Towards a general approach for tailoring the hydrophobic binding site of phenylalanine ammonia-lyases

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Unnatural substituted amino acids play an important role as chiral building blocks, especially for pharmaceutical industry, where the synthesis of chiral biologically active molecules still represents an open challenge. Recently, modification of the hydrophobic binding pocket of phenylalanine ammonia-lyase from Petroselinum crispum (PcPAL) resulted in specifically tailored PcPAL variants, contributing to a rational design template for PAL-activity enhancements towards the differently substituted substrate analogues. Within this study we tested the general applicability of this rational design model in case of PALs, of different sources, such as from Arabidopsis thaliana (AtPAL) and Rhodosporidium toruloides (RtPAL). With some exceptions, the results support that the positions of substrate specificity modulating residues are conserved among PALs, thus the mutation with beneficial effect for PAL-activity enhancement can be predicted using the established rational design model. Accordingly, the study supports that tailoring PALs of different origins and different substrate scope, can be performed through a general method. Moreover, the fact that AtPAL variants I461V, L133A and L257V, all outperformed in terms of catalytic efficiency the corresponding, previously reported, highly efficient PcPAL variants, of identical catalytic site, suggests that not only catalytic site differences influence the PAL-activity, thus for the selection of the optimal PAL-biocatalysts for a targeted process, screening of PALs from different origins, should be included.

The current state of art of the PAL mediated biotransformations revealed several synthetically useful PALs of eukaryotic (plant and yeast) or bacterial origins1–4. Their substrate scope has been intensively studied within the last decade, with several PALs, such as those originary from Petroselinum crispum (PcPAL)2,5, Anabaena variabilis (AvPAL)7,8, Rhodotorula glutinis (RgPAL)9,10, Arabidopsis thaliana (AtPAL)11, Planctomyces brasiliensis (PbPAL)12, Kangiella koreensis (KkPAL)13, Pseudozyma antarctica (PzaPAL)14, shown to possess broad substrate scope. However, all these studies also revealed significant differences in their catalytic efficiencies towards specific substrates2,7,11,12,14. As example, PbPAL and the recently explored AL-11 PAL15 transformed substrates with electron-donor substituents, previously shown to be poor substrates for other PALs, such as PcPAL, AtPAL and AvPAL. Comparison of the catalytic sites of PALs of different origins shows a highly conserved polar substrate binding region responsible for the fixation of substrate’s carboxyl- and NH2- group (Fig. 1, Fig. S1), which also embeds the catalytically essential 3,5-dihydro-5-methylene-4H-imidazol-4-one MIO-group16,17. Besides, the residues of the polar binding region form an essential H-bond network18 (Fig. S2). The differences within the hydrophobic substrate-binding region of PALs (Fig. 1, Fig. S1), responsible for the facile active site accommodation of the substrate’s aromatic ring, supposedly contribute to the different substrate specificities observed among aromatic ammonia lyases14,15. Interestingly, several hydrophobic active site residues, such as those corresponding to I460 and L256 of PcPAL are highly conserved, while large diversification can be observed at positions homologue with 137 and 138 of PcPAL (Fig. 1, Fig. S1). These later residues are well-known for their substrate-specificity modulator effect, specific polar residues (e.g. histidine) at position corresponding to 137 of PcPAL provide also tyrosine ammonia-lyase (TAL) activity, such as in case of RgPAL and RtPAL from Rhodotorula sp., with reported TAL/PAL activities19. Histidine ammonia-lyases (HALs) show a characteristic His

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residue at position corresponding to 138 of PcPAL.\textsuperscript{19,21} Exploration and characterization of novel PALs from different origins is continuously expanding\textsuperscript{12–15} driven by the aim to find PALs of increased operational and/or thermostability, or of activity towards substrates hardly transformed by existing PALs.

Protein engineering efforts on PALs of different origins, such as Pc\textsuperscript{PAL}\textsuperscript{2,6,18,22}, Av\textsuperscript{PAL}\textsuperscript{7,23}, Rg\textsuperscript{PAL}\textsuperscript{24}, Pb\textsuperscript{PAL}\textsuperscript{12}, AL-1\textsuperscript{15}, also focused to provide variants of expanded substrate scope or increased operational and thermal stability. Diversification at positions 137 and 138 has been performed at several PALs, such as Pc\textsuperscript{PAL}\textsuperscript{2,6,18}, Av\textsuperscript{PAL}\textsuperscript{7,8,23}, Rt\textsuperscript{PAL}\textsuperscript{24} and Pb\textsuperscript{PAL}\textsuperscript{12}, and provided variants of improved catalytic performance, with modifications especially at analogue positions of 137 of Pc\textsuperscript{PAL}, highlighting the substrate specificity modulator effect of this residue. Our recent mutational analysis of the hydrophobic binding pocket of Pc\textsuperscript{PAL}, revealed other specificity modulator active site residues and depicted their specific interaction with the differently positioned \textit{ortho}-, \textit{meta}-, \textit{para}-substituents of the non-natural substrates, thus providing excellent tool for the rational protein engineering of Pc\textsuperscript{PAL}. Considering the differences in the hydrophobic substrate binding pocket of PALs of different origins, that strongly influence their substrate scope, the general validity of our recently developed rational engineering strategy among PALs of diverse origins, hence of diverse substrate scope, should be assessed. This might provide a desirable general rational design strategy among PALs, allowing facile development of substrate-tailored PALs of various origins. Accordingly, we tested whether the mutational strategy developed for Pc\textsuperscript{PAL} applies to other PALs, such as At\textsuperscript{PAL} and Rf\textsuperscript{PAL}, of different sequence similarities to Pc\textsuperscript{PAL}. At\textsuperscript{PAL} possessing high, 81% sequence identity and identical catalytic site with Pc\textsuperscript{PAL}, while Rf\textsuperscript{PAL} shares lower, 38% sequence identity with Pc\textsuperscript{PAL}, and contains specific ‘TAL-activity provider’ His and Gln residues at positions analogue to 137 and 138 of Pc\textsuperscript{PAL} (Fig. 1). Notable, that \textit{wild-type} At\textsuperscript{PAL} and Pc\textsuperscript{PAL} show very similar catalytic efficiencies (k\textsubscript{cat}), while in comparison Rf\textsuperscript{PAL} shows ~ twofold increased k\textsubscript{cat} values, but lower specificity constants (k\textsubscript{cat}/K\textsubscript{M}) in the natural PAL-reaction (deamination of L-Phe) and also within the reverse ammonia addition route of trans-cinnamic acid\textsuperscript{11}.

