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Peroxidase 4- Based Enzymatic Synthesis of Stilbene Oligomers in Methyl Jasmonate-Elicited Grapevine Cell Suspensions

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Abbreviations: CD: cyclodextrins, HRP: horseradish peroxidase, LB: lysogeny broth, MeJA: methyl jasmonate, PAL: phenylalanine ammonia lyase, PRX: peroxidase, PRX4: peroxidase 4, STS: stilbene synthase, UV: ultraviolet

ABSTRACT: Stilbenes are specialized metabolites that are particularly abundant in *Vitis* species. Although the biosynthetic pathways of stilbenes have been well-characterized, the role of specific peroxidases in stilbene oligomerization remains to be investigated. In this study, we used grapevine cell cultures to characterize the functional role of *Vitis vinifera* peroxidase 4 (VvPRX4) in the production of resveratrol oligomers after elicitation with methyl jasmonate (MeJA). We showed that MeJA triggers the accumulation of *t*-resveratrol, resveratrol dimers, and predicted resveratrol trimers in culture medium. This accumulation was correlated with upregulation of the *PRX4* gene in grapevine cells. Using bacterial crude extracts containing VvPRX4, we demonstrated that VvPRX4 converts *t*-resveratrol into dimers, *t*- ϵ -viniferin into tetramers, and both combined substrates into resveratrol trimers. Additionally, VvPRX4 mediates the formation of glycosylated dimers using *t*-piceid and *t*-resveratrol as substrates. These results highlight the functional role of VvPRX4 in stilbene oligomerization in MeJA-elicited grapevine cell cultures

INTRODUCTION

Stilbenes are plant-specialized metabolites produced by more than 70 plant species^{1,2} and are particularly abundant in grapevine tissues, where over a hundred stilbene-core compounds have been identified.³ Stilbenes are phytoalexins synthesized in response to pathogen exposure^{4–6} but also abiotic stresses such as ultraviolet (UV) exposure⁷ and water deficit.⁸ Accumulation of stilbenes in grapevine cells, leaves, and berries is also induced in response to various biobased elicitors.^{9–12} Furthermore, their production is enhanced in primed plants with beneficial microbes after pathogen challenges.^{10,13–15} *trans*-Resveratrol (3,4',5-trihydroxy-*trans*-stilbene) is the primary stilbene synthesized in grapevines through the phenylpropanoid pathway. Its biosynthesis begins with phenylalanine, which undergoes three enzymatic steps catalyzed by phenylalanine ammonia-lyase, cinnamate 4-hydroxylase, and *p*-coumarate CoA-ligase, leading to the production of *p*-coumaric acid. This compound is then condensed with three molecules of malonyl-CoA by stilbene synthase to produce *trans*-resveratrol.¹⁶ Various enzymes, including decorating enzymes such as glycosyltransferases, hydroxylases, and methyltransferases, further modify resveratrol into several derivatives including a monoglycosylated monomer (e.g., piceid), a hydroxylated monomer (e.g., piceatannol), a methylated monomer (e.g., pterostilbene), resveratrol dimers (e.g., δ -viniferin, ϵ -viniferin, pallidol, leachianol F-G, and ampelosin A), resveratrol trimers (e.g., α -viniferin and miyabenol C), and resveratrol tetramers (e.g., vitisin B and hopeaphenol). In *Vitis* spp., 13 enzymes have been identified as responsible for the production of resveratrol derivatives, peroxidases being the most extensively studied so far.¹⁶

Resveratrol oligomers are prominently neosynthesized among other derivatives following pathogen infection^{4,17} and exhibit a very high biological activity against a broad range of phytopathogenic microbes compared to monomers.^{18,19} Moreover, resveratrol oligomers display higher anti-inflammatory²⁰ and anticancer²¹ activities compared to resveratrol. This underscores the potential interest of these oligomers in enhancing plant resistance to pathogens as well as in therapeutic applications. However, the biosynthetic pathways of these oligomers are poorly understood. Previous studies have reported that extracellular laccases released from pathogens are able to catalyze the oxidation and polymerization of various phenolic substrates including resveratrol.^{22–25} Stilbene oligomers can also be generated through oxidative dimerization catalyzed by plant enzymes, including peroxidases.^{26,27} However, it remains unclear which peroxidase may be involved in the production of different resveratrol oligomers and whether the enzyme uses resveratrol as the unique substrate or not.

