imidazole/ 200 mM imidazole/ 250 mM imidazole or 500 mM imidazole,
335 respectively). The eluates of each step were collected in separate fractions and analyzed by SDS PAGE.
336 MxSafC containing fractions with low protein background were pooled and dialyzed overnight at 4°C
337 against storage buffer (20 mM Tris/HCl pH 7.4, 50 mM NaCl, 0.2 mM MgCl₂, 2 mM DTT). The protein
338 concentration was determined by absorbance at 280 nm (NanoDrop, ThermoFisher Scientific, USA)
339 before the purified enzyme was aliquoted and stored at -70°C.

340 *In vitro* OMT reaction

341 The *in vitro* OMT reaction was adapted from the conditions used by Siegrist et al.³². To minimize
342 pipetting errors, a master mix including all reaction components, but the enzyme was prepared (50 mM
343 HEPES/NaOH pH 7, 20 mM MgCl₂, 2 mM SAM, 2 mM substrate (from 40x stock in DMSO)). The total
344 reaction volume was 42 μL, with 2 μL of purified enzyme/ TXTL reaction used in the initial experiment
345 and later 5 μL of a diluted stock (2.5-fold dilution in OMT reaction buffer) to further minimize pipetting
346 errors. After aliquoting the master mix into 96-well microtiter plates (200 μL round-bottom plates), the
347 TXTL samples were added and mixed by carefully pipetting up and down. Purified MxSafC enzyme was
348 included in one well as a positive control. The “no OMT” controls (see section “*In vitro*
349 transcription/translation”) were treated like the other enzyme samples. The sealed plates were
350 incubated at 30°C for 24 h before the reactions were quenched with HClO₄ (final 2% v/v from a 10% v/v
351 stock) and centrifuged. The supernatants were analyzed by Time Resolved-Fluorescence Energy
352 Resonance Transfer (TR-FRET) and (optionally) by High Performance Liquid Chromatography (HPLC).

353 TR-FRET assay for SAM detection

354 To detect the consumption of the SAM co-factor as a measure of OMT reactivity, we used the TR-FRET
355 Bridge-It® S-Adenosyl Methionine (SAM) Fluorescence Assay Kit from Mediomics LLC (St. Louis, Missouri)
356 according to the manufacturer’s instructions with slight modifications. In brief, we thawed the assay
357 solution at 37°C for 30 min and transferred 18 μL into the wells of a white 384-well round-bottom
358 polystyrene plate (Corning, NY, USA). We diluted the quenched OMT reactions 21-fold by mixing 2 μL of
359 the reaction with 40 μL of water by pipetting up and down, and transferred the samples to the 384-well
360 plate without bubbling. In addition to the “no OMT” controls (no SAM consumption expected), one or
361 two wells were measured with only the TR-FRET assay solution (20 μL, “blank”). The plate was incubated
362 in the dark for 30 min at room temperature before measuring the TR-FRET signal in a Tecan Infinite-200
363 plate reader with the following settings: *mode*: fluorescence top reading, *excitation wavelength*:
364 340 nm, *emission wavelength*: 667 nm, *excitation bandwidth*: 9 nm, *emission bandwidth*: 20 nm, *gain*:
365 220 (manual), *number of flashes*: 100, *integration time*: 400 μs, *lag time*: 50 μs, *settle time*: 150 ms. The
366 ratio of the acceptor channel counts to the donor channel counts was calculated for all measured wells
367 (FRET), baseline corrected with the FRET ratio of the “blank” and normalized to the average of the FRET
368 ratio of the “no OMT” controls to obtain the relative SAM detection (Eq. 1). In the initial experiment, the
369 samples were handled with single-channel pipettes, whereas in the later experiments, multichannel
370 pipettes were used throughout to minimize pipetting errors.

371 Eq 1: relative SAM detection= $(\text{FRET}-\text{FRET}_{\text{blank}})/\text{Average}(\text{FRET}_{\text{noOMT}}-\text{FRET}_{\text{blank}})$ with
372 $\text{FRET}=\text{counts}_{667}/\text{counts}_{620}$

373 Fermentation

374 The OMT encoding plasmids were transformed into chemically competent³³ *E. coli* K12 MG1655(DE3)³⁴
375 already bearing the plasmids c84 and c86 encoding for CYP199A2 F185L NΔ7 and its redox partners
376 putidaredoxin (Pux) and putidaredoxin reductase (PuR). All strains generated in this way are listed in
377 Extended Data Table 3. The final strains were maintained on selective media with carbenicillin,
378 spectinomycin and kanamycin at all times. Starter cultures were prepared from three individual colonies
379 of the final strains in 5 mL Lysogeny broth (LB) supplemented with carbenicillin (100 μg/mL),
380 spectinomycin (50 μg/mL) and kanamycin (50 μg/mL) in round-bottom polystyrene tubes, incubated
381 over night at 37°C with agitation and used to inoculate the main cultures (7 mL LB with antibiotics;
382 round-bottom polystyrene tubes). After 4 h of growth at 37°C, 250 rpm, OD₆₀₀ was measured and the
383 appropriate volume of each culture pelleted and resuspended in modified, selective M9 medium
384 including substrates and 4% glucose to obtain 15 mL cultures at OD₆₀₀ of 0.7 in sterile glass tubes. These
385 cultures were incubated at 26°C, 160 rpm for 96 h. Samples of 200 μL were taken after 96 h and
386 quenched with 50 μL of HClO₄ (10 % (v/v) stock), spun for 10 min at 20,000 x g and the supernatants
387 were analyzed by HPLC. Media optimization was performed in small scale (5 mL in round-bottom
388 polystyrene tubes) throughout the entire experiment.