![Figure 1. Catalytic site of Pc\textsuperscript{PAL}, with key residues from the hydrophobic substrate-binding region marked based on their proximity to the differently positioned (\textit{ortho}-blue, \textit{meta}-orange and \textit{para}-green) aromatic substituents of the substrates and the homologue active site residues in Rf\textsuperscript{PAL} (P11544) and At\textsuperscript{PAL} (P45724) based on sequence alignments with Pc\textsuperscript{PAL} (P24481) (right Table) with colour-marked residues subjected to mutagenesis, generating the focused PAL variant-library (bottom Table).](image-url)
Results and discussion

Mutant library generation and enzyme activity screens. Recent mapping of the hydrophobic binding pocket of PcPAL, revealed mutant variants, obtained by mutagenesis of residues L256, L134, F137 and I460, of enhanced catalytic activity to ortho-, meta-, para- substituted substrates, respectively. Accordingly, the homologues of all four substrate specificity modulating residues were similarly replaced in AtpAL and RtPAL (Fig. 1), initially obtaining four AtpAL variants (L257V, L133A, F136V and I461V) and four RtPAL variants (L266V, L134A, H137V and I472V). Notable, that residue H137 of RtPAL, homologue of F137 of PcPAL, is most probably involved in H-bonding with Q138, homologue of L138 of PcPAL. Since H137V RtPAL variant showed reduced activity within the activity screens, we presumed that the presence of residue Q138 within a complete hydrophobic active site environment is non-favourable. Thus, the mutational strategy was adapted, and within the ‘PcPAL-like’ RtPAL variants, besides the specific mutation of residues involved in aromatic substituent accommodation, additional mutations H137F and Q138L have also been included. Thus, RtPAL variant H137F/Q138L resembles the catalytic site of wt-PcPAL, variants H137F/Q138L/L266V and H137F/Q138L/I472V being homologues to L256V and I460V PcPAL, respectively, and variant H137V/Q138L RtPAL to F137V PcPAL variant. Despite our efforts, including different PCR protocols for mutagenesis, RtPAL variant L134A/H137F/Q138L resembling the catalytic site of L134A PcPAL could not be obtained, thus only L134A RtPAL was employed within the activity tests. The slight variations in thermal unfolding temperatures (Tm) of the purified enzyme variants indicated that mutations did not affect the protein folding (Figs. S3, S4 and Table S2), the only significant modification being observed in case of variant H137F/Q138L/I472V RtPAL, with Tm value decreased with ~6 °C compared to the wild-type RtPAL.

Activity assessments of the PcPAL, AtpAL and RtPAL variant library. The activities of the obtained RtPAL and AtpAL variants were assessed and compared with those of the corresponding PcPAL variants within the deamination and amination reactions of phenylalanines rac-1a–l and cinnamic acids 2a–l, monosubstituted at ortho-, meta-, para- positions of their aromatic ring.

Activity assessments for ortho-substituted substrates. Generally, within the whole-cell mediated biotransformations of ortho-substituted substrates 1,2a–d, mutations L257V, L133A in case of AtpAL and mutation L266V in case of RtPAL provided similar enhancement of the conversion-based enzyme activity, relatively to their wild-type variants, as the one reported for L256V PcPAL1 (Table 1). Additional general tendency can be observed among the results obtained with wild-type PALs, AtpAL outperforming in terms of conversion the corresponding PcPAL, while wt-RtPAL provided the lower conversions in both reaction routes (Table 1).

