Identification of the amino acid position controlling the different enzymatic activities in walnut tyrosinase isoenzymes (jrPPO1 and jrPPO2)

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Polyphenol oxidases (PPOs) are ubiquitously distributed among plants, bacteria, fungi and animals. They catalyze the hydroxylation of monophenols (monophenolase activity) and the oxidation of o-diphenols (diphenolase activity) to o-quinones. PPOs are commonly present as an isoenzyme family. In walnut (Juglans regia), two different genes (jrPPO1 and jrPPO2) encoding PPOs have been identified. In this study, jrPPO2 was, for the first time, heterologously expressed in E. coli and characterized as a tyrosinase (TYR) by substrate scope assays and kinetic investigations, as it accepted tyramine and L-tyrosine as substrates. Moreover, the substrate acceptance and kinetic parameters ($k_{cat}$ and $K_m$ values) towards 16 substrates naturally present in walnut were assessed for jrPPO2 (TYR) and its isoenzyme jrPPO1 (TYR). The two isoenzymes prefer different substrates, as jrPPO1 shows a higher activity towards monophenols, whereas jrPPO2 is more active towards o-diphenols. Molecular docking studies performed herein revealed that the amino acid residue in the position of the 1st activity controller (HisB1 + 1; in jrPPO1 Asn240 and jrPPO2 Gly240) is responsible for the different enzymatic activities. Additionally, interchanging the 1st activity controller residue of the two enzymes in two mutants (jrPPO1-Asn240Gly and jrPPO2-Gly240Asn) proved that the amino acid residue located in this position allows plants to selectively target or dismiss substrates naturally present in walnut.
In 2016 Martínez-García et al. identified a second tyrosinase gene within the walnut genome, encoding a putative TYR (jrPPO2), which is, for the first time, investigated within this study. jrPPO1 (C0L1U17) and jrPPO2 (A0A2I4DDQ0) share an amino acid sequence identity of 73% (Fig. S2) and especially the active centers show a high level of conservation (Fig. S3). The most striking difference between the two active sites is the amino acid in the position of the 1st activity controller: jrPPO1 features an asparagine (Asn240), whereas jrPPO2 displays a glycine (Gly240). Asn in the position of the 1st activity controller (HisB1 + 1) has previously been associated with increased monophenolase activity as it stabilizes a water molecule which is activated by a conserved glutamic acid and reacts as a base for the deprotonation of incoming substrates. The deprotonation of the substrate is imperative for the hydroxylation of monophenols. Thus, an asparagine in the position of the 1st activity controller is also present in the sequences of MdPPO2 (Malus domestica; TYR) and VvPPOg2 (Vitis vinifera, TYR). In contrast, the deprotonation of a substrate can also occur by a hitherto unknown mechanism as glycine in the position of the 1st activity controller is compatible with monophenolase activity as well. This has already been proven by the sequences of MdPPO3 (Malus domestica; TYR), ToPPO1, ToPPO2, (Taraxacum officinale, TYR) and VvPPOcs-3 (Vitis vinifera, TYR), which all accept the standard substrates L-tyrosine and/or tyramine.

Walnut (Juglans regia) is known for being rich in phenolic compounds valuable to the cosmetic and pharmaceutical industries, however, PPO side reactions in walnut limit the availability of these compounds. The rich abundance of phenolic compounds in walnut has been investigated thoroughly within the last years, leading to the identification of numerous small phenolic compounds (e.g. pyrogallol, benzoic acid derivatives, phenylacetic acid derivatives and cinnamic acid derivatives; Fig. S4). Besides, flavonoids represent a prominent group of phenolics in walnut (Fig. S5), represented by the flavonols kaempferol, quercetin and myricetin and the flavanol taxifolin, which are associated with positive health effects, such as antioxidative, antibacterial, antitumor and anti-inflammatory activity. Thus, their preservation is desired upon storage. Moreover, naphthoquinones, with juglone (5-hydroxy-1,4-naphthoquinone; Fig. S5) being the most abundant one, are phenolic compounds characteristic for Juglans regia. They have been demonstrated to show allelopathic, insecticidal and anthelmintic effects and are, therefore, proposed as biological insecticides and herbicides. Moreover, the cultivation of walnut has gained economic relevance within the last decades due to timber production as well as the high nutritional value of walnut kernels. However, little is known about the tyrosinase activity towards the vast spectrum of phenolic compounds naturally present in walnut. Thus, understanding the reactivities of jrPPO1 and jrPPO2 offers the possibility of controlling the PPO activity in walnut.

Herein, we report the cloning of the gene encoding latent jrPPO2, the recombinant expression of soluble protein as well as its biochemical characterization. Moreover, the activity of recombinantly expressed jrPPO1 and jrPPO2 towards natural walnut substrates was assessed and the activities of the two enzymes clearly showed different substrate preferences. Kinetic investigations supplemented with docking studies identified the 1st activity controller residue (jrPPO1: Asn240, jrPPO2: Gly240) as the cause for the different reactivities in these two enzymes, which was further substantiated by kinetic measurements and docking studies using two mutants targeting the 1st activity controller residues of the two isozymes (jrPPO1-Asn240Gly, jrPPO2-Gly240Asn).