389 M9 medium composition (1x) prepared from sterile stocks: M9 salts (Millipore-Sigma, used as 5x stock),
390 Trace Mineral Supplement (ATCC® MD-TMS™, used as 200x stock), vitamin mix (from 100x stock; final:
391 riboflavin 0.84 mg/L, folic acid 0.084 mg/L, nicotinic acid 12.2 mg/L, pyridoxine 2.8 mg/L, and
392 pantothenic acid 10.8 mg/L), biotin (from 1000x stock; final: 0.24 mg/L), thiamine (from 1470x stock;
393 final: 340 mg/L), δ-Aminolevulinic acid (from 1000x stock in MeOH, final: 7.5 μg/mL), IPTG (from 1000x
394 stock, final: 1 mM), carbenicillin (from 1000x stock, final: 100 μg/mL), spectinomycin (from 1000x stock,
395 final: 50 μg/mL), kanamycin (from 1000x stock, final: 50 μg/mL, 4% (w/v) glucose (from 50% w/v stock).
396 Additives for media optimization experiments: caffeic acid (from fresh 100x stock in MeOH, final 2 mM)
397 and either a) no further additives, b) MgCl₂ (from 500x sterile stock in water, final 2 mM) or c) MgCl₂
398 (from 500x sterile stock in water, final 2 mM) and L-methionine (from fresh 100x stock in 1M HCl, final
399 10 mM). Additives for all other experiments: MgCl₂ (from 500x sterile stock in water, final 2 mM) and L-
400 methionine and L-tyrosine (from fresh joined 100x stock in 1M HCl, final 10 mM and 3 mM,
401 respectively).

402 HLPC analysis

403 The supernatants of the quenched *in vitro* OMT reactions and fermentation samples were analyzed by
404 reversed-phase HPLC (instrument: Agilent 1100; autosampler: HiP sampler G1367A, T=4°C, 10 µL
405 injection; column: Agilent Zorbax Eclipse XDB-C18 80Å, 4.6 x 150 mm, 5µm, T=30°C; detector: Agilent
406 diode array detector G1315B, λ=275 nm (catechol and methylated products) and λ=310nm ((iso-)ferulic
407 acid and pathway intermediates); gradient: 10% to 35% Acetonitrile with 0.1% Trifluoroacetic acid over
408 17 min). The peaks for products and intermediates were identified by comparing the retention times to
409 authentic standards. The integrated peak areas were converted to concentrations in mM based on
410 calibration curves generated with authentic standards.

411 Sequence analysis of putative OMTs

412 We aligned the sequences with mafft v7.310³⁵b (--genafpair), inferred the maximum likelihood
413 phylogenies with FastTree v2.1.10³⁶ and visualized the tree and the corresponding activity heat map in
414 Figure 4 with the R packages ggplot2³⁷ and ggtree³⁸.

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418 Author Contributions

419 KH conceived the study, selected the putative OMTs, performed all experiments and wrote the
420 manuscript with support and guidance by K.L.J.P. KH and TH analyzed the data and created figures. TH
421 provided bioinformatics support. All authors read and approved the final version of this manuscript.

422 Competing Interests statement

423 The authors declare that they have no competing interests.

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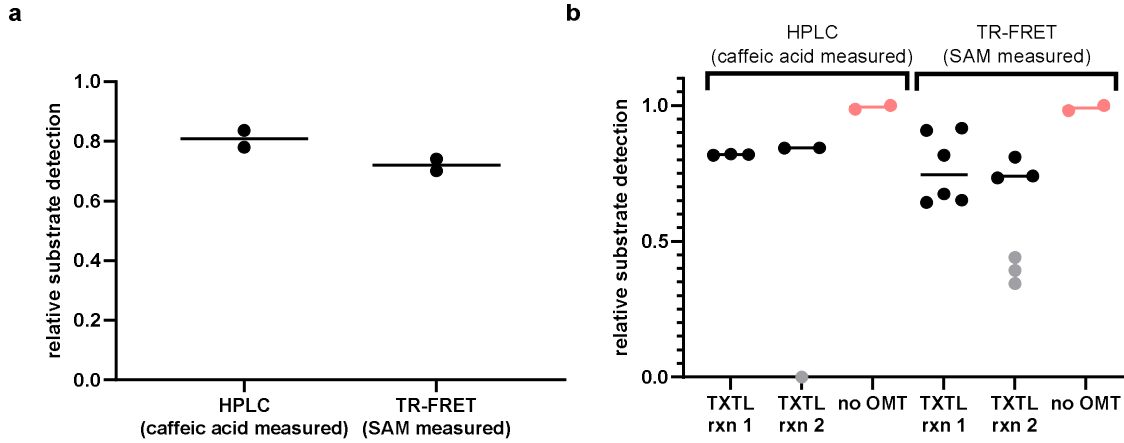
550 Tables