Plant peroxidases (PRXs) are known to catalyze oxidoreduction reactions involving hydrogen peroxide and various substrates including specialized metabolites. These enzymes are typically secreted into the cell wall or the vacuole²⁸ and belong to pathogenesis-related protein family 9. They play significant roles in plant defense mechanisms, germination and development,²⁹ and lignification.³⁰ The horseradish peroxidase (HRP), a class III plant peroxidase (EC 1.11.1.7), was the first enzyme used to demonstrate the oxidative dimerization of resveratrol into δ -viniferin *in vitro*.^{26,31} This enzyme, as all

class III peroxidases, forms radical intermediates from resveratrol, which can self-couple to form oligomers.¹⁶ A large diversity of oligomers has been identified in grapevine,³ and their production can be induced by various elicitors.¹⁶ MeJA,^{32,33} combined or not with cyclodextrins (CD)^{12,34} or stevioside,³⁵ has been identified to stimulate the biosynthesis of resveratrol dimers in grapevine cell cultures, including δ -viniferin, ϵ -viniferin, pallidol, leachianol F-G, and labruscol,^{12,36} while higher oligomers have not been detected. In grapevines, 10 peroxidases, mainly identified in grapevine cell cultures, were shown to use resveratrol as a substrate.¹⁶ However, the reaction products were not always identified, and the formation of higher oligomers was not described.¹⁶

In plants, peroxidases are encoded by a large multigene family.³⁷ *Vitis vinifera* is thought to have 97 PRX genes.³⁷ Transcriptome analysis revealed the existence of seven upregulated peroxidase genes in the MeJA- and stevioside-elicited grapevine cell cultures.³⁸ The authors reported that *VIPRX21* and *VIPRX35* encode functional grapevine class III peroxidases catalyzing the oxidative dimerization of *trans*-resveratrol to form δ -viniferin.³⁸ A similar study has shown that nine class III peroxidase genes including *VvPRX4* and *VvPRX65* were upregulated in *V. vinifera* cell cultures after MeJA treatment in combination or not with CD.³⁹ Various proteins, including peroxidase 4, were also expressed in the secretome of grapevine cell cultures following treatment with MeJA alone or in combination with CD.^{40,41} In addition, conditioned culture medium containing peroxidases from elicited grapevine cell cultures was shown to convert *trans*-resveratrol into resveratrol dimers in the presence of H₂O₂.⁴² However, direct involvement of the culture-medium-secreted class III *VvPRX4* in resveratrol oligomerization remains to be clarified.

In this study, we used *Vitis labrusca* cell cultures to characterize the functional role of the MeJA-upregulated *VvPRX4*³⁹⁻⁴¹ in the production of resveratrol oligomers. We first analyzed the stilbene profile obtained in the MeJA-elicited grapevine cell cultures. We also studied the expression of related biosynthetic genes, including *PAL*, *STS*, and *PRX4*. To further characterize the enzymatic activity of *VvPRX4*, we performed a heterologous expression in *Escherichia coli* and subsequent bioconversion assays using stilbenoids (resveratrol, piceid, and ϵ -viniferin) as substrates. To the best of our knowledge, this is the first study to explore the enzymatic activity of grapevine PRX4. This study aims to understand the functional role of *VvPRX4* in the biosynthesis of resveratrol oligomers, providing new insights into the biosynthesis pathways of these promising molecules.

MATERIALS AND METHODS

Plant Material.

Cultures of *V. labrusca* (L.) cv. Concord were maintained in Gamborg B5 medium containing vitamins, 30 g/L sucrose, 0.2 mg/L kinetin, and 0.1 mg/L naphthalene acetic acid. Cell cultures were maintained in the dark at 26 °C in 300 mL Erlenmeyer flasks containing 100 mL of cell suspension in an orbital shaker at 110 rpm as previously described.¹² Suspension cells were subcultured every week by diluting them to 1/3 (v/v) into fresh medium.

Elicitation of Grapevine Cell Cultures.

Fresh cultures were established by diluting 11 g of cells from seven-day-old flasks into 90 mL of Gamborg B5 medium in 300 mL Erlenmeyer flasks. Cell suspensions were treated 3 days later with 500 μ M of MeJA (Sigma- Aldrich) or EtOH for control. The MeJA solution was prepared in 95% ethanol to achieve a final ethanol concentration of 0.25% in the culture medium (with or without MeJA). Culture cells were sampled at 0, 12, 24, 48, and 72 h post-treatment for gene expression and stilbene analysis. Cultures were filtered under reduced pressure to separate the cells from the culture media. Cells were then frozen in liquid nitrogen and stored at -80 °C, and the medium was kept at -20 °C for stilbene analysis. Two biological replicates, each containing three technical replicates, were performed to ensure the reproducibility of the experiments.

RNA Extraction and qRT-PCR.