**Results and discussion**

**Genomic DNA extraction and cloning of the jrPPO2 gene.** Genomic DNA (gDNA) was isolated from walnut leaves using a cetyltrimethylammonium bromide (CTAB) assisted cell lysis method, which produced a total yield of 250 ng gDNA (~40,000 base pairs)/g frozen plant material. The co-extraction of phenolic compounds substantially decreases downstream applicability (PCR) of DNA extracts. Thus, 2% (w/v) PVP (polyvinylpyrrolidone) was added to the DNA extraction buffer as well as 20 mM sodium ascorbate to suppress PPO side reactions since in situ produced quinones oxidize DNA.

The predicted jrPPO2 gene encompasses an N-terminal chloroplast transit peptide, an active domain and a C-terminal domain. Using Q5 High-Fidelity DNA polymerase and specific primers (Table S1) a ~1,700 base pair amplicon was obtained, cloned in the pENTRY-IBA51 vector and sequenced to reveal the sequence of the predicted JrPPO2. Compared to the sequence published by Martínez-García et al., the gene sequence herein contained the following mutations: Asp256Asn, Phe293Leu, Ser296Pro and Asp477Asn, which are all located...
mass of corresponding to the formation of the thioether bridge and one of the two disulfide bonds being closed. The dependence was assessed in increments of 0.5 pH units ranging from pH 3.0 to pH 8.0 (pH 3.0–pH 5.5: sodium citrate buffer). Varying numbers of closed disulfide bonds due to ESI–MS investigations have already been reported for E. coli is impeded by the reducing environment of the cytosol. However, disulfide bonds can be present as an artifact of the electrospray ionization process, during which thiyl radicals, formed via one-electron oxidation of thiol groups, dimerize rapidly. The thioether bridge is formed independently in the bacterial cytosol via an artifact of the expression process. In plant PPOs ranging from pH 4.5 (sodium citrate buffer) to pH 8.0 (sodium phosphate buffer). Thus, the pH optima of plant PPOs ranging from pH 4.5 (sodium citrate buffer) to pH 8.0 (sodium phosphate buffer) have already been reported for PPO1. The formation of the disulfide bonds during the recombinant expression process in E. coli is impeded by the reducing environment of the cytosol. However, disulfide bonds can be present as an artifact of the expression process. The thioether bridge is formed independently in the bacterial cytosol via an artifact of the expression process.

Expression of jPPPO1, jPPPO1-Asn240Gly, jPPPO2 and jPPPO2-Gly240Asn. As described previously for PPO1 and other plant PPOs, expression at low temperatures (~ 20 °C) in combination with prolonged expression times and the usage of a nutrient-rich medium (2xYT) results in an increased overall yield. jPPPO2 produced the highest yield with 70 mg/l purified, latent enzyme, followed by jPPPO2-Gly240Asn (63 mg/l), jPPPO1 (41 mg/l) and jPPPO1-Asn240Gly (34 mg/l). All enzymes were expressed at a purity level of at least 95% (Fig. S6) and were stored in 50 mM Tris–HCl pH 7.5 and 200 mM NaCl and were immediately used for kinetic measurements.

Molecular mass determination. ESI-LTQ-MS revealed the masses of recombinant jPPPO1, jPPPO1-Asn240Gly, jPPPO2 and jPPPO2-Gly240Asn. The crystal structure analysis of jPPPO1 exhibits one thioether bridge and two conserved disulfide bonds which are, due to the similar spatial arrangement of the amino acids involved in the formation of the disulfide and the thioether bridge, most probably also in vivo present in jPPPO2 (Fig. S1). The mass of jPPPO1, jPPPO2 and jPPPO2-Gly240Asn matched with the calculated masses corresponding to the formation of the thioether bridge and one of the two disulfide bonds being closed. The mass of jPPPO1-Asn240Gly indicated the formation of the thioether bridge and both disulfide bonds (Table 1 and Fig. S7) being closed. Varying numbers of closed disulfide bonds due to ESI–MS investigations have already been reported for PPO1. The formation of the disulfide bonds during the recombinant expression process in E. coli is impeded by the reducing environment of the cytosol. However, disulfide bonds can be present as an artifact of the expression process. The thioether bridge is formed independently in the bacterial cytosol via an artifact of the expression process.

Characterization of jPPPO1 and jPPPO2. jPPPO was characterized in terms of its pH optimum and activation by SDS using 1 mM dopamine (Fig. S8) as a substrate. Different pH values for maximum activity have been reported for plant PPOs ranging from pH 4.5 (sodium citrate buffer) to pH 8.0 (sodium phosphate buffer). Thus, the pH dependence was assessed in increments of 0.5 pH units ranging from pH 3.0 to pH 8.0 (pH 3.0–pH 5.5: sodium citrate buffer, pH 6.0–pH 8.0: sodium phosphate buffer). The maximum activity was observed at pH 6.0 (Fig. 2), which follows the pH optimum of jPPPO1 (pH 6.0) previously reported.