551 Table 1: List of putative OMT selected for experimental characterization.

protein name in this study	Uniprot name	donor organism	Domain	Phylum (class)	HMMsearch		cat. triad
HMMsearch 1 (plant caffeic acid OMTs as seed sequences)					E-value	Score	H-E-E
AlkOMT	A0A251WJU7_9CYAN	<i>Alkalinema sp.</i> CACIAM 70d	Bacteria	Cyanobacteria (Melainabacteria group)	4.10E-44	162	H-E-E
BraLOMT	A0A0R3MQ32_9BRAD	<i>Bradyrhizobium lablabi</i>	Bacteria	Proteobacteria (Alpha)	2.10E-33	126.7	H-E-E
BucROMT	A0A091HDB2_BUCRH	<i>Buceros rhinoceros silvestris</i>	Eukaryota	Metazoa	5.70E-27	105.6	H-E-E
CanNOMT	A0A0N9Y1E2_9ARCH	<i>Candidatus Nitrocosmic oleophilus</i>	Archaea	Thaumarchaeota	4.90E-32	122.2	H-E-E
CreAOMT	A0A1Q7MH91_9CREN	<i>Crenarchaeota archaeon</i> 13_1_40CM_3_52_17	Archaea	Crenarchaeota	7.50E-31	118.3	H-G-E
DicDOMT	OMT12_DICDI	<i>Dictyostelium discoideum</i>	Eukaryota	Mycetozoa	7.70E-31	118.3	H-D-E
GloKOMT	U5QFM0_9CYAN	<i>Gloeobacter kilaeensis</i> JS1	Bacteria	Cyanobacteria (Melainabacteria group)	4.20E-45	165.2	S-E-E
GloOMT	K9XAK2_9CHRO	<i>Gloeocapsa sp.</i> PCC 7428	Bacteria	Cyanobacteria (Melainabacteria group)	7.80E-37	138	H-Q-W
HalOMT	U1MFJ5_9EURY	halophilic archaeon J07HX5	Archaea	Halobacteria	5.60E-30	115.4	H-E-E
HymGOMT	A0A212T1X1_9BACT	<i>Hymenobacter gelipurpurascens</i>	Bacteria	Bacteroidetes (Chlorobi group)	7.70E-42	154.5	H-E-E
LegHOMT	A0A0A8UVF9_LEGHA	<i>Legionella hackeliae</i>	Bacteria	Gammaproteobacteria	9.50E-29	111.4	H-E-Q
MesMOMT	A0A1G9C2B4_9RHIZ	<i>Mesorhizobium muleiense</i>	Bacteria	Alphaproteobacteria	9.40E-34	127.9	H-E-E
SapPOMT	A0A067BNB9_SAPPC	<i>Saprolegnia parasitica</i> (strain CBS 223.65)	Eukaryota	Oomycetes	3.70E-28	109.5	H-D-E
TielOMT	A0A151ZKG1_9MYCE	<i>Tieghemostelium lacteum</i>	Eukaryota	Mycetozoa	5.50E-28	108.9	H-D-E
HMMsearch 2 (bacterial OMTs as seed sequences)							K-N-D
AciOMT	A0A178GH82_9GAMM	<i>Acinetobacter sp.</i> SFD	Bacteria	Gammaproteobacteria	5.60E-27	105.4	R-N-A
ChiCOMT	A0A1M6USV2_9FLAO	<i>Chishuiella changwenlii</i>	Bacteria	Bacteroidetes (Chlorobi group)	2.20E-54	195	K-N-D
DesAOMT	Q1JXV1_DESA6	<i>Desulfuromonas acetoxidans</i> (strain DSM 684)	Bacteria	Proteobacteria (delta/epsilon)	3.70E-30	115.8	R-N-K
KibpOMT	A0A0N9HPV5_9PSEU	<i>Kibdelosporangium phytohabitans</i>	Bacteria	Actinobacteria	5.10E-41	151.3	K-N-D
OmnOMT	A0A1G1JPP6_9BACT	<i>Omnitrophica bacterium</i> GWA2_52_8	Bacteria	(PVC group) Candidatus Omnitrophica	2.40E-54	194.9	K-N-D
PhoAOMT	A0A1U7IJN5_9CYAN	<i>Phormidium ambiguum</i> IAM M-71	Bacteria	Cyanobacteria (Melainabacteria group)	2.00E-93	322.7	K-N-D
RetFOMT	X6M5Z7_RETFI	<i>Reticulomyxa filosa</i>	Eukaryota	Foraminifera	4.90E-54	193.9	K-N-D
SalOMT	R4W9N9_9EURY	<i>Salinarchaeum sp.</i> Harcht-Bsk1	Archaea	Halobacteria	2.30E-25	100.1	K-N-D
SarHOMT	G3WFI7_SARHA	<i>Sarcophilus harrisii</i>	Eukaryota	Metazoa	2.80E-54	194.7	K-N-D
SelSOMT	A0A1T4QLE1_9FIRM	<i>Selenihalanaerobacter shriftii</i>	Bacteria	Firmicutes	2.90E-42	155.4	K-N-D