Total RNA was extracted from 100 mg of frozen cells using the Extract'all kit (Eurobio) and quantified with a Nanodrop spectrophotometer (Thermo Fisher Scientific). Reverse transcription was performed on 150 ng of RNA using the Verso cDNA synthesis kit (Thermo Fisher Scientific). Four blind RNAs, 150 ng each, were also submitted to a reverse transcription program without the RT enzyme (NRT samples). qRT-PCR was performed using a CFX384 real-time system (Bio-Rad) and the qPCR BIO SyGreen Blue mix. PCRs were carried out in triplicate in 384-well white plates (Brand) with a final volume of 5 μ L per well containing the 1X SYBR Green mix (Taq polymerase and dNTPs), forward and reverse primers (0.3 μ M for all selected genes except *VvSTS* primers at 0.6 μ M), and reverse-transcribed RNA (1:10 diluted). Primers (Table S1) were designed with Primer-Blast (NCBI). Their efficiency was calculated with qRT-PCR on serial dilutions using the amplified fragments of the concerned genes. PCR conditions were 95 °C for 3 min followed by 40 cycles of 5 s at 95 °C and 30 s at 60 °C. For each reaction, a single amplicon with the expected melting temperature was obtained. Quantification of transcripts was normalized using both *VvEF1 α* (elongation factor 1 α ; XM_002284888.3) and *Vv60SRP* (60S ribosomal protein L18; XM_002270599.3) using Bio-Rad CFX Manager software v.4.1. Their stabilities were confirmed by the geNorm algorithm. The cycle threshold (Ct) of each amplification curve was calculated by Maestro software using the second derivative maximum method. Relative expression was calculated with the $\Delta\Delta$ Cq method compared to the control sample at 0 h of treatment.

Heterologous Expression of *VvPRX4* in *E. coli*.

The *PRX4* gene was amplified from reverse-transcribed RNA by PCR using primers 5'-ATGGCCATGGGCCAACTCTCGACCAACTTT- TACTCT-3' and 3'-GGTGCTCGAGTTAAT-TCACCTCCACAGCTC-5', cloned into the bacterial vector pET-22b(+) containing a histidine tag using NcoI (Invitrogen) and XhoI (Invitrogen) restriction enzymes, and sequenced. *E. coli* BL21 (DE3) star cells were transformed by thermal shock with the vector pET-22b(+)-*VvPRX4* or pET-22b(+) (as a control) for protein expression. Baffled shake flask cultures of *E. coli* BL21(DE3) carrying pET-22b(+)-*VvPRX4* or pET-22b(+) were grown at 30 °C in a lysogeny broth (LB) low-salt medium supplemented with 100 mg/L ampicillin at 180 rpm. After overnight preculture for 16 h, the strains were subcultured at 1/100 (v/v) in LB medium. Protein production was induced with 0.5 mM IPTG for 3 h at the exponential phase when OD₆₀₀ reached 0.6. After centrifugation, cell pellets were recovered and lysed with the extraction buffer (0.25 M Tris-HCl, 0.25 M NaCl, 5 mM dithiothreitol, 5% of glycerol, 1 mM PMSF, pH 7.4) with 3 mL of buffer for 1 g of cell pellet. The crude extracts of *E. coli* BL21(DE3) star carrying pET-22b(+)-*VvPRX4* or pET-22b(+) were analyzed by SDS-PAGE and by Western blotting.

SDS-PAGE and Western Blotting

Proteins (20 μg) were incubated for 5 min at 95 °C in Laemmli buffer (800 mM Tris-HCl, 2% SDS, 10% glycerol, 0.005% bromophenol blue, 1% dithiothreitol) and then separated into 12% polyacrylamide SDS-PAGE gel using a Mini-PROTEAN 3 cell electrophoresis system (Bio-Rad). One gel was stained with GelCode Blue Safe (Thermo Scientific) for 1 h and then observed. The other gel was transferred onto a PVDF membrane (Thermo Scientific) by using the iBlot 2 system (Invitrogen). The membrane was blocked for 30 min in Tris-buffered saline solution (Tris-HCl 25 mM, NaCl 137 mM, KCl 2.7 mM) containing 3% low-fat dried milk and 0.5% Tween 20 and washed for 5 min in washing buffer (Tris-buffered saline containing 0.1% low-fat dried milk and 0.5% Tween 20). The membrane was then incubated for 1 h at room temperature in washing buffer containing primary antibodies (6x-histidine tag monoclonal antibody, Invitrogen). After a 5 min wash, the membrane was incubated with secondary antibodies (antimouse IgG, HRP-linked antibody, Cell Signaling). The membrane was washed for 5 min in washing buffer, and the detection was performed using a SuperSignal West Pico PLUS kit (Thermo Scientific) with an Odyssey XF imaging system (LI-COR).

Guaiacol Peroxidase Activity.