A general characteristic of plant PPOs is their latency as activity can be measured only in the presence of an additional activator. SDS has been proven suitable in activating plant PPOs and was previously shown to overcome their latency. Thus, the activation of PPOs is achieved with SDS molarities ranging from 0.35 mM to 4.0 mM. We tested the concentration-dependent activation of jPPPO1 with SDS molarities ranging from 0.5 to 5.0 mM. The highest activity was observed at 2.5 mM SDS (Fig. 2), compared to 2.0 mM for jPPPO1. The respective pH optima and SDS optima of jPPPO1 and jPPPO2 were used for substrate scope assays and the kinetic measurements.

jPPPO2 was characterized kinetically using the monophenolic substrates tyramine and L-tyrosine and the diphenolic substrates dopamine and L-DOPA (Fig. S8). jPPPO2 showed activity towards both monophenolic and both diphenolic substrates and, therefore, was classified as a TYR. $k_{cat}$ (s⁻¹) and $K_m$ (mM) values were determined for tyramine, L-tyrosine, dopamine and L-DOPA (Table 2).

$k_{cat}$ values were higher for the diphenols ($k_{cat}$ dopamine = 186 s⁻¹; $k_{cat}$ L-DOPA = 132 s⁻¹) compared to the monophenols ($k_{cat}$ tyramine = 9.14 s⁻¹; Table 2). Moreover, the catalytic efficiency ($k_{cat}/K_m$) of jPPPO2 was considerably higher for the less polar substrate tyramine ($k_{cat}/K_m$ = 18.7 s⁻¹ mM⁻¹) compared to the carboxylic substrate L-tyrosine ($k_{cat}/K_m = 0.69$ s⁻¹ mM⁻¹), which held also true for the diphenolic substrates (Table 2).

| Enzyme          | Mass calculated (Da) | Mass measured (Da) | Δmass (Da) |
|-----------------|----------------------|--------------------|-----------|
| jPPPO1          | 56,359.37 (–4H)     | 56,358.81          | − 0.56    |
| jPPPO1-Asn240Gly| 56,300.31 (–6H)     | 56,300.58          | + 0.27    |
| jPPPO2          | 56,790.82 (–4H)     | 56,790.56          | − 0.26    |
| jPPPO2-Gly240Asn| 56,847.87 (–4H)     | 56,847.58          | − 0.29    |

Table 1. Calculated and measured molecular weights of jPPPO1, jPPPO1-Asn240Gly, jPPPO2 and jPPPO2-Gly240Asn.
Figure 2. The pH (A) and SDS (B) profile of jrPPO2. The error bars indicate ± one standard deviation. Activities towards the standard substrate dopamine are plotted in relation to the maximum activity set to 100%. Measurements were performed in triplicates. Detailed information about the experimental setup is provided in the materials and methods section.

Table 2. Enzymatic parameters of standard substrates and natural substrates for jrPPO1, jrPPO2, jrPPO1-Asn240Gly and jrPPO2-Gly240Asn. Values are reported ± one standard deviation. *Represents parameters that could not be measured due to low activity and limited substrate solubility. †Indicates values previously reported10 and added to this Table. K_{cat} and k_{cat}/K_{m} values were determined as described in the materials and methods section.
higher catalytic efficiency towards activity ratios (PPO1, compared to jPPO2 over monophenols than jPPO2 (Table 2). The same trend held true for the monophenolase/diphenolase efficiency ratios (jPPO1 and jPPO2: pyrogallol, protocatechuic acid, gallic acid, ethyl gallate, 4-hydroxyphenylacetic acid, 9 = coumaric acid, 10 = caffeic acid, 11 = ferulic acid, 12 = juglone, 13 = kaempferol, 14 = quercetin, 15 = taxifolin, 16 = myricetin (Figs. S4 and S5). The control lane contained no enzyme. Photos were taken after 5 minutes, 2 hours and 24 hours and edited using GIMP 2.10.18 (https://www.gimp.org). Detailed information about the experimental setup is provided in the materials and methods section.

A substrate scope assay shows varying substrate scopes for jPPO1 and jPPO2. The activity of recombinantly expressed jPPO1 and jPPO2 towards 16 aglyconic, phenolic compounds naturally present in walnut29–33 (Table S2 and S3) was tested. Eleven small phenolic compounds (pyrogallol, 4-hydroxybenzoic acid, protocatechuic acid, gallic acid, salicylic acid, vanillic acid, ethyl gallate, 4-hydroxyphenylacetic acid, coumaric acid, caffeic acid and ferulic acid; Fig. S4), four flavonoids (kaempferol, quercetin, taxifolin, myricetin; Fig. S5) and the naphthoquinone juglone (Fig. S5) were tested.

Substrate-enzyme combinations leading to a visually detectable change in color within 24 hours were flagged as active, whereas substrate-enzyme combinations remaining colorless after this time were flagged as inactive (Fig. 3).