StiAOMT	E3FEM3_STIAD	<i>Stigmatella aurantiaca</i>	Bacteria	Proteobacteria (delta/epsilon)	6.20E-20	82.4	K-N-S
StyLOMT	A0A078AUZ0_STYLE	<i>Stylonychia lemnae</i>	Eukaryota	Ciliophora	2.40E-54	194.9	K-N-D
TheMOMT	E6SHY4_THEM7	<i>Thermaerobacter marianensis</i> (strain ATCC 700841)	Bacteria	Firmicutes	6.90E-64	226.1	K-N-D
VerLOMT	A0A0G4MCD6_9PEZI	<i>Verticillium longisporum</i>	Eukaryota	Fungi	7.00E-21	85.5	K-N-D

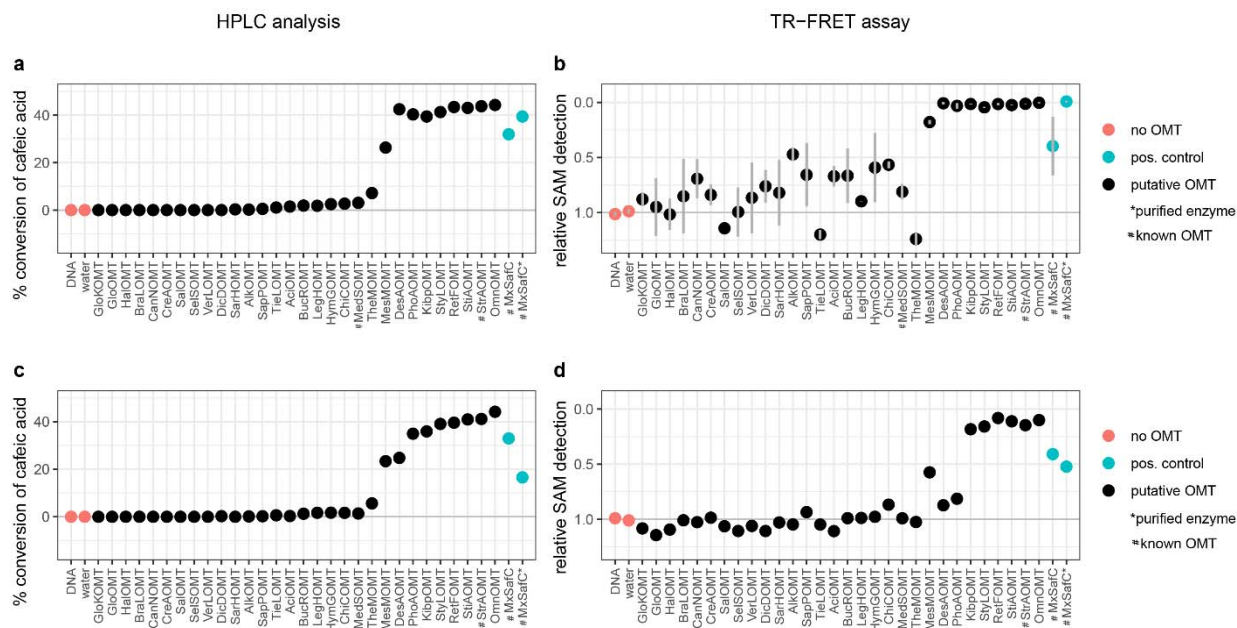
552 **Figures**



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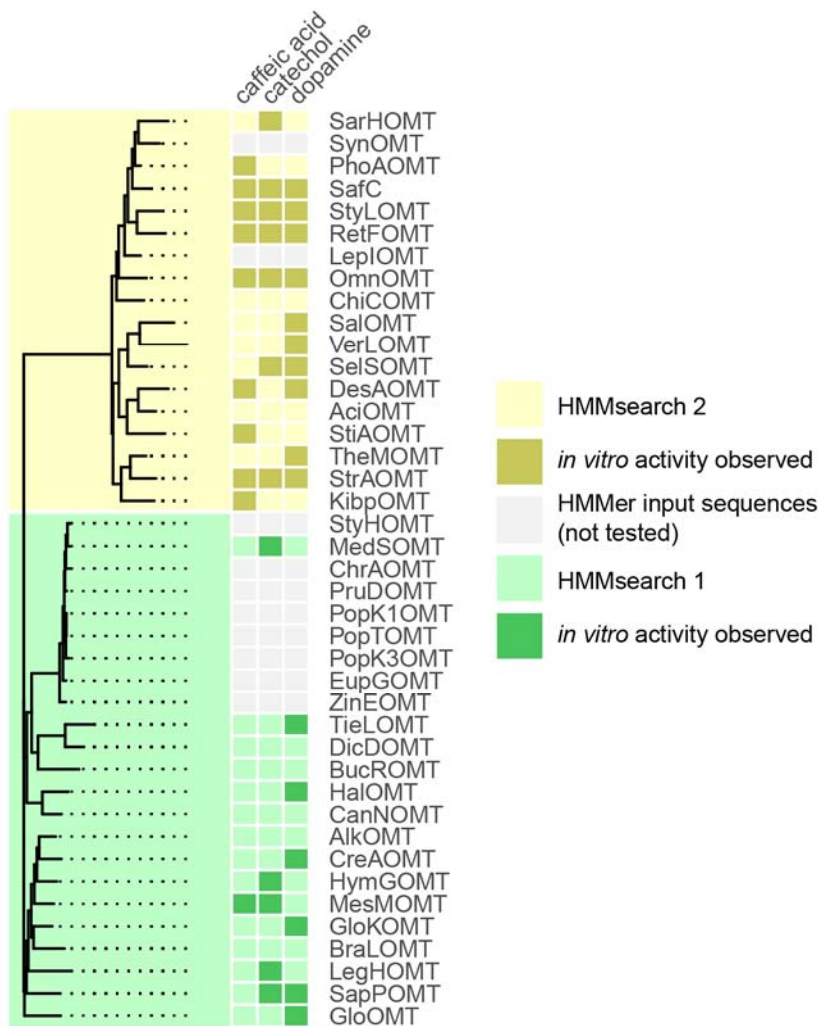
554 *Figure 1: Substrate detection at the end of the OMT reaction with MxSafC as a catalyst relative to the negative controls*
555 *measured by HPLC (caffeic acid concentration measured) and TR-FRET (SAM concentration measured). a) OMT reaction*
556 *performed with recombinantly expressed and purified MxSafC (data points are biological replicates (n=2, median)). b) OMT*
557 *reaction performed with MxSafC expressed from linear DNA in the myTXTL in vitro transcription/translation kit (two biological*