Peroxidase activity was determined by measuring the oxidation of guaiacol to tetraguaiacol in the presence of H_2O_2 over 5 min, as previously described.³⁸ Ten microliters of crude extract were transferred to a 96-well microplate to which was added 200 μL of reaction mixture (100 mM phosphate potassium buffer (pH 5), 25 mM guaiacol, and 25 mM of H_2O_2). Absorbance was measured at 470 nm at 25 °C every 30 s for 5 min using a Spark multimode microplate reader (TECAN). To determine the optimum pH, the reaction mixture pH was varied between 3 and 8.5, and the activity was measured in the same conditions. Enzymatic activity was calculated using the extinction coefficient (26.6 $\text{mM}\cdot\text{cm}^{-1}$ at 470 nm) with the following formula, as previously described:⁴³ activity ($\text{U}\cdot\text{mL}^{-1}$) = $(\Delta\text{OD}\cdot\text{min}^{-1} \times \text{TV} \times \text{dF})/(\epsilon_{470} \times \text{EV})$, where $\Delta\text{OD}\cdot\text{min}^{-1}$ = change in absorbance per minute, TV = total volume (mL), dF = dilution factor, ϵ_{470} = molar absorptivity of tetraguaiacol at 470 nm ($\text{mL}\cdot\mu\text{mol}^{-1}\cdot\text{cm}^{-1}$), and EV = enzyme volume (mL).

In Vitro Bioconversion of Stilbenes.

Crude extracts were concentrated using 10K Microsep centrifugal filters (Cytiva) in a 50 mM sodium acetate buffer (pH 5.5) until the enzymatic activity, measured using a guaiacol assay, exceeded 0.2 $\text{U}\cdot\text{mL}^{-1}$. The bioconversion of stilbenes was performed in test tubes, each containing a magnetic stirrer. To perform the oligomerization reaction, crude extracts were diluted in sodium acetate buffer (pH 5.5) to reach a final peroxidase activity of 0.1 $\text{U}\cdot\text{mL}^{-1}$ in the reaction mixture. Resveratrol (previously prepared in EtOH 95%) was then added to the reaction mixture to reach 0.1 U (enzyme activity) per 1 mg of resveratrol, followed by the addition of 10 mM of H_2O_2 . The reaction was performed for 1 h under magnetic stirring (600 rpm). Optimization of the reaction conditions was performed with resveratrol as a substrate using a design-of-experiment approach (MODDE, Sartorius) where temperature (25, 37, and 50 °C) and enzyme/substrate ratios (0.01, 0.05, and 0.1 $\text{U}\cdot\text{mg}^{-1}$ of resveratrol) were varied (Figure S1). Further experiments were carried out under optimized conditions (35.4 °C, 0.1 $\text{U}\cdot\text{mg}^{-1}$) in test tubes under magnetic stirring (600 rpm). Different stilbenes were tested as substrates at the same molar concentration as that for resveratrol (4.38 mM, equivalent to 1 mg of resveratrol). These included *t*-piceid, *t*- ϵ -viniferin, *t*- δ -viniferin, labruscol, and leachianol F-G, as well as combinations of substrates such as resveratrol with piceid or resveratrol with ϵ -viniferin. Each substrate was prepared in EtOH 95%. The bioconversion rate was evaluated as a percentage (%) of the amount of substrate remaining in the reaction medium by UPLC-FL/ HPLC-DAD. Newly formed monomers and oligomers were identified by UPLC-MS. Resveratrol and piceid were purchased from Interchim. *t*- ϵ -Viniferin was purchased from Sigma-Aldrich. Labruscol, leachianol F-G, and *t*- δ -viniferin were synthesized and purified as previously described.²⁴

Stilbene Analysis by UPLC-FL/HPLC-DAD.

t-Resveratrol, *t*-piceid, *t*- ϵ -viniferin, *t*- δ -viniferin, and labruscol were quantified by UPLC (Waters). Culture media were diluted with methanol (1/9: v/ v) and filtered through 0.2 μm polytetrafluoroethylene (PTFE) filters in subdued light conditions before UPLC analysis. Samples were injected onto an Acquity UPLC Cortex C18 reverse phase (1.6 μm 2.1 \times 100 mm) (Waters, St-Quentin en Yvelines, France) equilibrated with a 90:10 (v/v) H_2O /acetonitrile (ACN) mobile phase. Stilbenes were eluted with a linear gradient of 10 to 100% ACN containing 0.1% orthophosphoric acid at a flow rate of 0.5 $\text{mL}\cdot\text{min}^{-1}$ as previously described.¹⁴ The stilbenes were detected with a fluorometer ($\lambda_{\text{ex}} = 330$, $\lambda_{\text{em}} = 375$ nm) and quantified based on standard calibration curves. Leachianol F-G (nonfluorescent) was quantified using a Vanquish HPLC system (Thermo Scientific) equipped with a DAD and an Accucore aQ column (100 \times 3 mm, 2.6 μm). The eluant system was flowed at 0.8 $\text{mL}\cdot\text{min}^{-1}$ and set as follows: 30% of formic acid 0.1% solution, 20 to 45% ACN from 0 to 3 min, 45% ACN from 3 to 4.5 min, 45 to 50% ACN from 4.5 to 5 min, 50 to 55% ACN from 5 to 6.5 min, 55 to 60% ACN from 6.5 to 7 min, 60% ACN from 7 to 7.5 min, 60 to 20% ACN from 7.5 to 8 min, and finally 20% ACN from 8 to 9 min and complete by Milli-Q water. Leachianol F-G was detected at 0.75 min at 210 nm.

