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COMMUNICATION

Catechol Oxidase versus Tyrosinase Classification Revisited by Site-Directed Mutagenesis Studies

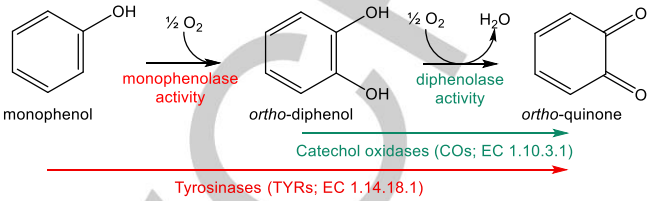
Sarah M. Prexler[a], Martin Frassek[a], Bruno M. Moerschbacher*[a] and Mareike E. Dirks-Hofmeister[b]

Abstract: Catechol oxidases (COs) and tyrosinases (TYRs) are both polyphenol oxidases (PPOs) that catalyze the oxidation of *ortho*-diphenols to the corresponding quinones. By the official classification, only TYRs can also catalyze the preceding hydroxylation of monophenols to *ortho*-diphenols. As both enzymes cause undesired browning of damaged fruits and vegetables and are of significant interest for biotechnological applications, researchers have been trying to find the molecular reason for the mono-/diphenolase specificity for decades. However, the much-discussed hypotheses for the lack of monophenolase activity of plant COs are almost exclusively based on crystal structures so far, lacking experimental evidence. To experimentally test these hypotheses, we used dandelion PPOs offering high phylogenetic diversity to perform site-directed mutagenesis studies. Our experimental and phylogenetic analyses refute the crystal structure-based hypotheses. We found that while plant PPOs of phylogenetic group 2 solely exhibit diphenolase activity, plant PPOs of phylogenetic group 1 unexpectedly also show monophenolase activity. This finding sheds new light upon the long-discussed molecular basis for mono-/diphenol substrate specificity and challenges the current practice of naming plant PPOs, as is the rule, COs.

Catechol oxidases (COs; EC 1.10.3.1) and tyrosinases (TYRs; EC 1.14.18.1) are ubiquitously distributed enzymes that are e.g. responsible for the undesired browning of damaged fruits and vegetables. Although they are of significant interest for biotechnological applications, the molecular basis for their different activities - either only on *ortho*-diphenols (COs) or additionally on monophenols (TYRs) (Scheme 1) - is still unknown.

So far, COs and TYRs are considered as indistinguishable by their amino acid sequences and physico-chemical properties[1,2]. Both enzyme classes belong to the super family of type-3 copper proteins[3] characterized by the two copper binding motifs CuA and CuB, each of them holding three histidine residues (H_{A1}, H_{A2}, H_{A3} and H_{B1}, H_{B2}, H_{B3}) able to coordinate one copper atom[4–6]. Together, the copper ions CuA and CuB can bind one oxygen molecule.

COs were long believed to be solely plant enzymes, and all plant PPOs were believed to be COs. Also, for a long time, TYRs had only been found in fungi, animals and bacteria[7]. However, a publication from 2010 was the first one to classify a fungal enzyme from *Aspergillus oryzae* as a CO[8]. In 2014, the first plant TYR



Scheme 1. Reactions catalyzed by catechol oxidases and tyrosinases.

was described in walnut (*Juglans regia*), ultimately invalidating the hypothesis of these kingdom-specific enzymes[9]. Much effort has since been made to elucidate the molecular differences between enzymes showing both mono- and diphenolase activity (i.e. TYRs) and enzymes showing only diphenolase activity (i.e. COs).