Nine substrates were accepted by both enzymes (jPPO1 and jPPO2): pyrogallol, protocatechuic acid, gallic acid, ethyl gallate, 4-hydroxyphenylacetic acid, coumaric acid, caffeic acid, quercetin and taxifolin (Figs. S4 and S5) and showed a clearly visible change in color within 24 hours (Fig. 3). Substrates carrying a 3-methoxy group (vanillic acid and ferulic acid) were rejected by both enzymes (jPPO1 and jPPO2), in contrast to their non-methoxylated homologs (vanillic acid / 4-hydroxybenzoic acid and ferulic acid/coumaric acid) (Fig. 3). Consequently, the 3-methoxy group is incompatible with the enzymatic activity of jPPO1 and jPPO2. Moreover, salicylic acid, which carries a 2-hydroxy group, kaempferol, myricetin and the naphthoquinone juglone were rejected by both jPPO1 and jPPO2 (Fig. 3). Several substrates showed varying reaction rates for jPPO1 compared to jPPO2, however, the differences were most prominent for the two benzoic acid derivatives protocatechuic acid and gallic acid. Protocatechuic acid (Fig. S4) was accepted by jPPO2 (after ~2 hours) but rejected by jPPO1 (after 24 hours). Similarly, gallic acid (Fig. S4) was oxidized by jPPO2 within minutes, whereas activity towards jPPO1 was detected only after 24 hours (Fig. 3).

Kinetic measurements of jPPO1 and jPPO2 identify different substrate preferences. To further investigate the kinetic behavior of jPPO1 and jPPO2, and values were determined for substrates that showed activity towards jPPO1 and/or jPPO2. Molar extinction coefficients were reported previously52 or were determined herein (see supplementary information; Table S2). The flavonoid substrates (quercetin and taxifolin; Fig. S5) were assayed in a solution containing 10% DMSO due to their limited water solubility. The effects of 10% DMSO on the activities of jPPO1 and jPPO2 were assessed using dopamine. In the presence of 10% DMSO, jPPO1 retained 72% activity and jPPO2 retained 75% activity, compared to enzymatic tests without additional DMSO. Thus, both enzymes are similarly affected by the addition of 10% DMSO. jPPO1 and jPPO2 were more active towards diphenols than towards the corresponding monophenols (Table 2), as in general reported for PPOs10,17,21,44. However, jPPO2 showed a stronger preference for diphenols over monophenols than jPPO1, as diphenolic and triphenolic substrates showed higher activity values (kcat value) and higher efficiency values (kcat/Km ratio) towards jPPO2 than towards jPPO1. The only exception was protocatechuic acid (Fig. S4), which was more active (higher kcat value) with jPPO1. However, since the Km value for protocatechuic acid (Fig. S4) increased for jPPO1 (compared to jPPO2), it showed a substantially higher catalytic efficiency towards jPPO2 (kcat/Km = 109 s⁻¹ mM⁻¹), compared to jPPO1 (kcat/Km = 3.95 s⁻¹ mM⁻¹) (Table 2). In contrast, all monophenolic substrates (4-hydroxyphenylacetic acid, coumaric acid, L-tyrosine, and tyramine) showed a higher turnover rate towards jPPO1, compared to jPPO2. Monophenolase/diphenolase activity ratios (kcat monophenol/kcat diphenol) of corresponding mon- and diphenols were higher for jPPO1 than for jPPO2. The activity ratio of tyramine/dopamine for jPPO1 was 0.27, compared to 0.05 for jPPO2 (Table 2). The same trend held true for the monophenolase/diphenolase efficiency ratios (kcat monophenol/kcat diphenol).

Thus, our data show that jPPO1 favors monophenolic substrates, whereas jPPO2 targets diphenolic substrates. This trend also correlates with the flavonoid substrates and was particularly pronounced for the diphenolic substrate taxifolin (Fig. S5), which was 19-times more active towards jPPO2 (kcat = 19.6 s⁻¹), compared to...
jrPPO1 \( (k_{cat} = 1.04 \text{ s}^{-1}; \text{Table } 2) \). An asparagine in the position of the 1st activity controller residue (Asn240) has previously been proven to increase monophenolase activity\(^{10,25} \), which explains the higher activity \( (k_{cat} \text{ values}) \) of jrPPO1 towards monophenols, compared to jrPPO2. To clarify the molecular cause for the increased diphenolase activity of jrPPO2, compared to jrPPO1, docking studies were employed.

Docking studies illustrate the molecular cause of the different reactivities of jrPPO1 and jrPPO2. For the docking studies, a homology model of jrPPO2 was built using the SWISS-MODEL server\(^{53,54} \) (Fig. S3) and the crystal structure of jrPPO1 (PDB entry 5CE9) as a template. Molecular docking was performed for jrPPO1 as well as jrPPO2. Binding poses were calculated for all kinetically investigated substrates, which included the standard substrates tyramine, l-tyrosine, dopamine and l-DOPA and the natural substrates pyrogallol, protocatechuic acid, gallic acid, ethyl gallate, 4-hydroxyphenylacetic acid, coumaric acid, caffeic acid, quercetin and taxifolin. The results offered highly valuable information detailing the molecular basis for the different reactivities of jrPPO1 and jrPPO2.

The homology model of jrPPO2 exhibited a high level of structural homology, compared to the crystal structure of jrPPO1 (RMSD = 0.487 Å). However, the amino acid in the position of the 1st activity controller residue represents a notable difference between the architectures of the active centers of jrPPO1 and jrPPO2 (Figs. S2 and S3). jrPPO1 features a Gly in this position, whereas jrPPO2 features a spatially more demanding Asn, which is protruding directly into the active center (Fig. S9).