Identification of Stilbenes by UPLC-MS Analysis.

Stilbenes were identified by UPLC-MS analysis using an ACQUITY ultra-performance liquid chromatography system coupled to a photodiode array detector (PDA) and a Xevo TQD mass spectrometer (Waters, Milford, MA) equipped with an electrospray ionization (ESI) source controlled by Masslynx 4.1 software (Waters, Milford, MA). The metabolite separation was performed using a Waters Acquity HSS T3 C18 column (150 \times 2.1 mm, 1.8 μm) with a flow rate of 0.4 $\text{mL}\cdot\text{min}^{-1}$ at 55 °C. Chromatograms were analyzed by selected ion monitoring (SIM) at m/z 227 (*t*-resveratrol RT = 10.7 min), 389 (*t*-piceid RT = 8.2 min), 453 (*t*- ϵ -viniferin RT = 13.4 min, *t*- δ -viniferin RT = 15.1 min, pallidol RT = 9.9 min), 471 (leachianol F-G RT = 7.2 min), 615 (monoglycosylated resveratrol dimer), 679 (resveratrol trimer), 777 (diglycosylated-*t*- δ -viniferin RT = 11.3 min), and 905 (hopeaphenol RT = 11.8 min, resveratrol tetramers). *t*-Resveratrol, *t*-piceid, and *t*- ϵ -viniferin were purchased from Sigma-Aldrich standards (St. Louis, MI, USA). Pallidol and hopeaphenol were obtained as previously described.⁴⁴ *t*- δ -Viniferin diglycosylated was synthesized and purified as previously described.⁴⁵ δ -Viniferin, leachianol F-G, and labruscol were synthesized and purified as the ones used for in vitro bioconversion tests.

Statistical Analysis.

Multiple *t*-tests were performed to statistically analyze the results of Figures 1A and 2 using Prism6 (GraphPad Software) and the Holm-Sidak method. Asterisks denote the significant differences between MeJA treatment and control: *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; and ns, nonsignificant difference.