The initial explanation for why COs lack monophenolase activity was a bulky phenylalanine (F; H_{B3}-13) atop of CuA conserved in plant PPOs[10]. As deduced from the crystal structure of sweet potato (*Ipomoea batatas*) PPO (*Ib*CO; PDB ID: 1BT1), this F may sterically hinder monophenols to turn for binding in the catalytic cavity. An alternative hypothesis evolved from analyzing the crystal structures of *Bacillus megaterium* TYR, where a conserved water molecule was suggested to be activated by a highly conserved glutamate (E; H_{B1}-4) and an asparagine (N; H_{B1}+1)[11]. The activated water is thought to mediate deprotonation of the monophenolic substrate necessary for its hydroxylation. This observation was recently generalized to plant PPOs, suggesting the E at position H_{B1}-4 and the N at position H_{B1}+1 to be generally necessary and sufficient for monophenolase activity[12]. This hypothesis was experimentally approached by replacing G_{241} with N in *Vv*PPOcs-3 (*Vitis vinifera* 'Cabernet Sauvignon') in an attempt to convert a CO into a TYR. However, the qualitative, at best semi-quantitative in-gel activity assay used showed that *Vv*PPOcs-3-WT already possessed some monophenolase activity[12,13]. Overall, both hypotheses for the missing monophenolase activity of most plant PPOs require further experimental evidence.

To biochemically assess the molecular basis for mono-/diphenolase specificity, we performed site-directed mutagenesis and detailed kinetic studies with well-characterized dandelion (*Taraxacum officinale*) PPOs. Dandelion PPOs form one of the largest PPO families ever discovered[14–21] consisting of eleven isoenzymes separating into two different phylogenetic groups (group 1 and group 2)[22]. Recently, it has been proposed that plant PPOs in general divide into these two groups[23] whose members differ in catalytic cavity architecture, kinetic parameters, and substrate specificity. The latter has recently been shown to be significantly impacted by the 'substrate selector' residue at position H_{B2}+1 in the active site[24]. We utilized the dandelion PPOs' phylogenetic diversity to experimentally investigate the hypotheses for the lack of monophenolase activity of plant PPOs.

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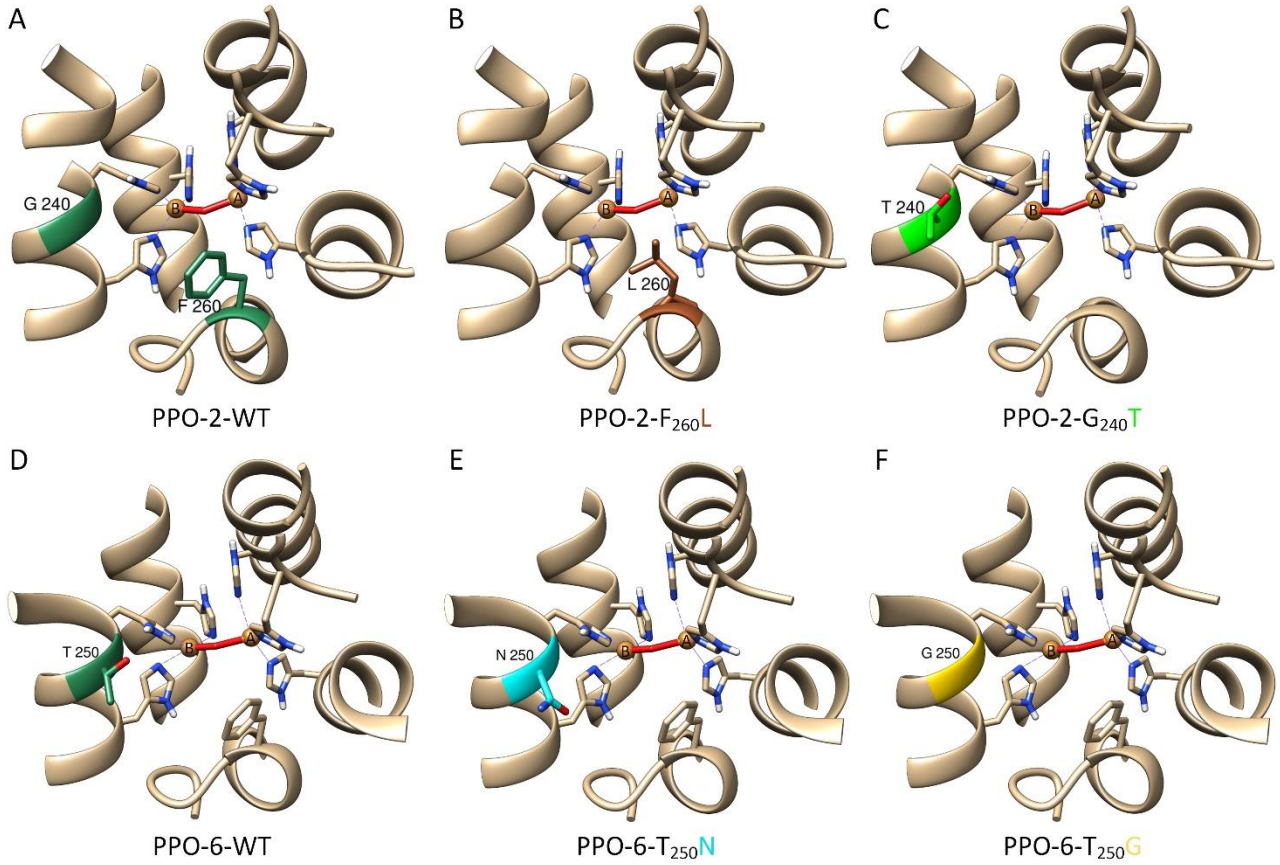
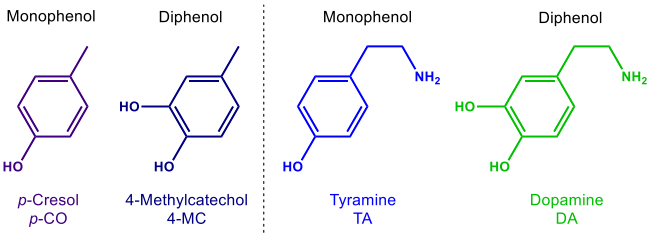