558 *replicates are shown in separate columns; data points within each column are technical replicates of the OMT reaction and the*
559 *TR-FRET assay; “no OMT” data points are biological replicates; outliers (grey data points) are likely caused by a pipetting error in*
560 *the OMT assay and were excluded from determining the median).*

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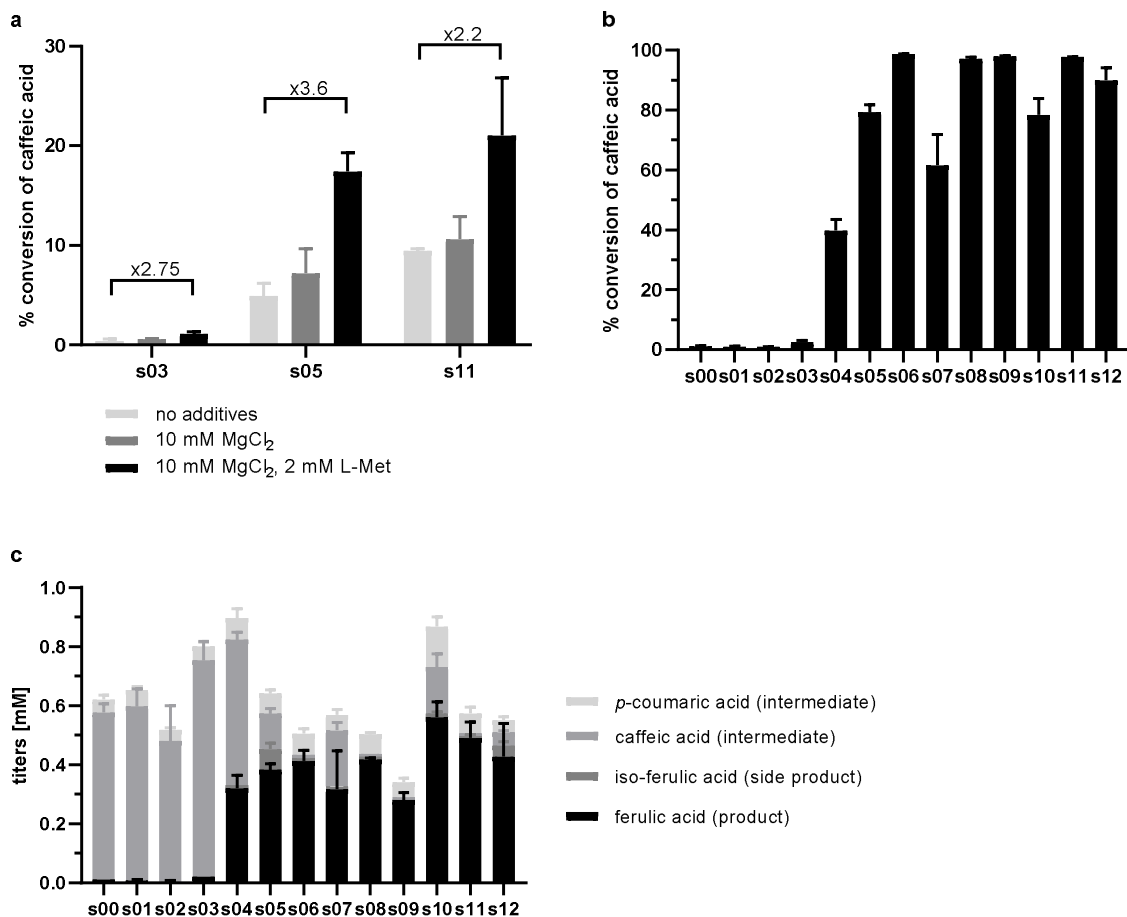
562
 563 *Figure 2: Screening of 30 enzymes of interest against caffeic acid as substrate and SAM as co-substrate in two independent*
 564 *experiments. OMT reaction stopped after 24h. a) and c) Samples analyzed by HPLC, expressed as percent caffeic acid converted.*
 565 *b) and d) Samples analyzed by TR-FRET expressed as SAM levels detected relative to the “no OMT” controls; b) technical*
 566 *replicates of the TR-FRET assay shown as mean +/- SD, n=2; d) single measurement. Data points sorted by increasing substrate*
 567 *turnover based on panel c; Red data points – negative controls, blue data points – positive controls.*