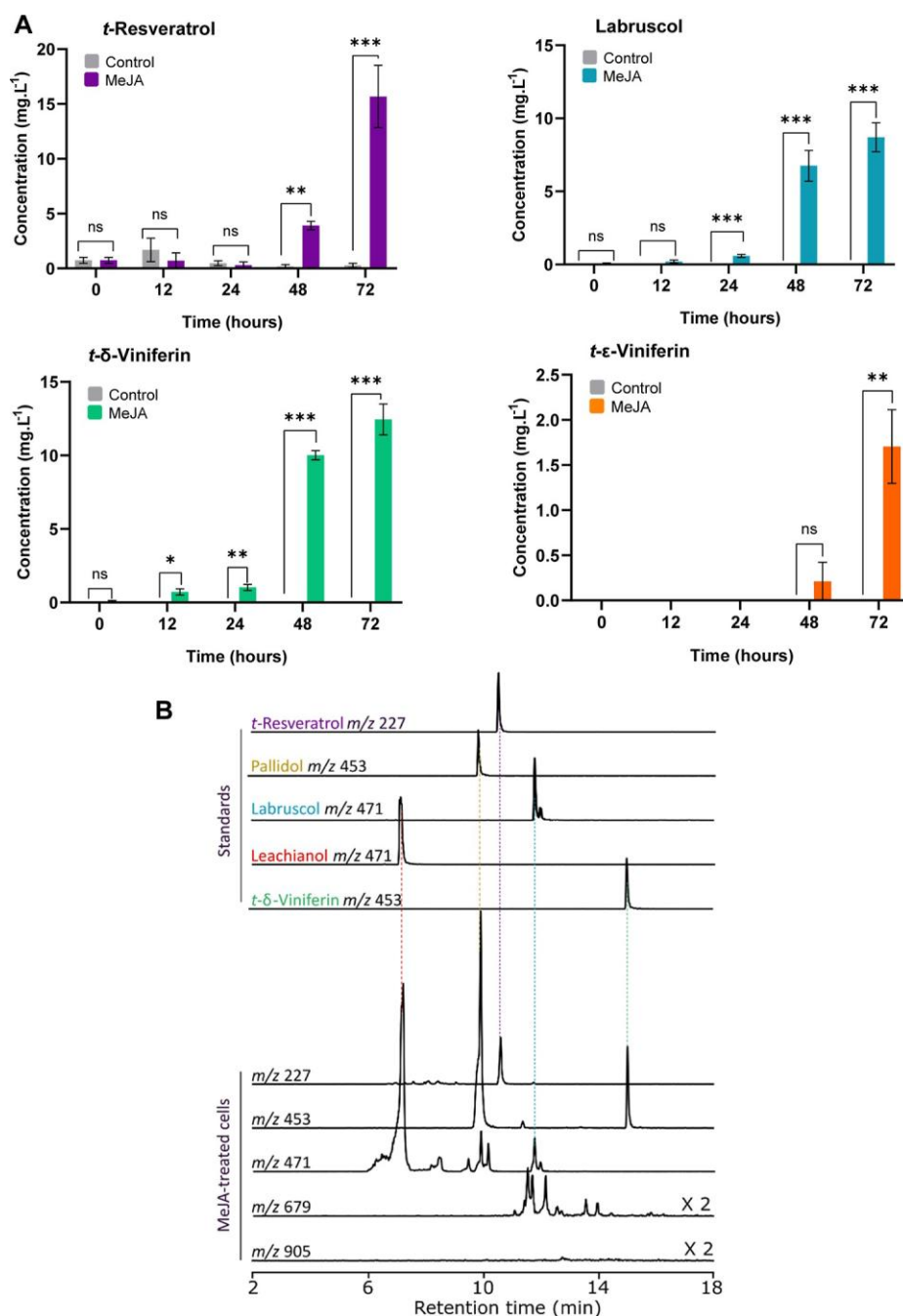


Figure 1. Resveratrol and its oligomers accumulate in the cell culture medium of *V. labrusca* following MeJA treatment. (A) Concentration ($\text{mg} \cdot \text{L}^{-1}$) of *t*-resveratrol, labruscol, *t*- δ -viniferin, and *t*- ϵ -viniferin in culture medium after MeJA treatment. *Vitis labrusca* cell cultures were treated with MeJA ($500 \mu\text{M}$) or EtOH (0.25%) as a negative control 3 days after subculture. Data are means of two biological replicates with three technical repeats each. Bars indicate the standard error of the mean (SEM). Statistical significance is denoted by asterisks (*) based on multiple *t*-test statistical tests (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, nonsignificant difference). (B) Stilbenes identified by UPLC-MS at 72 h post-treatment with MeJA. Upper SIM chromatograms represent the standards of *t*-resveratrol, pallidol, labruscol, leachianol F-G, and *t*- δ -viniferin. Lower SIM chromatograms represent stilbene identified in MeJA-treated cells. Compounds at five mass-to-charge (*m/z*) ratios were detected corresponding to resveratrol (*m/z* 227), resveratrol dimers (*m/z* 453 and *m/z* 471), resveratrol trimers (*m/z* 679), and resveratrol tetramers (*m/z* 905).

RESULTS

MeJA Induces Resveratrol and Oligomer Accumulation in Cell Culture Medium.

To study the production of stilbenes in MeJA-elicited grapevine cell cultures, the culture medium was collected and quantified by UPLC at different time points (0, 12, 24, 48, and 72 h) (Figure 1A). Cells were treated with $500 \mu\text{M}$ MeJA as this concentration was more efficient to enhance the accumulation of stilbenes compared to 100 and $200 \mu\text{M}$ (Figure S2). Forty-eight h after MeJA treatment, *t*-resveratrol significantly increased compared to the control, reaching about $15.7 \text{ mg} \cdot \text{L}^{-1}$ at 72 h (Figure 1A). Significant production of *t*- δ -viniferin, labruscol, and *t*- ϵ -viniferin was observed at 12, 24, and 72 h, respectively, with levels

reaching approximately 12.5, 8.70, and 1.70 mg·L⁻¹ at 72 h, respectively (Figure 1A). Simultaneously, MeJA considerably slowed down cell growth (Figure S3) without affecting cell viability (Figure S4) and induced a small accumulation of the four stilbenes at the cellular level (including the cell wall), representing less than 15% of total stilbenes identified (Figure S5).

In order to identify major peaks in the resulting chromatograms, analysis was performed with UPLC-MS at 72 h post-treatment (Figure 1B). UPLC-MS analysis showed the presence of two molecular ions of *m/z* 453 corresponding to pallidol and *t*- δ -viniferin after comparison with pure standards (Figure 1B). Additionally, several molecular ions of *m/z* 471, including leachianol F-G and labruscol, were identified. UPLC-MS also revealed several molecular ions of *m/z* 679, specific to resveratrol trimers (Figure 1B). No molecular ions of *m/z* 905, which would indicate the presence of resveratrol tetramers, were detected (Figure 1B).

MeJA Upregulates VvPAL15, VvSTS3, and VvPRX4 Gene Expression.