The calculated binding poses clearly show that in jrPPO2 all diphenolic substrates are preferentially oriented in a lying down position (orienting the 3′-hydroxy group toward the di-copper center; Figs. 4, S10, S11), whereas, in jrPPO1, diphenolic substrates have to approach the di-nuclear center in an upright orientation (orienting the 4′-hydroxy group toward the di-copper center; Fig. 4). Orienting diphenolic substrates in a lying down position in jrPPO1 is prevented by Asn240, which overlaps with the tails of diphenolic substrates in jrPPO2. Alternatively, diphenolic substrates can be oriented in jrPPO1 in an upright position as well (data not shown). Thus, orienting substrates into the active center of jrPPO2 with the phenolic ring facing the di-copper center appears to be entropically more favorable, compared to jrPPO1, due to the spatially less demanding 1st activity controller residue (Gly240). This explains the significantly higher turnover rates and efficiency values of diphenolic substrates for jrPPO2, compared to jrPPO1 (Table 2).

In contrast, monophenolic substrates featuring a 4′-hydroxy group are oriented exclusively in an upright position in jrPPO1 and jrPPO2, as demonstrated by the molecular docking poses (Figs. 5, S10, S11). Thus, the entropic advantage of the more spacious active center of jrPPO2 does not come into effect for monophenolic substrates. Moreover, the asparagine present in the position of the 1st activity controller in jrPPO1 has been shown to facilitate monophenolase activity by aiding in the imperative abstraction of the phenolic proton from incoming monophenolic substrates\(^{20} \). The resulting phenolate substrate, carrying a negative charge, exhibits an increased affinity towards the positively charged di-copper center, compared to the corresponding not-dissociated phenol\(^{20} \). This was first demonstrated for VvPPOcs-3\(^{30} \), which features a glycine in the position of the

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**Figure 4.** Docking poses of protocatechuic acid, ethyl gallate, and caffeic acid. The transparency was set to 0.3, the copper ions are displayed as brown spheres. (A–C) (red) represent the active center of jrPPO1. (D–F) (blue) represent the active center of jrPPO2, with substrates docked as follows: A = protocatechuic acid, B = ethyl gallate, C = caffeic acid, D = protocatechuic acid, E = ethyl gallate, F = caffeic acid. The active centers of jrPPO1 and jrPPO2 are shown as seen by incoming substrates. The images were created using PyMOL 2.3\(^{56} \) and edited using GIMP 2.10.18 (https://www.gimp.org).
Asn240Gly = 300 s\(^{-1}\), His204 (HisB1) is located at a distance of 2.7 Å from the amide group of Asn204 (1st activity controller residue). Thus, in

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k_{\text{cat}} \text{reduced activity towards dopamine (}\text{jr}\text{PPO1} = 92.5 s^{-1}, \text{jr}\text{PPO2-Gly240Asn} = 66.3 s^{-1})
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Figure 5. Docking poses of 4-hydroxyphenylacetic acid, coumaric acid, and tyrosine. The transparency was set to 0.3, the copper ions are displayed as brown spheres. (A–C) (red) represent the active center of \text{jr}\text{PPO1}, (D–F) (blue) represent the active center of \text{jr}\text{PPO2}, with substrates docked as follows: A = 4-hydroxyphenylacetic acid, B = coumaric acid, C = tyrosine, D = 4-hydroxyphenylacetic acid, E = coumaric acid, F = tyrosine. The active centers of \text{jr}\text{PPO1} and \text{jr}\text{PPO2} are viewed as seen by incoming substrates. The images were created using PyMOL 2.3\(^{38}\) and edited using GIMP 2.10.18 (https://www.gimp.org).

1st activity controller residue (Gly241). Semiquantitative in-gel activity tests demonstrated that the mutant VvPPOcs-3-Gly241Asn (1st activity controller: Asn241) showed increased activity rates towards the monophenolic substrates tyramine and p-tyrosol (compared to the native enzyme; 1st activity controller: Gly241)\(^ {25}\), which is in accordance with our results. Moreover, it has been demonstrated, based on the crystal structure of the bacterial tyrosinase from \textit{Bacillus megaterium} (\textit{Bm}TYR)\(^{55}\), which also features an Asn in the position of the 1st activity controller (His81 + 1 = Asn205), that Asn205 forms a polar bond with the first CuB coordinating histidine (His81 = His204) and, thereby, stabilizes His204\(^ {55}\). In \textit{Bm}TYR, the N\(_\text{δ1}\) atom of the imidazole ring of His204 (His81) is located at a distance of 2.7 Å from the amide group of Asn204 (1st activity controller residue). Similarly, the N\(_\text{δ1}\) atom of the imidazole ring of His239 (His95) in \textit{jr}\text{PPO1} is located at a distance of 2.9 Å from the amide group of Asn240 (1st activity controller residue)\(^ {55}\). Thus, in \textit{jr}\text{PPO1} Asn240 probably shows a stabilizing effect on His239. The combination of these effects explains the higher activity rates of monophenolic substrates with \textit{jr}\text{PPO1}, compared to \textit{jr}\text{PPO2} (Table 2).