Figure 1. Locations of the mutated amino acid residues in the active site of PPO-2 (A-C) and PPO-6 (D-F), respectively. The PPO active site incorporates the copper atoms A and B, each coordinated by three histidine residues (bright blue). In the illustrated met-state, the copper atoms together bind one hydroxide ion (red sticks). A: PPO-2-WT with the wildtype amino acids that have been changed at position H_{B1}+1 (G_{240}) and at the 'gate residue' position H_{B3}-13 (F_{260}) in dark turquoise; B: 'gate residue' mutant PPO-2-F_{260}L (for PPO-2-F_{260}P and PPO-2-F_{260}G see Figure S1 in Supporting Information); C: H_{B1}+1 mutant PPO-2-G_{240}T (for PPO-2-G_{240}N see Figure S1 in Supporting Information); D: PPO 6-WT with the wildtype amino acid that has been changed at position H_{B1}+1 (T_{250}) in dark turquoise; E: H_{B1}+1 mutant PPO-6-T_{250}N; F: H_{B1}+1 mutant PPO-6-T_{250}G. Brown: leucine (L); green: threonine (T); cyan: asparagine (N); yellow: glycine (G).

For this purpose, we chose one well-characterized enzyme from each group (PPO-2 from group 1 and PPO-6 from group 2) for heterologous expression in *E. coli*[25], site-directed mutagenesis studies (Figure 1 and Figure S1 in Supporting Information), and detailed kinetic characterization. The latter was performed with the two mono-/diphenolic substrate pairs *p*-CO/4-MC and TA/DA (Scheme 2), where the mono- and the diphenol were tested separately.

Interestingly, PPO-2-WT showed clear activity with both monophenolic substrates, whereas PPO-6-WT showed no monophenolase activity at all (Figure 2, Table S2 in Supporting Information). PPO-2-WT showed higher diphenolase than monophenolase activity; the same was observed for *Agaricus bisporus* TYR (*Ab*TYR), the positive control enzyme for monophenolase activity (Figure S2 and Table S2 in Supporting Information). As PPO-2-WT possesses F at the 'gate' position H_{B3}-13 and no N at position H_{B1}+1, this result already questions both existing crystal structure-based hypotheses for the lack of monophenolase activity. Still, we wanted to further test the hypothesis that an N at position H_{B1}+1 in combination with the conserved E at position H_{B1}-4 leads to monophenolase activity[12].