More detailed, in case of o-Br-substituted substrates excellent, equilibrium-approaching conversions are obtained with the wild-type PcPAL (87% for 2a and 42% for 1a after 6 h and 24 h reaction times) and AtpAL (94% for 2a after 1 h reaction time and 48% for 1a after 6 h reaction time), thus the increased catalytic efficiency of variants L256V PcPAL and L257V AtpAL is less reflected within the conversion-based enzyme activities. However, the 2.3- and 2.1-fold increased kcat values, comparatively to the wt-variant’s (Table 1), support the beneficial effect of the mutations. Similar behaviour can be observed in case of substrates 1b and 2b, with high conversions of similar range being registered for both wt- or mutant variants of Pc/PcPAL, but 3.5- and 5.5-fold increased catalytic efficiencies (kcat) of the corresponding L256V and L257V variants. AtpALs provided stationery conversions of ~86% for 2b and ~50% for rac-1b within significantly shorter reaction times of 3 h and 30 min, respectively, in comparison with similar conversions obtained only after 24 h reaction times using Pc/PcPALs. Interestingly, while in case of 1a the Km value was not significantly altered upon mutations analogue to L256V, in case of 1b...
the mutation resulted in highly decreased substrate affinity (increased $K_M$ values) for all three PALs of different origin, supporting a more relaxed accommodation of $1b$ within the modified active site.

### Table 1. Activity assessment of the different PAL variants within the ammonia addition and ammonia elimination reactions of ortho-substituted cinnamic acids $2a$–$d$ and rac-phenylalanines $1a$–$d$, respectively.

| Subst. | Enzyme | Variant | Conversion -% (Reaction time - h) | $K_M$ (μM) | $k_{cat}$ (s$^{-1}$) | Conversion -% (Reaction time - h) |
|--------|--------|---------|----------------------------------|------------|-----------------|----------------------------------|
| $\alpha$-Br 1a, 2a | $P_c$-PAL | wt | 86.6 (6 h) | 95.7 (24 h) | 95.7 (24 h) | 153 | 0.157 | 31.9 (6 h) | 42.4 (24 h) |
|       |        | L256V | 89.6 (6 h) | 95.7 (24 h) | 92.7 (6 h) | 199 | 0.21 | 36.7 (3 h) | 47.6 (6 h) |
| $\alpha$-CF$_3$ 1b, 2b | $R_t$-PAL | wt | 6.3 (3 h) | 34.8 (24 h) | 49.2 (3 h) | 51.0 (0.5 h) | 0.177 | 50.6 (0.5 h) | 42.4 (24 h) |
|       |        | H137F/Q138L/L266V | 41.4 (0.5 h) | 86.3 (24 h) | 75.4 (3 h) | 240 | 0.032 | 37.1 (0.5 h) | 49.7 (24 h) |
| $\alpha$-OCH$_3$ 1c, 2c | $P_c$-PAL | wt | 5.5 (24 h) | n.d. | n.d. | 36.5 (24 h) | 8.9 (24 h) | 0.108 | 15.6 (24 h) | 43.9 (6 h) |
|       |        | L134A | 40.8 (24 h) | n.d. | n.d. | 1254 | 0.18 | 15.6 (24 h) | 43.9 (6 h) |
|       |        | L256V | 7.5 (24 h) | - | - | - | - | - | - |
| $\alpha$-CH$_3$ 1d, 2d | $P_c$-PAL | wt | 30.4 (3 h) | 78.5 (24 h) | 55.3 (3 h) | 60.7 (3 h) | 86.6 (24 h) | 96 | 0.115 | 20.7 (6 h) | 47.7 (24 h) |

Figure 3. 2-Methoxycinnamic acid $2c$ docked into the active site of: (A) wt-$R_t$PAL (green, $-4.2$ kcal/mol), L134A $R_t$PAL (purple, $-6.9$ kcal/mol), and H137F/Q138L/L266V $R_t$PAL (grey, $-8$ kcal/mol); (B) wt-$P_c$PAL (green, $-7.3$ kcal/mol) and L134A $P_c$PAL (purple, $-7.7$ kcal/mol). Steric clashes between the ortho-methoxy group and side chains of residues L266 and L134, respectively, are highlighted with red dashed lines. The modified residues and the active site orientation of $2c$ within the corresponding PAL variant is marked with similar colour.

the mutation resulted in highly decreased substrate affinity (increased $K_M$ values) for all three PALs of different origin, supporting a more relaxed accommodation of $1b$ within the modified active site. Wild-type $R_t$PAL in case
### Table 2. Activity assessment of the different PAL variants within the ammonia addition and ammonia elimination reactions of meta-substituted cinnamic acids 2e–h and rac-phenylalanines 1e–h, respectively. n.d. not determinable, during enzyme kinetics the non-linear range of the Michaelis–Menten curve was not obtained using substrate concentration allowed by the solubility of the tested compounds. n.a. no activity detected. "–" no determination/measurement was performed. ⁶During the ammonia additions the enantiomeric excess of the obtained l-phenylalanine analogues was also monitored, in all cases ee > 99% have been obtained. *Except in case of the l-1e (ee = 93.6%) produced within the ammonia addition reaction of 2e catalyzed by L133A AtPAL.