Mutagenesis studies confirm the pivotal influence of the 1st activity controller on enzymatic activity. The mutants \textit{jr}\text{PPO1-Asn240Gly} and \textit{jr}\text{PPO2-Gly240Asn} were generated by site-directed mutagenesis (Table S1) to further prove the influence of the amino acid residue present in the position of the 1st activity controller. Now, in the position of the 1st activity controller, \textit{jr}\text{PPO1-Asn240Gly} resembles \textit{jr}\text{PPO2}, whereas \textit{jr}\text{PPO2-Gly240Asn} resembles \textit{jr}\text{PPO1}. A substrate scope assay revealed the preferences of each mutant towards natural substrates and proved that \textit{jr}\text{PPO1-Asn240Gly} resembles \textit{jr}\text{PPO2} in terms of substrate preferences as it accepts 4-hydroxybenzoic acid and showed activity with gallic acid (Fig. S4) and ethyl gallate (Fig. S4) within minutes (Figs. 3 and 6). In contrast, \textit{jr}\text{PPO2-Gly240Asn} rejected 4-dihydroxybenzoic acid, which was rejected by \textit{jr}\text{PPO1} but accepted by \textit{jr}\text{PPO2}. Gallic acid and ethyl gallate were both oxidized by \textit{jr}\text{PPO2-Gly240Asn} after several hours, which corresponds to the substrate scope assay of \textit{jr}\text{PPO1} (Figs. 3 and 6).

Furthermore, kinetic parameters were determined for the monophenolic substrate tyramine and the diphenoic substrate dopamine for both mutants (Table 2). In accordance with our previous results, \textit{jr}\text{PPO1-Asn240Gly} showed a considerably increased activity towards the diphenol dopamine, compared to \textit{jr}\text{PPO1} (\(k_{\text{cat}} \text{jr}\text{PPO1-Asn240Gly} = 300 s^{-1}, k_{\text{cat}} \text{jr}\text{PPO1} = 92.5 s^{-1}\)), whereas the activity towards the monophenol tyramine was reduced (\(k_{\text{cat}} \text{jr}\text{PPO1-Asn240Gly} = 7.60 s^{-1}, k_{\text{cat}} \text{jr}\text{PPO1} = 24.7 s^{-1}\)) (Table 2). In contrast, \textit{jr}\text{PPO2-Gly240Asn} showed a reduced activity towards dopamine (\(k_{\text{cat}} \text{jr}\text{PPO2-Gly240Asn} = 66.3 s^{-1}, k_{\text{cat}} \text{jr}\text{PPO2} = 186 s^{-1}\)) and an increased activity towards tyramine (\(k_{\text{cat}} \text{jr}\text{PPO2-Gly240Asn} = 10.9 s^{-1}, k_{\text{cat}} \text{jr}\text{PPO2} = 9.14 s^{-1}\)). Accordingly, \textit{jr}\text{PPO1-Asn240Gly} (tyramine/dopamine activity ratio = 0.03) had a stronger preference of diphenols over monophenols than \textit{jr}\text{PPO2-Gly240Asn} (tyramine/dopamine activity ratio = 0.16; Table 2).

Docking studies were performed for \textit{jr}\text{PPO1-Asn240Gly} and \textit{jr}\text{PPO2-Gly240Asn} using tyramine and L-tyrosine (Fig. 7) and all substrates investigated during the previous docking experiments (Figs. 4, 5, S10–S13). Our data show that diphenoic substrates are oriented in a laying down position in \textit{jr}\text{PPO1-Asn240Gly} (as observed for \textit{jr}\text{PPO2}; Figs. 4, 7, S12 and S13). In contrast, \textit{jr}\text{PPO2-Gly240Asn} diphenoic substrates must approach...
the di-nuclear copper center in an upright position, since Asn240 now blocks substrates from orienting in the laying down orientation (Figs. 7, S12 and S13). Monophenolic substrates enter the active center of both mutants (jrPPO1-Asn240Gly and jrPPO2-Gly240Asn) in an upright orientation. This data show that the amino acid residue in the position of the 1st activity controller is responsible for the different substrate preferences of jrPPO1 (targeting monophenols) and jrPPO2 (targeting diphenols). The substrate scope is dependent on the amino acid present in the position of the 1st activity controller. Moreover, an asparagine in the position of the 1st activity controller increases monophenolase activity, whereas diphenolase activity is reduced (compared to the presence of glycine in the same position). Substituting asparagine with a spatially less demanding amino acid (such as glycine) increased the $k_{cat}$ value of dopamine considerably. Our results lead to the conclusion that jrPPO1 and jrPPO2 in vivo target different substrates and, thus, most probably fulfill different physiological tasks. The common appearance of plant PPOs as a family of isoenzymes suggests that they are involved in several cellular pathways, covering a diverse spectrum of functionalities. We hope that our work will inspire the deciphering of the different tasks assigned to PPOs, thereby illuminating their elusive reactivities.

Figure 6. Substrate scope assay of jrPPO1-Asn240Gly and jrPPO2-Gly240Asn including 16 natural substrates: 1 = pyrogallol, 2 = 4-hydroxybenzoic acid, 3 = protocatechuic acid, 4 = gallic acid, 5 = salicylic acid, 6 = vanillic acid, 7 = ethyl gallate, 8 = 4-hydroxyphenylacetic acid, 9 = coumaric acid, 10 = caffeic acid, 11 = ferulic acid, 12 = juglone, 13 = kaempferol, 14 = quercetin, 15 = taxifolin, 16 = myricetin (Figs. S4 and S5). Photos were taken after 5 minutes, 2 hours and 24 hours and edited using GIMP 2.10.18 (https://www.gimp.org). Detailed information about the experimental setup is provided in the materials and methods section.