Scheme 2. Structures of the tested mono-/diphenol substrate pairs.

When we replaced E_{235} of PPO-2 with the sterically conservative, but uncharged glutamine (Q), the enzyme lost all its activity (Figure S3 in Supporting Information). When we replaced the same residue with the similarly (i.e. negatively) charged aspartate (D), activity was maintained (Figure S3 in Supporting Information). This outcome confirms - in accordance with a study by Hu *et al.*[26] - that a negatively charged residue is needed at position H_{B1}-4 in the active site of PPOs to act as catalytic base.

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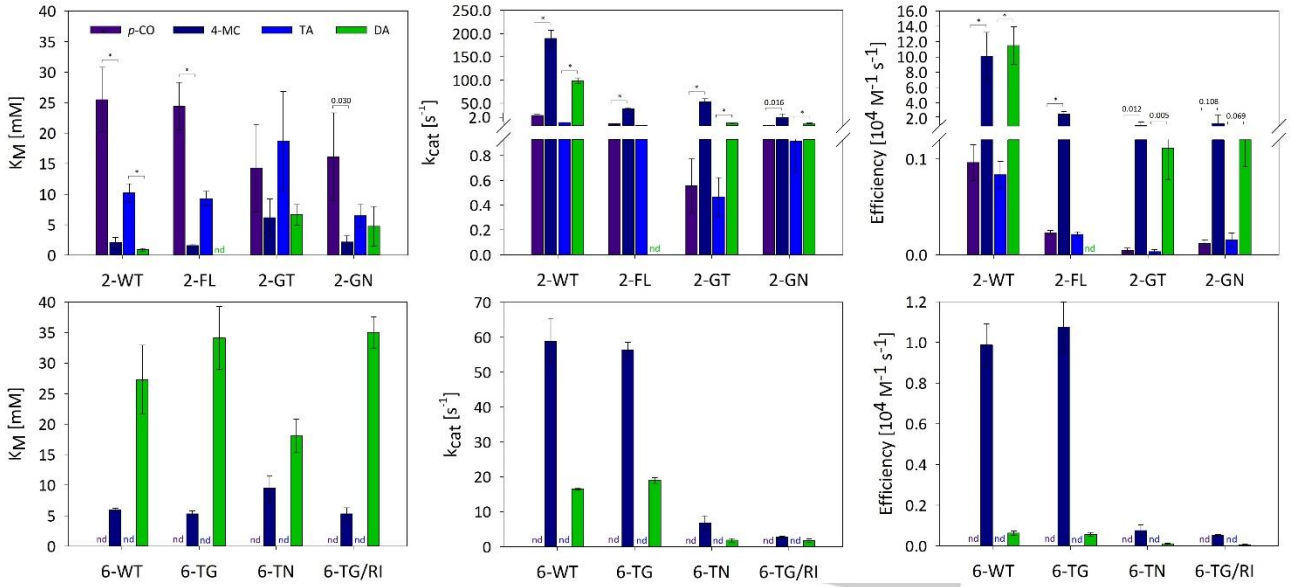

Figure 2. Kinetic parameters of WT and mutein enzymes with the tested mono-/diphenol substrate pairs. All values (except for 2-WT and 2-FL) represent means ± standard deviations of the means calculated for three independent enzyme batches (see also Table S2 in Supporting Information). With each enzyme batch, kinetics were performed three times, each in triplicates. The values of 2-WT and 2-FL represent the means ± standard deviations of three triplicate measurements with one enzyme batch, as example of the performed measurements with two independent enzyme batches. For statistical evaluation, all values underwent a t-test in SigmaPlot 12.5. Monophenols: *p*-cresol (*p*-CO) and tyramine (TA); diphenols: 4-methylcatechol (4-MC) and dopamine (DA); nd: not detected. *: $p < 0.001$.