To further understand the effect of MeJA on the stilbene biosynthetic pathway and the oligomer production, the expression of genes encoding phenylalanine ammonia lyase 15 (*VvPAL15*), stilbene synthase3 (*VvSTS3*), and peroxidase 4 (*VvPRX4*) was analyzed in grapevine cell culture at 0, 12, 24, 48, and 72 h using RT-qPCR and specific primers (Table S1). Data showed that in control cells, the transcript accumulation of the different genes was very low (Figure 2). In grapevine cells treated with MeJA, *VvPAL15* and *VvSTS3* genes were rapidly upregulated. The levels of mRNA for *VvPAL15* and *VvSTS3* increased 12 h after MeJA was supplied and were maximal at 24 h post-treatment. Thereafter, transcripts of both genes remained constant or slowly declined. The *VvPRX4* gene was also significantly upregulated at 24 h post-treatment and reached high expression at 48 h after MeJA treatment (Figure 2). These kinetics results showed that the *VvPRX4* transcript accumulation preceded the accumulation of resveratrol dimers, *t*- δ -viniferin, and labruscol in cell culture medium (Figure 1A).

Expression and Peroxidase Activity of Recombinant VvPRX4 in E. coli.

To evaluate the capacity of VvPRX4 to oligomerize stilbenes, the *VvPRX4* gene was cloned into the pET-22b(+) vector and expressed in the *E. coli* heterologous system. The histidine-tagged VvPRX4 production was confirmed by Western blotting (Figure 3B). Several bands of different masses were revealed by Coomassie blue (Figure 3A), including a 34 kDa band, corresponding to the size of VvPRX4 in the bacterial protein extract. The same band of 34 kDa was also detected by Western blot (Figure 3B). Bands of smaller sizes were also observed, indicating the presence of degradation products. No bands were detected in the negative control sample (Figure 3B). To investigate the role of VvPRX4 in stilbene oligomer production, purification of the histidine-tagged target protein was assessed using a nickel resin column (Figure S6). However, this method did not result in a successful protein isolation due to the low amount of soluble PRX4 protein produced in *E. coli*, as it was previously reported for horseradish peroxidase expression in *E. coli*,⁴⁶ and the binding of native *E. coli* protein on the column (identified as *S*-adenosylmethionine synthase in this study). Thus, the experiments were performed using crude protein extracts from bacterial lysates of *E. coli* BL21(DE3) star containing either pET22b(+)-VvPRX4 or pET22b(+) as a negative control. Crude extracts from heterologous expression were also used to characterize peroxidase activity as previously described.³⁸ To further assess the enzyme function, peroxidase activity was measured using a guaiacol assay (Figure 3C). The crude extract containing VvPRX4 showed peroxidase activity across a pH range of 3.5 to 8.5, with peak activity observed at pH 5.5 and 6 (Figure 3C). No activity was observed in the crude extracts containing an empty vector (Figure 3C).

Stilbene monomers (*t*-resveratrol and *t*-piceid) as well as stilbene dimers (*t*- ϵ -viniferin, labruscol, leachianol F-G, and *t*- δ -viniferin) were tested as substrates to evaluate their conversion by crude extract containing VvPRX4. The remaining amounts of all substrates were evaluated using UPLC-FL, except for leachianol, which was quantified using HPLC-DAD. After 1 h of incubation under optimal conditions determined previously (Figure S1), the entire amount of *t*-resveratrol or *t*-piceid was consumed in the reaction media (Figure 3D), indicating a strong affinity of VvPRX4 for these two stilbene substrates. Approximately 40% of *t*- ϵ -viniferin remained after its incubation with VvPRX4 (Figure 3D), suggesting that this dimer is partially used by the enzyme to form oligomers. However, 100% of leachianol F-G and *t*- δ -viniferin and about 90% of labruscol remained in the reaction medium after 1 h of enzymatic reaction (Figure 3D).

In Vitro Bioconversion of Stilbenes by VvPRX4.

Products formed from reactions involving *t*-resveratrol, *t*-piceid, and *t*- ϵ -viniferin as substrates were identified by UPLC-MS (Figure 4). None of the three molecules were used or converted in the negative controls (empty vector; Figure 4). However, using *t*-resveratrol as a substrate in the presence of VvPRX4, UPLC-MS identified two molecular ions of *m/z* 453 corresponding to pallidol and *t*- δ -viniferin along with one molecular ion of *m/z* 471 corresponding to leachianol F-G. This indicates that the entire resveratrol used by VvPRX4 can be converted at least into 3 different resveratrol dimers (Figure 4A). Various molecular ions of *m/z* 905 were also identified from *t*- ϵ -viniferin in the presence of VvPRX4 (Figure 4B), including hopeaphenol. With *t*-piceid as the substrate, one molecular ion of *m/z* 777, corresponding to diglycosylated-*t*- δ -viniferin, was generated in the presence of VvPRX4 (Figure 4C).