| Subst. | Enzyme | Variant | Conversion - % (Reaction time - h) | Kₘ (μM) | kcat (s⁻¹) | Conversion - % (Reaction time - h) |
|--------|--------|---------|----------------------------------|--------|-----------|----------------------------------|
| m-Br   | Pc/PAL | wt 1e, 2e | 31.8 (6 h) | 153 | 0.095 | 31.8 (6 h) |
|        |        | 1460V   | 51.7 (3 h) | 51.4 | 0.154 | 30.4 (6 h) |
|        |        | 1L33A   | 51.0 (4.5 h) | 51.4 | 0.154 | 37.2 (6 h) |
|        | At/PAL | 1e4161 | 24.2 (0.5 h) | 76.5 (0.5 h) | 76.5 (0.5 h) | n.d. |
|        |        | 1L33A   | 65.6 (6 h) | 363 | 0.343 | 40.0 (3 h) |
|        | R/PAL  | 1e472V | 11.7 (6 h) | 11.7 (6 h) | 87.3 (1 h) | 87.3 (1 h) |
|        |        | 1L33A   | 11.7 (6 h) | 11.7 (6 h) | 39.1 (24 h) | 39.1 (24 h) |
|        |        | H137F/Q138L/I472V | 73.3 (3 h) | 73.3 (3 h) | 88.2 (6 h) | 88.2 (6 h) |
|        |        |         | <1 (6 h) | 4.1 (4.2 h) | n.d. |

Activity assessments for meta-substituted substrates. Interestingly, in case of phenylalanine/cinnamic acid analogues with substituents in meta-position 1e, 1f, 1h and 2e, 2f, 2h, the wild-type variant R/PAL showed superior catalytic efficiency in comparison to wild-type Pc/PAL and At/PAL, supported by its higher kcat values within the ammonia eliminations of 1e, 1f, 1h or conversion values in both reaction routes of m-CF₃- and m-Me-substituted substrates. Of both substrates 2a, 2b provided only moderate conversion of 30% (6 h) and 35% (24 h), respectively, thus the beneficial effect of mutation L266V was clearly visible also within the conversion-based enzyme activity, with 94% (6 h) and 82% (24 h) conversion for 2a and 2b, respectively. In case of o-OCH₃-, substituted substrates 1c, 2c, the beneficial effect of mutations analogue with L134A from Pc/PAL was also observed in case of At/PAL, where the corresponding L133A variant provided high conversions of 95% and 3.1-fold increased kₘ values. In case of L134A, but also wild-type R/PAL, very low/no conversions of < 1–3% were detected, while enzyme kinetics also revealed low initial velocities and substrate affinities. Interestingly, in this case the "Pc/PAL-like" R/PAL L266V variant, with mutations H137F/Q138L/I472V provided increased conversions of 19% for 1c and 17% for 2c after 16 h reaction time. Indeed, in this particular case, due to the bend caused by the oxygen atom of the o-OCH₃ substituent, the methyl group positions between residues 134 and 266 (Fig. 3A,B), while the increased hydrophobicity induced by mutation H137F and Q138L most probably facilitates the accommodation of the substrate’s aromatic moiety. In accordance with the experimental results, both flexible and rigid docking of 2c within the active sites of wild-type, L134A and H137F/Q138L/L266V R/PAL revealed substrate orientations of significantly lower energy for both mutant variants in comparison with those obtained for the wild-type R/PAL (Fig. 3A). In case of o-CH₃-, substituted substrates 1d, 2d the wild-type variants of all three PALs provided high conversion in both reaction routes, the best performer At/PAL reaching in shortest reaction time of 3 h 83% conversion of 2a. Similarly to the case of the o-Br-substituted substrates 1a, 2a, the increased catalytic efficiency of the variants bearing mutations analogue to L256V of Pc/PAL is supported by their increased kₘ values in comparison to their wild-type variants. The less significant, only 1.1–1.3-fold increase in kₘ values, than in case of 1a–c, is expectable based on the smallest sterical requirement of the methyl group, which seemingly, when ortho-positioned on the substrate, is favourably accommodated within the active site of all PAL variants.
Activity assessments for para-substituted substrates. In case of para-substituted phenylalanines 1j, 1k, 1l and cinnamic acids 2i–2l very low (<10%) or no conversion was detected when using wild-type Pc/PAL and Rp/PAL variants, in accordance with the reported steric clashes between the p-substituent and active site residues. Interestingly, wt-Rt/PAL afforded close to maximum conversion of all para-substituted phenylalanines, except for p-OCH₃-phenylalanine, where similarly to Pc/PALs, low conversion of 14% and kₐ value of 0.007 s⁻₁ were obtained within the ammonia elimination of rac-1k and no conversion within the ammonia addition to 2k.

Related to the effect of the mutational strategy, we observed that in case of At/PAL variants I461V and F136V, provided important conversion and activity enhancements for all substrates 1i–2l. Accordingly, while in case of p-Br- and p-CF₃-substituted substrates the mutation-induced increase in the conversions is less significant, due to the well-performing wild-type variant, the 2.9-fold and 3.4-fold increased kₐ values of variant I461V for 1i and 1j support the beneficial effect of the mutation. In case of substrates 1k, 1l and 2k, 2l, p-substituted with the electron-donating -OCH₃ and -CH₃ groups, the superior catalytic efficiency of I461V variant to wt-At/PAL is also resembled within the highly increased conversion values. While mutation F136V of At/PAL also induced significant increase in the conversions of all substrates, in case of substrates 1i, 1j and 2i, 2j even surpassing the conversions registered with I461V variant, however the enantiomeric excess (ee) of the l-phenylalanines 1i, 1j and 1k produced within the ammonia additions, were of lower value (ee of 92%, 83% and 97%, respectively) in comparison with the highly enantiopure forms (ee > 99%) produced by