Furthermore, we i) replaced the amino acid at position H_{B1}+1 in PPO-2 (G_{240}) and in PPO-6 (T_{250}) with N (leading to the muteins PPO-2-G_{240}N and PPO-6-T_{250}N) and ii) exchanged the amino acids at position H_{B1}+1 of PPO-2 and PPO-6, leading to the muteins PPO-2-G_{240}T and PPO-6-T_{250}G. Additionally, we replaced the arginine (R) at position H_{B2}+1 of PPO-6-T_{250}G with isoleucine (I) leading to the mutein PPO-6-T_{250}G/R_{254}I. The reason for the construction of this double mutant was the strong impact of R_{254} on the substrate specificity of PPO-6 recently revealed[24].

All WT and mutein enzymes were successfully expressed in *E. coli* in an active form and purified via *Strep*-tag affinity chromatography (Figure S4 in Supporting Information). As already observed for the WT enzymes, all PPO-2-based muteins showed activity with both monophenolic substrates, whereas all PPO-6-based muteins indicated no monophenolase activity at all (Figure 2, Table S2 in Supporting Information). Still, the amino acid residue at position H_{B1}+1 seems to impact the enzymes' total activity and their ability to oxidize monophenols: Whereas G_{250} introduced into PPO-6 did not have any harmful effect on diphenolase activity, T_{240} and N_{240} introduced into PPO-2 as well as N_{250} introduced into PPO-6 reduced the total activity by two-thirds. The total activity of PPO-6-T_{250}G/R_{254}I was even further minimized. Regarding monophenolase activity, PPO-2-G_{240}T showed strongly reduced activity, both with *p*-CO and TA (Figure 2, Table S2 in Supporting Information). The introduction of N_{240} did not increase PPO-2 monophenolase activity, and the introduction of N_{250} into PPO-6 did not lead to its conversion from a CO to a TYR.

To also approach the hypothesis about the bulky F preventing monophenolase activity in plant PPOs, we replaced F_{260} of PPO-2 with three amino acids occurring in other PPOs at the 'gate residue' position: a leucine (L) found in wheat and pineapple PPO as well as in filamentous fungus TYRs[19], a proline (P) found in mushroom TYRs[19], and a glycine (G) found in bacterial TYRs[27]. Again, all enzymes were successfully expressed in *E. coli* in an active form and purified via *Strep*-tag affinity chromatography (Figure S4 in Supporting Information). Whereas the introduction of P_{260} and G_{260} led to total activity loss, the F_{260}L mutation reduced the total activity by two-thirds (Figure 2, Table S2 in Supporting Information). Interestingly, compared to PPO-2-WT, PPO-2-F_{260}L showed the same K_M values and relative activities with all tested mono- and diphenolic substrates except with DA (Figure 2, Table S2 and Figure S5 in Supporting Information); with DA, PPO-2-F_{260}L did not have any activity. Hence, our results biochemically prove that the much-discussed 'gate residue' F in plant PPOs does not affect monophenolase activity at all. By contrast, the F at position H_{B3}-13

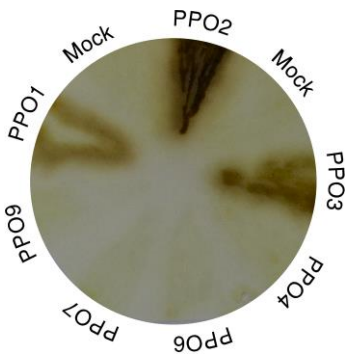

Figure 3. Monophenolase activity screening of *E. coli* strains expressing dandelion PPOs. Mock: vector control.