Figure 7. Docking poses calculated for diphenolic substrates for jrPPO1-Asn240Gly and jrPPO2-Gly240Asn. The transparency was set to 0.3. The copper ions are displayed as brown spheres. (A, B) (red) represent the active center of jrPPO1-Asn240Gly. (C, D) (blue) represent the active center of jrPPO2-Gly240Asn, with substrates docked as follows: A = tyramine, B = dopamine, C = tyramine, D = dopamine. The active centers of jrPPO1-Asn240Gly and jrPPO2-Gly240Asn are viewed as seen by incoming substrates. The images were created using PyMOL 2.3 (https://www.gimp.org).
Conclusions

In this study, jrPPO2 was, for the first time, heterologously expressed, purified and characterized. Activity tests using standard substrates (tyramine, L-tyrosine, dopamine, L-DOPA) clarified that jrPPO2 is a TYR, as it accepted L-tyrosine and tyramine. Moreover, substrate scope assays using 16 natural substrates showed a more expansive substrate scope for jrPPO2 as it accepted 4-hydroxybenzoic acid and gallic acid, compared to jrPPO1, which was inactive with 4-hydroxybenzoic acid and showed only marginal activity towards gallic acid. Kinetic parameters were determined for jrPPO2 and its isozymes jrPPO1, which pointed towards differences in substrate preference. jrPPO2 showed a higher catalytic efficiency for diphensols whereas jrPPO1 was more active on monophenols. Docking studies revealed that the amino acid in the position of the 1st activity controller can increase the activity towards monophenolic substrates, as it has previously been proposed, by stabilizing a conserved water molecule or reduce enzymatic activity towards diphensolic substrates by sterically impeding substrate orientation. The two mutants jrPPO1-Asn240Gly and jrPPO2-Gly240Asn proved the key role of the 1st activity controller as jrPPO1-Asn240Gly showed an enzymatic profile similar to jrPPO2, whereas jrPPO2-Gly240Asn resembled jrPPO1.

Our results demonstrate that, in vivo, different PPOs within the same plant target different substrates, which is achieved by the variability of one crucial amino acid residue (1st activity controller). This novel understanding of the functionality of PPO isoenzymes in plants will hopefully allow controlling their reactivity and, thereby, enhance the nutritional and economic value of plant products.

Materials and methods

Isolation of genomic DNA and cloning of the jrPPO2 gene. Walnut leaves were collected from naturally grown trees around Vienna and stored at -80 °C. Two g of frozen leaves were ground in liquid nitrogen. The frozen paste was mixed with 2 ml extraction buffer (100 mM HEPEs, 0.5 M NaCl, 20 mM sodium ascorbate, 2% PVP and 1% cetyltrimethylammonium bromide (CTAB), pH 8.0) [2]. The mixture was incubated in a 70 °C water bath for one hour followed by centrifugation at 20.000 rpm for 10 min. The supernatant was extracted with 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1, pH 7.8) and subsequently the aqueous layer was washed two times with 1 volume 100% chloroform. The aqueous layer was precipitated by adding 1 volume of EtOH (96%) and incubation at 0 °C for 2 hours. The pellet resulting from centrifugation at 20.000 rpm for 10 min at 4 °C was washed twice with EtOH (70%) at 0 °C, dried and resuspended in 100 µl TE buffer (10 mM Tris—HCl, 1 mM EDTA, pH 8.0). The quality and quantity of the DNA extract were checked by 0.6% agarose gel electrophoresis (Fig. S14).

The first pair of primers binding outside of the open reading frame of the active domain and the C-terminal domain was designed (using the NEB Tm calculator v1.12.0) from the sequence of jrPPO2 published previously [20] (Table S1). Q5 High-Fidelity DNA polymerase (NEB, Ipswich, USA) was used for the amplification and a ~ 1,700 base pair amplicon was produced (for detailed PCR setup see supplementary information). The PCR product was cloned into the pENTRY-IBA51 vector and sequenced to reveal the full-length sequence of jrPPO2. Thereafter, the gene encoding jrPPO2 (active and C-terminal domain) was amplified with the second pair of primers (designed based on the sequencing results; Table S1). Using Q5 High-Fidelity DNA polymerase a ~ 1,500 base pair amplicon was obtained, cloned into the pENTRY-IBA51 vector and again sequenced. The sequence-verified construct was sub-cloned into the open reading frame of a pGEX-6P-1 based expression vector using the Esp3I restriction enzyme (Thermo Fisher, Waltham, USA) and transformed into E. coli BL21 (DE3) cells.

Construction of the mutants jrPPO1-Asn240Gly and jrPPO2-Gly240Asn. The genes encoding jrPPO1 and jrPPO2, cloned into the pENTRY-IBA51 donor vector, respectively, served as templates for the mutagenesis experiments. Q5 High-Fidelity DNA polymerase was used to introduce the mutations into the sequence by back to back annealing primers (Table S1) with the forward primer carrying the desired mutation. T4 Polynucleotide Kinase (NEB) and T4 DNA Ligase (NEB) were used to create cyclic plasmids (pENTRY-IBA51). The open reading frames were then sub-cloned into the pGEX-6P-1 expression vector using the Esp3I restriction enzyme and expressed as described previously [21] (see supplementary information).