**Figure 4.** Active orientations of meta-substituted substrates m-Br-cinnamic acid 2e (cyan), m-CF₃-cinnamic acid 2f (magenta), m-OCH₃-cinnamic acid 2g (green) and m-CH₃-cinnamic acid 2h (orange) within (A) wt-Rt/PAL and (B) wt-Pc/PAL.
Table 3. Activity assessment of the different PAL variants within the ammonia addition and ammonia elimination reactions of para-substituted cinnamic acids 2i–1 and rac-phenylalanines 1i–1, respectively. n.d. not determinable, during enzyme kinetics the non-linear range of the Michaelis–Menten curve was not obtained using substrate concentration allowed by the solubility of the tested compounds; n.a. no activity detected; *; ** no determination/measurement was performed. *During the ammonia additions the enantiomeric excess of the obtained l-phenylalanine analogues was also monitored, in all cases ee > 99% have been obtained, with marked exceptions. *1. In case of 2i: PcPAL F137V variant provided l-1i with ee = 93%; in case of 1a: H137F/Q138L/I472V PAL variant provided l-1i with ee = 97%; in case of 1b: H137F/Q138L/I472V PAL variant provided l-1j with ee = 82%; **During the kinetic resolution-type ammonia eliminations in case of high enantioselectivity the maximal conversion values of rac-phenylalanines is 50%, conversions exceeding this value, support the low enantioselectivity of the process.

| Subst. | Enzyme | Variant | Conversion - % (Reaction time - h) | K<sub>v</sub> (µM) | k<sub>catalytic</sub> (s<sup>-1</sup>) | Conversion - % (Reaction time - h) |
|--------|--------|---------|-----------------------------------|----------------|--------------------------------|----------------------------------|
|        |        |         | k<sub>cat</sub> (s<sup>-1</sup>) |                  |                             |                                   |
| 4-nitrophenol |        |         |                                  |                  |                             |                                   |
| p-Br    | 1–2 | wt       | 7.1 (24 h) | 269               | 0.165             | 49.9 (24 h) |
|         |     | I460V    | 58.4 (24 h) | 71               | 0.259             | 51.5 (24 h) |
|         |     | F137V    | 71.6 (24 h)* | -             | -                 | 59.8 (24 h)** |
|         |     | 1466V    | 84.7 (16 h) | 73               | 0.085             | 51.5 (6 h)  |
|         |     | F136V    | 90.5 (16 h) | 61               | 0.245             | 53.4 (6 h)  |
|         |     | -        | 89.4 (16 h)* | -             | -                 | 61.8 (6 h)** |
|         |     | H1377    | <1 (16 h) | 435 | 0.005 | <1 (16 h) |
|         |     | H1377    | <1 (16 h) | 2120 | 0.008 | <1 (16 h) |
|         |     | H1377    | 18.5 (16 h) | -             | -                 | 23.1 (6 h)  |
|         |     | H1377    | <1 (16 h) | n.a. | n.a. | <1 (16 h) |
|         |     | H1377    | 84.3 (16 h) | n.a. | n.a. | 50.3 (6 h)  |
| 3,4-dinitrophenol | 1–2 | wt       | 12.7 (24 h) | 2490 | 0.25 | 9.3 (24 h) |
|         |     | I460V    | 32.0 (6 h) 62.5 (24 h) | 901 | 0.55 | 37.3 (24 h) |
|         |     | F137V    | 45.9 (16 h) 65.3 (24 h)* | 151 | 0.42 | 52.9 (24 h)** |
| 3-iodophenol | 1–2 | wt       | 48.9 (16 h) | 1467 | 0.127 | 40.0 (6 h) |
|         |     | I461V    | 36.1 (6 h) 86.1 (16 h) | 275 | 0.428 | 50.7 (6 h) |
|         |     | F136V    | 92.8 (6 h) 93.8 (16 h)* | - | - | 80.8 (6 h)** |
|         |     | H137V    | <1 (16 h) | 6381 | 0.004 | <1 (16 h) |
|         |     | H137V    | <1 (16 h) | n.a. | n.a. | <1 (16 h) |
|         |     | H137V    | 10.5 (16 h) | 3231 | 0.032 | 7.9 (16 h) |
|         |     | H137V    | <1 (16 h) | n.a. | n.a. | <1 (16 h) |
|         |     | H137V    | 82.8 (16 h) | n.a. | n.a. | 50.9 (16 h) |
| 3-chlorophenol | 1–2 | wt       | <1 (24 h) | 1858 | 0.009 | <1 (24 h) |
|         |     | I460V    | 11.8 (24 h) | 265 | 0.103 | 17.0 (24 h) |
|         |     | F137V    | 4.1 (24 h) | - | - | 15.4 (24 h) |
|         |     | H137V    | <1 (24 h) | 1048 | 0.007 | 14.1 (16 h) |
|         |     | I461V    | 24.5 (24 h) | 132 | 0.112 | 42.0 (16 h) |
|         |     | F136V    | 23.7 (24 h)* | - | - | 38.4 (16 h) |
| 4-chlorophenol | 1–2 | wt       | <1 (16 h) | 1.7 (6 h) 4.0 (24 h) | 208 | 0.026 | 6.0 (10 h) |
|         |     | I460V    | 13.5 (6 h) 34.1 (24 h) | 107 | 0.092 | 10.7 (10 h) |
|         |     | F137V    | 16.6 (24 h) | - | - | 22.1 (10 h) |
|         |     | H137V    | <1 (16 h) | 3.8 (6 h) 29.6 (24 h) | 191 | 0.018 | 50.0 (16 h) |
|         |     | I461V    | 38.6 (6 h) 54.6 (24 h) | 144 | 0.1 | 50.1 (16 h) |
|         |     | F136V    | 29.2 (6 h) 33.3 (24 h) | - | - | 36.2 (16 h) |
| 4-bromophenol | 1–2 | wt       | <1 (16 h) | 1.7 (6 h) | 9902 | 0.003 | <1 (16 h) |
|         |     | I460V    | <1 (16 h) | n.a. | n.a. | <1 (16 h) |
|         |     | F137V    | <1 (16 h) | 7080 | 0.003 | <1 (16 h) |
|         |     | H137V    | <1 (16 h) | n.a. | n.a. | <1 (16 h) |
|         |     | H137V    | <1 (16 h) | n.a. | n.a. | <1 (16 h) |
|         |     | H137V    | <1 (16 h) | n.a. | n.a. | <1 (16 h) |
|         |     | H137V    | <1 (16 h) | n.a. | n.a. | <1 (16 h) |