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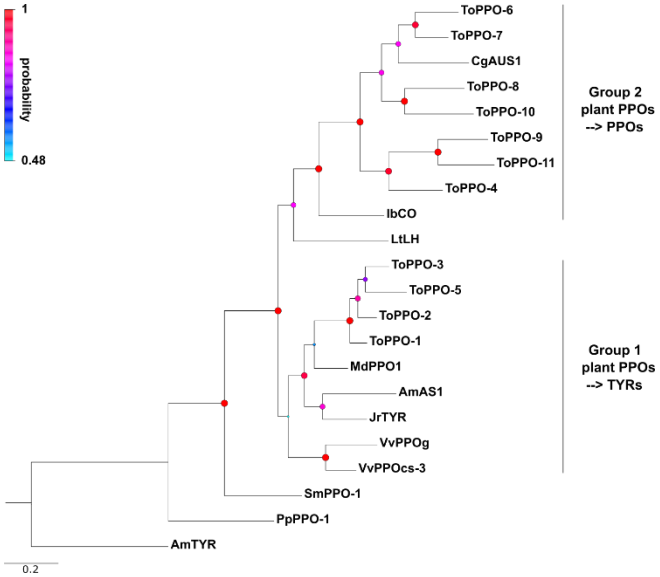

Figure 4. Phylogenetic tree of plant PPOs. For the analysis, we aligned the TYR domains of all plant PPOs that have, to date, been clearly biochemically characterized for their natural mono-/diphenolase specificity. The tree was rooted to a putative TYR sequence from the cyanobacterium *Acaryochloris marina* (GenBank: ABW32074.1) as well as to putative PPO sequences from the moss *Physcomitrella patens* (NCBI: XP_024397287.1) and the spike moss *Selaginella moellendorffii* (NCBI: XP_024519810.1). The reliability of internal branches was assessed by bootstrapping (1,000 replicates) and is indicated by colored circles, with big red circles indicating 100 % support and small blue circles indicating least support. All accession numbers are listed in Table S1 in Supporting Information.

of plant PPOs seems to be necessary for total enzyme activity. In our protein modeling studies, we observed a 'sandwich structure' of the second CuB coordinating histidine H_{B2}, the catechol ring of the substrate and the 'gate residue' F. Hence, we propose that hydrophobic interactions between the phenolic substrate's π electron system and the π electron systems of H_{B2} and F lead to correct positioning and stabilization of the substrate in the catalytic cavity (see also [24]). This deduction is in accordance with a hypothesis by Bijelic *et al*., who attributed the F at position H_{B3}-13 of walnut TYR to a 'substrate-guiding' function[28].

Our biochemical studies disprove the crystal structure-based hypotheses that have tried to explain why plant PPOs lack monophenolase activity. Our data in fact point out that phylogenetic group 1 plant PPOs seem to have monophenolase activity that is unaccounted for in their original classification, whereas group 2 plant PPOs seem not to have monophenolase activity. To check this hypothesis within the dandelion PPO family, we screened all PPOs available in expression strains for monophenolase activity. Indeed, PPO-1, PPO-2 and PPO-3 (group 1) showed clear activity with TA, whereas PPO-4, PPO-6, PPO-7 and PPO-9 (group 2) did not (Figure 3). Additionally, we quantitatively measured the specific activity of a second pair of purified group 1 and group 2 enzymes, PPO-1 and PPO-7, with the two mono-/diphenolic substrate pairs *p*-CO/4-MC and TA/DA: Whereas PPO-1 showed monophenolase activity comparable to the one of PPO-2, no monophenolase activity was detected for PPO-7 (Figure S6 in Supporting Information). This indication, that the mono-/diphenolase activity of dandelion PPOs is correlated to their phylogenetic grouping, fits previous reports on group-specific differences in substrate specificity[24].