Molecular mass determination via mass spectrometry. Mass spectra of jrPPO1, jrPPO2, jrPPO1-Asn240Gly and jrPPO2-Gly240Asn were measured on an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) equipped with a nanospray ion source using an ion transfer capillary temperature of 300 °C and an electrospray voltage of 2.1 kV. 5 µl of the sample was loaded on a trap column of an Ultimate 3000 nano HPLC-system (Dionex) using 0.1% trifluoroacetic acid. The separation was carried out at a flow rate of 300 nL/min on a C4 analytical column 50 cm x 75 µm Accucore C4, 150 Å, 2.6 µm (Thermo Fisher Scientific) using mobile phase A (2% acetonitrile, 0.1% formic acid and 98% H2O) and mobile phase B (0.1% formic acid, 20% H2O and 80% acetonitrile). Full MS scans were acquired in positive ion mode ranging from 400 to 2000 m/z at a resolution of 7,500 (FWHM at 400 m/z).

Characterization of jrPPO2, substrate scope assays and kinetic investigation of jrPPO1, jrPPO2, jrPPO1-Asn240Gly and jrPPO2-Gly240Asn. Kinetic measurements were performed in triplicates. Photometric measurements were all carried out on TECAN infinity M200 (Tecan, Salzburg, Austria) in 96 well plates at 25 °C using the latent enzyme and SDS as an activator. pH and SDS optima were determined for jrPPO2 using the diphenolic substrate dopamine. The highest activities were measured at pH 6.0 (50 mM sodium phosphate buffer) and 2.5 mM SDS. Kinetic measurements of jrPPO2 and jrPPO2-Gly240Asn were performed under these conditions by measuring the increase of the colored reaction products photometrically.
Molecular docking with \( \text{jrPPO1} \) and \( \text{jrPPO2} \).

Molecular docking was performed using AutoDock Vina\(^{46} \). The crystal structure of \( \text{jrPPO1} \) (PDB entry 5CE9) was prepared for molecular docking by adding missing side chains using Coot\(^{57} \). A homology model of \( \text{jrPPO2} \) was built using the SWISS-MODEL server\(^{58,59} \). The exhaustiveness was set to 100 and 20 poses were calculated for each target and substrate (Tables S4 and S5).

Structures of the substrates were obtained from PubChem and formatted into pdbqt files using AutoDockTools (ADT, v.1.5.6)\(^\text{60} \). Docking studies were performed with protonated, semi-protonated and deprotonated hydroxy-phenyl groups (generated by editing the substrate pdbqt files). Binding poses were searched in a grid box enclosing the two copper ions of the active site, the 1st and 2nd activity controller residues (Figs. S1 and S2). For \( \text{jrPPO1} \), \( \text{jrPPO1-Asn240Gly} \) and \( \text{jrPPO2} \), 200 µl solution at 25 °C. Due to their limited solubility, the assays for the flavonoid substrates (kaempferol, quercetin, taxifolin, myricetin; Fig. S5) and juglone were performed using 0.1 mM substrate, 100 µg enzyme, 50 mM sodium phosphate buffer, 2 mM (\( \text{jrPPO1} \) and \( \text{jrPPO1-Asn240Gly} \)) or 2.5 mM (\( \text{jrPPO2} \) and \( \text{jrPPO2-Gly240Asn} \)) SDS in 200 µl solution at 25 °C. Due to their limited solubility, the assays for the flavonoid substrates (kaempferol, quercetin, taxifolin, myricetin; Fig. S5) and juglone were performed using 0.1 mM substrate, 100 µg enzyme, 50 mM sodium phosphate buffer, 2 mM (\( \text{jrPPO1} \) and \( \text{jrPPO1-Asn240Gly} \)) or 2.5 mM (\( \text{jrPPO2} \) and \( \text{jrPPO2-Gly240Asn} \)) SDS and variable amounts of enzyme (Table S3). For quercetin and taxifolin, DMSO was added to a final concentration of 10% to increase the solubility of the substrates. The data were fitted to the Michaelis–Menten equation by non-linear curve fitting (OriginPro 8 software; Figs. S15–S18).

For calculating the kinetic parameters (\( k_\text{cat} \) and \( K_\text{m} \)), the maximum reaction rate was measured at 7–8 different substrate concentrations in a total volume of 200 µl containing 50 mM sodium phosphate buffer at pH 6.0, 2 mM (\( \text{jrPPO1} \) and \( \text{jrPPO1-Asn240Gly} \)) or 2.5 mM (\( \text{jrPPO2} \) and \( \text{jrPPO2-Gly240Asn} \)) SDS and variable amounts of enzyme ([Table S3]). For quercetin and taxifolin, DMSO was added to a final concentration of 10% to increase the solubility of the substrates. The data were fitted to the Michaelis–Menten equation by non-linear curve fitting (OriginPro 8 software; Figs. S15–S18).

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Author contributions
Design of the study (F.P., A.R.); conduction of the study, data collection, analysis and interpretation (F.P.); manuscript preparation and review (F.P., A.R.).

Competing interests
The authors declare no competing interests.

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