This is in accordance with the results from PcPAL, where mutation F137V also decreased the enantioselectivity of the enzyme with ee values of 97% and 82% being obtained for l-1i and l-1j, respectively.

RtPAL, in general, proved to be inefficient for the transformation of para-substituted amino acids, while the destabilization effect of mutation residue I472V, as described in case of meta-substituted substrates, resulted in no detectable activity. Instead, the mutation H137V, provided minor to moderate conversion increase of 7.9–23.1%, in case of p-Br- and p-CF<sub>3</sub>-substituted substrates 1i, 1j and 2i, 2j, where the beneficial effect of the mutation is also supported by the significantly increased k<sub>catalytic</sub> values. The lower catalytic efficiency of H137V RtPAL, reflected in significantly lower conversions compared to its homologue variants F136V, H137V PcPAL, might result from the presence of polar Q138 residue in the proximity of the hydrophobic, mutated V137 residue (Fig. 5), supported by the increased conversions provided by the ‘Pc-PAL-like′ H137V/Q138L RtPAL, approximating the conversions registered with the homologue At/Pc-PAL variants.

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Considering the above described conservation of the catalytic efficiency-enhancing effect (Fig. 6) of the mutational strategy developed for PcPAL, in case of AtPAL (81% sequence identity and identical catalytic site with PcPAL) and RtPAL (38% sequence identity and TAL-activity providing catalytic site, containing H137, Q138 residues at positions analogue to F137, L138 of PcPAL) the general applicability of the rational design strategy among PALs is supported. Notable, that in case of RtPAL, besides the modification of the substrate specificity-modulator residues, replacement of residue Q138, in proximity of position 137, to hydrophobic residues, further enhanced the catalytic properties of H137V RtPAL, supporting that the mutational strategy is adaptable for further additional mutations based on simple rational considerations, allowing facile development of substrate-tailored PALs of various origins. Despite the identical catalytic site residues of AtPAL and PcPAL, in several cases AtPAL variants in comparison with the corresponding PcPAL variants, showed higher catalytic efficiencies/ conversions (Fig. 6), highlighting that besides active site residues, other structural elements also determine the different enzyme activities/substrate specificities of PALs of different origins. Besides, the mutational approach revealed several PAL variants, such as L133A AtPAL, I460V AtPAL, L266V and H137V/Q138L RtPAL, which in comparison with their previously reported2 PcPAL homologues, possess enhanced catalytic efficiency within the various ammonia additions producing valuable L-phenylalanines (Fig. 6).

**Experimental part**

**Site-directed mutagenesis.** The codon optimized genes encoding PALs from *Arabidopsis thaliana* and *Rhodosporidium toruloides* were obtained through the synthesis services of GenScript, followed by their cloning into pET19b vector (using XhoI and 8pu1102I cloning sites for RtPAL and XhoI and NdeI cloning sites for AtPAL). The site-directed mutagenesis was performed following the protocol described by Naismith and...
37°C, 200 rpm until OD₆₀₀ reached 0.6–0.8 (approx. 3 h), when enzyme production was induced via the addition at 200 rpm. 2% (v/v) of the overnight culture was used to inoculate 50 mL LB medium. Cultures were grown at CR-I (+) chiral column (150 × 3 mm; 5 µm) and HClO₄ (pH = 1.5)/acetonitrile as mobile phase at a flow rate 1.0 mL/min. The enantiomeric excess values were determined by chiral HPLC separations, using Crownpak the pET-19b vector carrying the *E. coli* Rosetta (DE3) pLysS cells harboring the pET-19b vector carrying the wt- or mutant *atpal* gene. 2% (v/v) from the starter culture was used to inoculate 2 × 500 mL LB medium in 2 L flasks. The OD₆₀₀ was monitored and when a value of 0.45 was reached, the temperature was lowered from 37 to 25 °C, and the shaking continued till an OD₆₀₀ value of 0.6–0.8, when PAL expression was induced via IPTG (0.5 mM final concentration). The cell growth continued at 25 °C, 200 rpm for another 6 h, when cells were harvested by centrifugation at 4000 rpm (1751 × g), 4 °C for 20 min. The supernatant was discarded and the cell pellet was stored at −20 °C until further use or processed immediately using the optimized protein isolation protocol as described for *Pc* PAL²⁵.