568



573

574 Figure 4: Phylogenetic tree based on a multiple sequence alignment of the enzymes screened in this study and the HMMsearch
 575 input sequences (left; green: HMMsearch1; yellow: HMMsearch 2) side-by-side with the *in vitro* activity data on caffeic acid,
 576 catechol and dopamine (right, dark color=*in vitro* activity observed).



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578

579 *Figure 5: Fermentation of E. coli K12 MG1655(DE3) expressing a recombinant pathway to produce (iso-)ferulic acid. a) Media*
 580 *optimization performed with feeding of the pathway intermediate caffeic acid (2 mM); data displayed as percent conversion of*
 581 *fed caffeic acid. b) and c) Experiment performed with optimized media with glucose as a carbon source and L-Tyrosine (3mM) as*
 582 *a pathway precursor; data displayed as percent conversion of produced caffeic acid (panel b) or as stacked histogram of titers of*
 583 *product, side product and pathway intermediates (panel c). Mean +/- SD, n=3.*

584 **Extended Data Tables**

585 *Extended Data Table 1: Table of input sequences for HMMsearch1 and 2.*

GenBank	Uniprot	Uniprot name	Organism	Length in AA
HMM search 1 (plant caffeic acid OMTs as seed sequences)				
U16793	Q42653	OMT2_CHRAE	<i>Chrysosplenium americanum</i> (Golden saxifrage)	343
U13171	Q00763	COMT1_POPTM	<i>Populus tremuloides</i> (Quaking aspen)	365
M63853	P28002	COMT1_MEDSA	<i>Medicago sativa</i> (Alfalfa)	365
X74814	P46484	COMT1_EUCGU	<i>Eucalyptus gunnii</i> (Cider gum)	366
X83217	Q43609	COMT1_PRUDU	<i>Prunus dulcis</i> (Almond)	365
D49710	Q43046	COMT1_POPKI	<i>Populus kitakamiensis</i> (Aspen) (<i>Populus sieboldii</i> x <i>Populus grandidentata</i>)	365
D49711	Q43047	COMT3_POPKI	<i>Populus grandidentata</i>	364
L36109	/	COMT_STYH	<i>Stylosanthes humilis</i> (townsville stylo)	
U19911	Q43239	COMT1_ZINVI	<i>Zinnia elegans</i> (Garden zinnia)	354
HMM search 2 (bacterial OMTs as seed sequences)				
U24657	Q50859	Q50859_MYXXA	<i>Myxococcus xanthus</i>	220
BA000022	Q55813	Q55813_SYNY3	<i>Synechocystis sp.</i> (strain PCC 6803 / Kazusa)	220
AE010300	Q8F8Y3	Q8F8Y3_LEPIN	<i>Leptospira interrogans</i> serogroup Icterohaemorrhagiae serovar Lai (strain 56601)	231
BA000030	Q82B68	Q82B68_STRAW	<i>Streptomyces avermitilis</i> strain ATCC 31267	224

586 *Extended Data Table 2: List of plasmids used in this study with the enzymes expressed from the respective multiple cloning sites*
 587 *(MCS), the 5' primer sequences and the restriction sites used for subcloning.*