To view the correlation between mono-/diphenolase activity and phylogenetic grouping more generally, we aligned and phylogenetically analyzed the amino acid sequences of the TYR domains of all plant PPOs that have, to date, been clearly characterized for their natural mono-/diphenolase specificity (Figure 4). After the split-up of moss PPOs and PPOs of higher plants, the latter divide into two different phylogenetic groups: All enzymes that exhibit both monophenolase and diphenolase activity (except for *Lt*LH) group into group 1, whereas all enzymes showing only diphenolase activity group into group 2. The TYR (+)-larreatricin hydroxylase (*Lt*LH) specialized in the synthesis of precursors of 8–8' linked lignans specific to the creosote bush *Larrea tridentata*[29] integrates in between group 1 and group 2 enzymes (Figure 4). Interestingly, both PPOs from *Vitis vinifera* that have been characterized regarding mono-/diphenolase specificity[12,30], clearly belong to group 1 PPOs. Whereas *Vv*PPOg was described earlier to exhibit both mono- and diphenolase activity, *Vv*PPOcs-3 was described to exhibit only diphenolase activity. However, as mentioned before, monophenolase activity was apparent, though weak, in the in-gel activity assay used in the correspondent study[13].

In the amino acid alignment, we found clear differences between group 1 and group 2 enzymes, namely in the residues flanking the CuB coordinating histidines (H_{B1}+1 and H_{B2}+1) and in the residues H_{A1}+5, H_{A1}+9 and H_{B3}-8 (Figure S7 in Supporting Information). The residues located at H_{B1}+1 and H_{B2}+1 have previously been proposed to impact substrate specificity in plant PPOs[13,24]. Another distinctive feature in the amino acid alignment is an inserted stretch of four to seven (*Ib*CO: one) amino acids in front of the CuB motif of group 2 enzymes, as already described by Molitor *et al*.[23]. As the inserted residues form a surface-exposed loop structure near the active site, they are likely to influence enzyme-substrate interactions.

In summary, by site-directed mutagenesis and detailed kinetic characterization, we biochemically disproved the current hypotheses that suggest that plant PPOs lack monophenolase activity due to a missing N at position H_{B1}+1 and the hindering 'gate residue' F. Using the dandelion PPOs' phylogenetic diversity, we have shown that plant PPOs exhibiting monophenolase activity normally belong to phylogenetic group 1, whereas those exhibiting solely diphenolase activity generally belong to group 2. This finding adds new information that can be used in future studies to better assess the molecular basis for mono-/diphenol substrate specificity of PPOs; simultaneously, it challenges the current practice of naming plant type-3 copper enzymes, as is the rule, COs.

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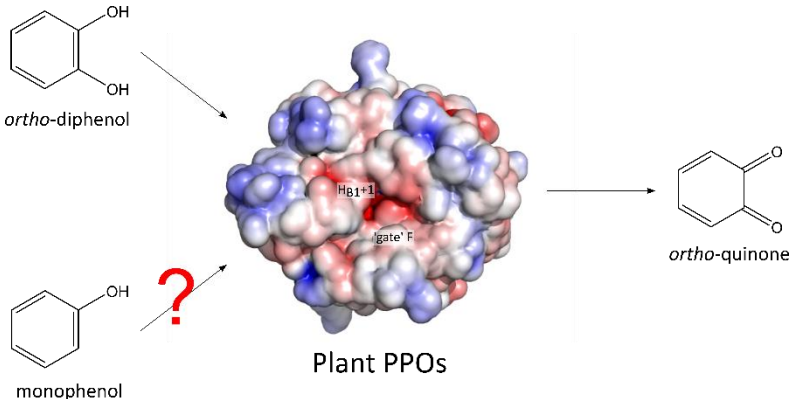

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Catechol Oxidase versus Tyrosinase Classification Revisited by Site-Directed Mutagenesis Studies

The much-discussed hypotheses about the residue at position H_{B1}+1 and the 'gate residue' phenylalanine being potentially responsible for the assumed general lack of monophenolase activity of plant polyphenol oxidases (PPOs) are tested by site-directed mutagenesis studies with dandelion (*Taraxacum officinale*) PPOs. The experimental results as well as additional phylogenetic analyses refute both hypotheses. Instead, we found that plant PPOs of phylogenetic group 2 solely exhibit diphenolase activity and, hence, are catechol oxidases (COs) as expected, while plant PPOs of phylogenetic group 1 additionally show monophenolase activity and, therefore, are actually tyrosinases (TYRs).