### Thermal unfolding profile of purified proteins

The thermal unfolding of all PALs was determined by nanoscale differential scanning fluorimetry measurements, using Prometheus NT.48 nanoDSF instrument (NanoTemper Technologies, München, Germany). PAL variant libraries were diluted with 20 mM Tris, 120 mM NaCl pH 8.8 buffer to a final concentration of 1 mg/mL. 10 µL of each sample were loaded into UV capillaries (NanoTemper Technologies) and unfolding of PAL enzymes was detected during heating in a linear thermal ramp of 1.5 °C/min between 20 and 95 °C, with an excitation power of 70%. Data analysis was performed using NT Melting Control software and melting temperature (T_m) was determined by fitting the experimental data using a polynomial function, in which the maximum slope is indicated by the peak of its first derivative (F350/F330). All measurements were performed in triplicate (Figs. S3, S4 and Table S2).

### Preparation of whole-cell PAL biocatalysts

The overnight precultures were prepared in 20 mL LB (Luria Bertani) medium supplemented with carbenicillin (50 µg/mL) and chloramphenicol (30 µg/mL) in 100 mL Erlenmeyer flasks, being inoculated with glycerol stocks of *E. coli* Rosetta (DE3) pLysS cells harboring the pET-19b vector carrying the wt or mutant *atpal* or *rtpal* gene, followed by incubation at 37 °C and shaking at 200 rpm. 2% (v/v) of the overnight culture was used to inoculate 50 mL LB medium. Cultures were grown at 37 °C, 200 rpm until OD₆₀₀ reached 0.6–0.8 (approx. 3 h), when enzyme production was induced via the addition of 0.5 mM IPTG (final concentration), and the cell growth was maintained at 20 °C, 200 rpm, overnight (approx. 17 h). The final OD₆₀₀ was measured for each mutant variant and wild-type PAL. The culture volumes required for the biotransformation screenings were harvested by centrifugation in 1.5 mL polypropylene tubes for 10 min at 13,300 rpm (12,000 × g). The required volume of bacterial culture, providing the amount of whole-cell pellet needed was calculated considering the volume of the reactions, the whole-cell biocatalysts concentration (with fixed cell density OD₆₀₀ of ~2), and the final OD₆₀₀ value of the induced cells. The harvested cells were washed with 500 µL PBS buffer (20 mM phosphate, 150 mM NaCl, pH 8.0) (13,300 rpm, 12,000 × g, 10 min) and stored at −20 °C until further use.

### Analytical scale ammonia addition and elimination reactions

The bacterial pellet of PAL-biocatalysts (prepared as described above, in 1.5 mL polypropylene tubes) was resuspended to an OD₆₀₀ of ~2, in 500 µL substrate solution (2 mM cinnamic acids 2a–l or 2 mM racemic amino acids rac-1a–l) prepared in 6 M NH₄OH buffer pH 10 adjusted with CO₂ (in case of ammonia addition) or 20 mM Tris.HCl, 120 mM NaCl buffer, pH 8.8 (in case of ammonia elimination). The reaction mixtures were incubated at 30 °C, 250 rpm. Reaction samples were taken after 3, 6, 16, and 24 h and quenched by adding an equal volume of MeOH, vortexed and centrifuged (13,400 rpm, 12,000 × g, 10 min). The supernatant was filtered through a 0.22 µm nylon membrane filter prior to analysis by HPLC. In order to determine the conversions values, a Gemini NX-C18 column (150 × 4.5 mm; 5 µm) was chosen, using as mobile phase: A: NH₄OH buffer (0.1 M, pH 9.0)/B: MeOH, with a flow rate of 1.0 mL/min. The enantiomeric excess values were determined by chiral HPLC separations, using Crownpak CR-I (+) chiral column (150 × 3 mm; 5 µm) and HClO₄ (pH = 1.5)/acetonitrile as mobile phase at a flow rate of 0.4 mL/min. HPLC methods and response factors used for the conversion value determinations, as well as retention times of the enantiomers of rac-1a–l can be consulted in our previous reports²⁵. All analytical scale biotransformations were performed in duplicates, while during the initial activity screens using a significantly sized reaction-subset the HPLC analysis have been performed for all samples within the duplicate set (see details in Supporting information, Chapter 6, Table S3).