Plasmid ID	backbone	enzyme expressed		5' primer for subcloning	restriction sites	source
		MCSI	MCSII			
c84	pCDFDuet	6His-Pux (putidaredoxin)	6His-CYP199A2 F185L NΔ7	/	/	³¹
c62	pETDuet	6His-Pux	PuR (putidaredoxin reductase)	/	/	³¹
c71	pRSFDuet	6His-FjTAL	/	/	NcoI, XhoI	This study
c157	pET21(+)	MxSafC	/	/	NcoI, XhoI	This study
c160	pET21b(+)	OmnOMT	/	/	NcoI, XhoI	This study
c161	pET21b(+)	DesAOMT	/	/	NcoI, XhoI	This study
c162	pET21b(+)	KibPOMT	/	/	NcoI, XhoI	This study
c163	pET21b(+)	MesMOMT	/	/	NcoI, XhoI	This study
c164	pET21b(+)	PhoAOMT	/	/	NcoI, XhoI	This study
c165	pET21b(+)	RetFOMT	/	/	NcoI, XhoI	This study
c167	pET21b(+)	StiAOMT	/	/	NcoI, XhoI	This study
c168	pET21b(+)	StrAOMT	/	/	NcoI, XhoI	This study
c169	pET21b(+)	StyLOMT	/	/	NcoI, XhoI	This study
c193	pET21b(+)	HalOMT	/	/	NcoI, XhoI	This study
c194	pET21b(+)	SalOMT	/	/	NcoI, XhoI	This study
c180	pRSFduet	6His-FjTAL	MesMOMT	CCATGCATatggacgagcgggatgcg	NdeI, XhoI	This study
c181	pRSFduet	6His-FjTAL	RetFOMT	CCATGCATatgggcaacagagcgacg	NdeI, XhoI	This study
c182	pRSFduet	6His-FjTAL	StrAOMT	CCATGCATatgggctcagaatcgcaac	NdeI, XhoI	This study
c183	pRSFduet	6His-FjTAL	MxSafC	CCATGCATatgggcatccatcatgtcg	NdeI, XhoI	This study
c184	pRSFduet	6His-FjTAL	KipBOMT	CCATGCATatgggcacgctgaatgg	NdeI, XhoI	This study
c186	pRSFduet	6His-FjTAL	OmnOMT	GCATGCATatgggcaaccaatccac	NdeI, XhoI	This study
c187	pRSFduet	6His-FjTAL	DesAOMT	GCATGCATatgggcaacaagaactgcac	NdeI, XhoI	This study
c188	pRSFduet	6His-FjTAL	PhoAOMT	GCATGCATatgggcaaaaaagaccttg	NdeI, XhoI	This study
c190	pRSFduet	6His-FjTAL	StiAOMT	GCATGCATatgggcaatgaaaaggatgg	NdeI, XhoI	This study
c191	pRSFduet	6His-FjTAL	StyLOMT	GCATGCATatggaagatcttaacaaagacaaatccg	NdeI, XhoI	This study
c197	pRSFduet	6His-FjTAL	HalOMT	GCATGCATatgggctcgacaagtctc	NdeI, XhoI	This study
c198	pRSFduet	6His-FjTAL	SalOMT	GCATGCATatgggcacgctgtgtcc	NdeI, XhoI	This study

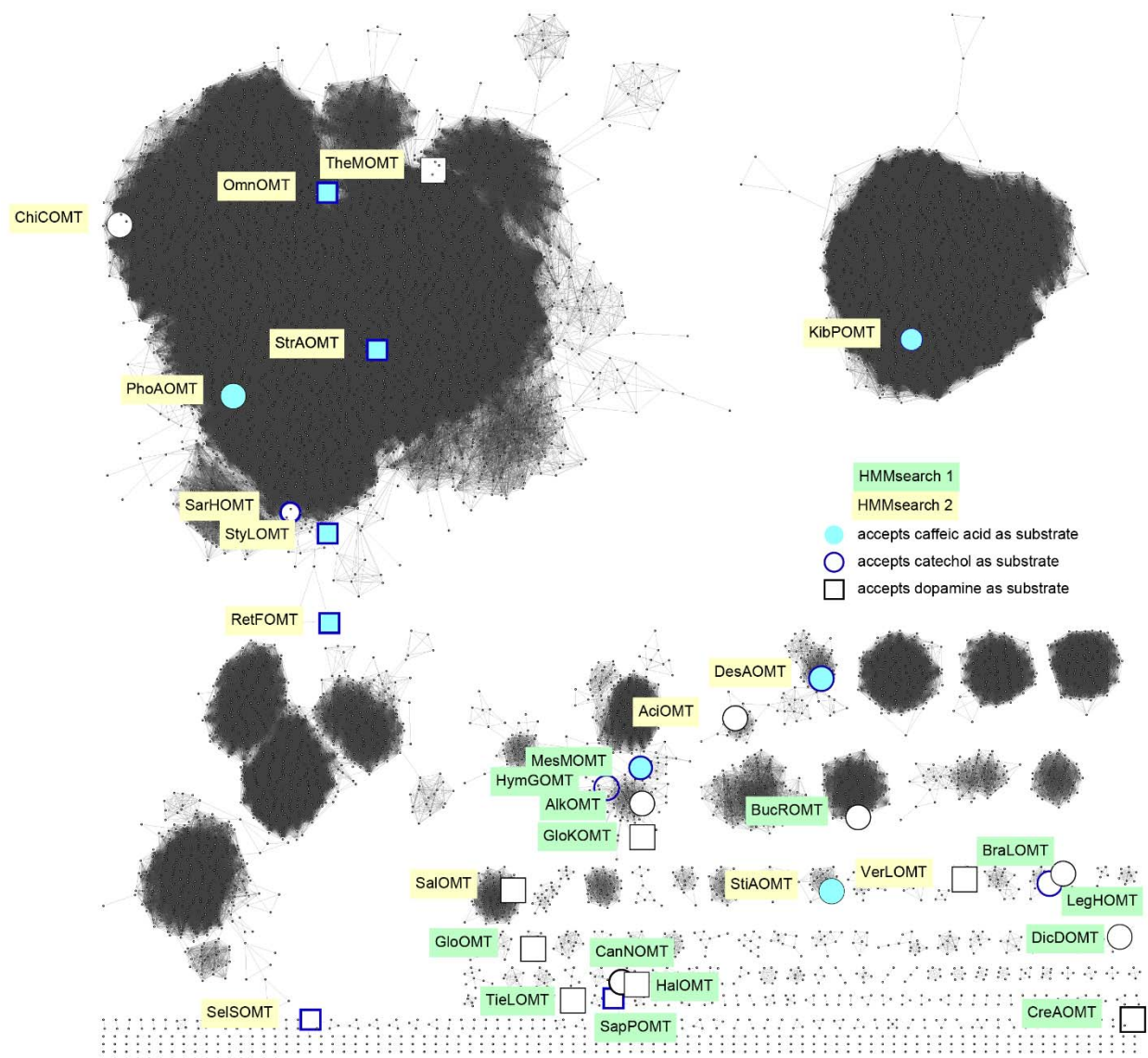
588 *Extended Data Table 3: List of bacterial strains used in this study to produce (iso-)ferulic acid from fed L-tyrosine or caffeic acid.*