### Enzyme kinetics

The initial enzyme activities were spectrophotometrically determined, using a Tecan Infinite Spark 10 M microplate reader and Corning 96-well Clear Flat Bottom UV-Transparent microplates. The kinetic measurements were performed in triplicate at 30 °C by monitoring the production of trans-cinnamic acid analogues 2a–l at 290 nm (wavelength where the corresponding amino acids rac-1a–l showed no absorption), using substrate concentrations of 0.1–20 mM of 1a–l, 100 mM Tris.HCl, 120 mM NaCl (pH 8.8) as buffer.
and purified PAL variants at fixed enzyme concentration of 0.322 μM. Kinetic constants (K<sub>Michaelis</sub>, v<sub>max</sub>) were obtained from the Michaelis–Menten curves by non-linear fitting. Standard deviations for the determined kinetic parameters are given within Tables S4–S6 (Supporting information).

**Computational studies.** The ground state geometries of the monosubstituted cinnamic acid derivatives 2a–l were obtained by calculations based on the density functional theory, performed using the Gaussian 09 software<sup>34</sup> by employing the B3LYP density functional and the 6-31G(d,p) basis set. Geometry optimizations were carried out in a water solvated environment using the Polarizable Continuum Model (PCM)<sup>35</sup>.

The molecular docking calculations were performed with the Autodock Vina software<sup>30</sup>, using flexible-ligand and rigid-receptor docking. The search space was defined by embedding the binding site residues and the M10 prosthetic group. In both cases the receptor grid was defined as a cubic box with the dimension of 20 Å × 20 Å × 20 Å. The exhaustiveness search parameter of Vina was increased to 100.

The crystal structure of P<sub>c</sub>PAL was retrieved from Protein Data Bank entry 6FGT<sup>35</sup>, whereas in case of R<sub>i</sub>PAL, the AlphaFold<sup>32</sup> predicted model was retrieved from the UniProt database (entry P11544)<sup>33</sup>. The assembled tetrameric structure was submitted for minimization using the YASARA web server<sup>34</sup>. Although crystal structures of R<sub>i</sub>PAL are available, PDB entries 1T6J and 1Y2M, both structures present the open conformation of the protein, missing the loop containing the Y110 residue, responsible for the catalytic site closure upon substrate binding.

**Conclusions**

Within this study we tested the applicability of the mutational strategy developed for PAL from Petroselinum crispum to other PALs with the aim to provide a general rational design strategy, highly desirable for developing substrate-tailored PALs of diverse substrate scope and origins. Accordingly, A<sub>i</sub>PAL and R<sub>i</sub>PAL, both well-characterized PAL representatives, that share different sequence identity (high degree of 81%, respectively low degree of 38%) to P<sub>c</sub>PAL, with R<sub>i</sub>PAL known to possess dual PAL/TAL-activity, were selected for this purpose. As expected, wild-type R<sub>i</sub>PAL with low sequence identity to P<sub>c</sub>PAL, showed different substrate specificity towards the substrate library, revealing its higher catalytic efficiency towards meta-substituted substrates in both ammonia elimination and ammonia addition reaction routes, while the substrate specificities of wt-P<sub>c</sub>/A<sub>i</sub>PAL have been found very similar. However, the enzyme activity tests of the generated focused A<sub>i</sub>PAL, R<sub>i</sub>PAL, P<sub>c</sub>PAL mutant library towards the mono-substituted substrates revealed that A<sub>i</sub>PAL variants, with some exceptions, surpassed in terms of conversion and catalytic efficiency the corresponding previously reported P<sub>c</sub>PAL homologues (L134A, L256V, F137V and I460V). Since their active sites possess identical residues, the results highlight that besides active site residues, other structural elements also determine the different enzyme activities/substrate specificities of PALs. Furthermore, the activity of PAL variants tailored towards substrates of different (ortho-, meta-, para-) substitution pattern, revealed that the mutational approach is applicable among different PALs, resulting the expected catalytic efficiency increase towards the targeted non-natural substrates, with minor sequence alignment-based rational refinements further improving its efficacy. Accordingly, in case of R<sub>i</sub>PAL, besides the modification of the substrate specificity modulator residues L266V, L134A, F137V and I472V, replacement of residue Q138, in proximity of mutated position 137, to hydrophobic residues, further enhanced the catalytic properties of R<sub>i</sub>PAL variants. In this context, the study paves the way and contributes for the development of the general rational design strategy among the PAL (E.C. 4.3.1.24) and PAL/TAL families (E.C. 4.3.1.25).

**Data availability**

The Uniprot identifiers of all protein sequences used within the alignments and experimental work and the Protein Data Bank (PDB) IDs for the protein structures used within the computational part are described within the manuscript, while other datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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Author contributions
S.D.T. and M.E.M. contributed to the work equally. S.D.T. was responsible for the preparation of whole-cell-biocatalysts, analytical scale biotransformations and their HPLC monitoring. M.E.M. was responsible for enzyme kinetic measurements. Isolation and purification of PAL variants, mutant library generation was performed by S.D.T., M.E.M. and A.F, while L.C. was involved in substrate synthesis and biotransformation-monitoring by HPLC. L.C.N. performed the computational studies and was responsible for the graphical artworks. L.C.B. conceived the project and was responsible for funding, supervised all experiments, data and wrote the paper together with F.D.I, S.D.T., M.E.M. All authors reviewed the manuscript.

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Competing interests
The authors declare no competing interests.

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