Strain ID	plamids	enzymes expressed	source
s00	c84 c62 c71	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL	³¹
s01	c84 c62 c197	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL, HalOMT	This study
s02	c84 c62 c198	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL, SalOMT	This study
s03	c84 c62 c180	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL, MesMOMT	This study
s04	c84 c62 c187	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL, DesAOMT	This study
s05	c84 c62 c183	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL, MxSafC	This study
s06	c84 c62 c188	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL, PhoAOMT	This study
s07	c84 c62 c184	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL, KipBOMT	This study
s08	c84 c62 c191	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL, StyLOMT	This study
s09	c84 c62 c181	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL, RetFOMT	This study
s10	c84 c62 c190	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL, StiAOMT	This study
s11	c84 c62 c182	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL, StrAOMT	This study
s12	c84 c62 c186	6His-Pux, 6His-CYP199A2F185L NΔ7 6His-Pux, PuR FJTAL, OmnOMT	This study

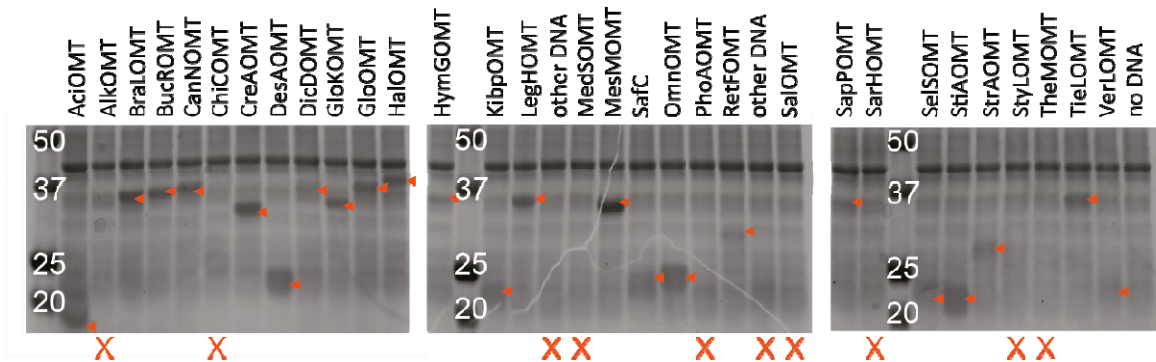
589 *Extended Data Table 4: Regioselectivity of OMT enzymes in vitro (pre-screen) and in vivo (fermentation) expressed as regio-*
 590 *isomeric excess of the meta- over the para-product ($RE = (c[meta] - c[para]) / (c[meta] + c[para]) * 100$).*

OMT enzyme	pre-screen		fermentation	
	RE mean	SD	RE mean	SD
HalOMT	n.d.	n.d.	n.d.	n.d.
SalOMT	n.d.	n.d.	n.d.	n.d.
MesMOMT	86.44	0.00	100.00	0.00
DesAOMT	32.09	7.01	93.87	0.76
MxOMT	-34.78	1.17	69.24	7.80
PhoAOMT	39.04	6.29	95.25	0.39
KibpOMT	46.40	5.92	93.13	2.88
StyLOMT	49.06	3.81	95.31	0.06
RetFOMT	88.04	9.95	100.00	0.00
StiAOMT	48.83	9.18	95.41	1.64
StrAOMT	62.38	9.45	97.27	2.41
OmnOMT	-2.96	3.41	84.65	2.58

591 Extended Data Figures



592
 593 Extended Data Figure 1: Sequence similarity network calculated with the EFI-EST webtool and visualized with the yFiles organic
 594 layout in Cytoscape with an edge cut-off at 50% sequence identity. Only nodes with an HMMsearch score of 70 or higher were
 595 included in the generation of the network. Enlarged nodes with label: enzymes tested in this study; Yellow box: enzymes with
 596 high sequence similarity to the bacterial input sequences, green box: enzymes with high sequence similarity to the plant input
 597 sequences; open black circle: inactive on tested substrates, filled symbol: active on caffeic acid, blue outline: active on catechol,
 598 rectangle: active on dopamine.



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600 *Extended Data Figure 2: Coomassie stained SDS PAGE of putative OMTs expressed in TXTL reactions. Orange arrows indicate*
 601 *protein bands suspected to represent the respective OMT. Lanes without obvious bands of the correct molecular weight are*
 602 *marked with X below.*