The combination of *t*-resveratrol and *t*- ϵ -viniferin in reaction medium in the presence of VvPRX4 led to the formation of two molecular ions of *m/z* 453, identified as pallidol and *t*- δ -viniferin, which are specific to the coupling of two *t*-resveratrol molecules (Figure 4D). One molecular ion of *m/z* 905 corresponding to hopeaphenol was detected from the specific VvPRX4-induced coupling of two *t*- ϵ -viniferin molecules (Figure 4D). Other molecular ions of *m/z* 679, specific to the combination of *t*-resveratrol and *t*- ϵ -viniferin, were also detected, indicating the presence of resveratrol trimers in the presence of VvPRX4 (Figure 4D). The combination of *t*-resveratrol and *t*-piceid in the VvPRX4 reaction medium resulted in the formation of two molecular ions of *m/z* 615, corresponding to two monoglycosylated resveratrol dimers, resulting from the coupling of *t*-resveratrol and *t*-piceid (Figure 4E). Pallidol, *t*- δ -viniferin, and leachianol F-G were also obtained from the *t*-resveratrol combination, as well as one molecular ion of *m/z* 777 corresponding to the same diglycosylated resveratrol dimer resulting from *t*-piceid dimerization (Figure 4E).

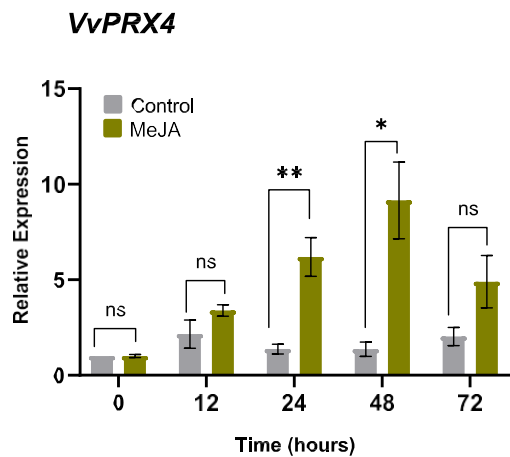
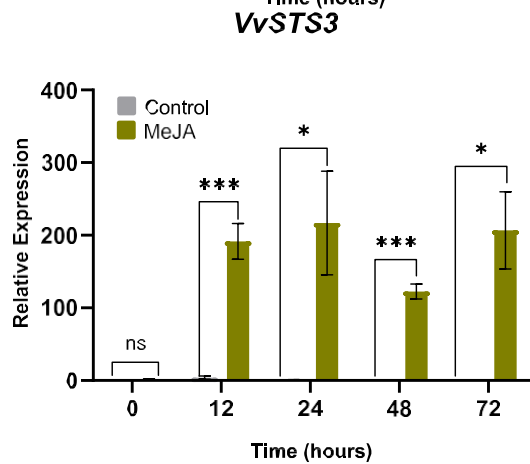
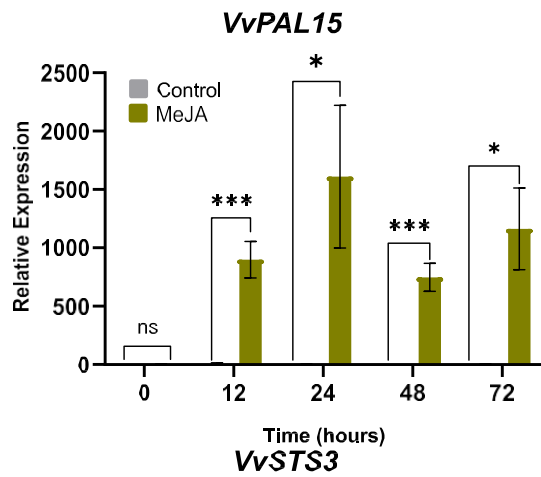


Figure 2. MeJA upregulates *VvPAL15*, *VvSTS3*, and *VvPRX4* expression in *V. labrusca* cell cultures. Cells were precultured for 3 days prior to MeJA treatment at 500 μ M and subsequently harvested at various time points for qRT-PCR analysis. EtOH (0.25%) was used as a negative control. Relative expression was normalized using *VvEF1* and *Vv60SRP* as housekeeping genes and compared to the control sample at 0 h. Data are means of two biological replicates with three technical repeats each. Bars represent the standard error of the mean (SEM). Statistical significance is denoted by asterisks (*) based on multiple *t*-test statistical tests (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ns, nonsignificant difference). *PAL* = phenylalanine ammonia-lyase; *STS* = stilbene synthase; *PRX* = peroxidase; *EF1* = elongation factor 1 α ; *60SRP* = 60S ribosomal protein.

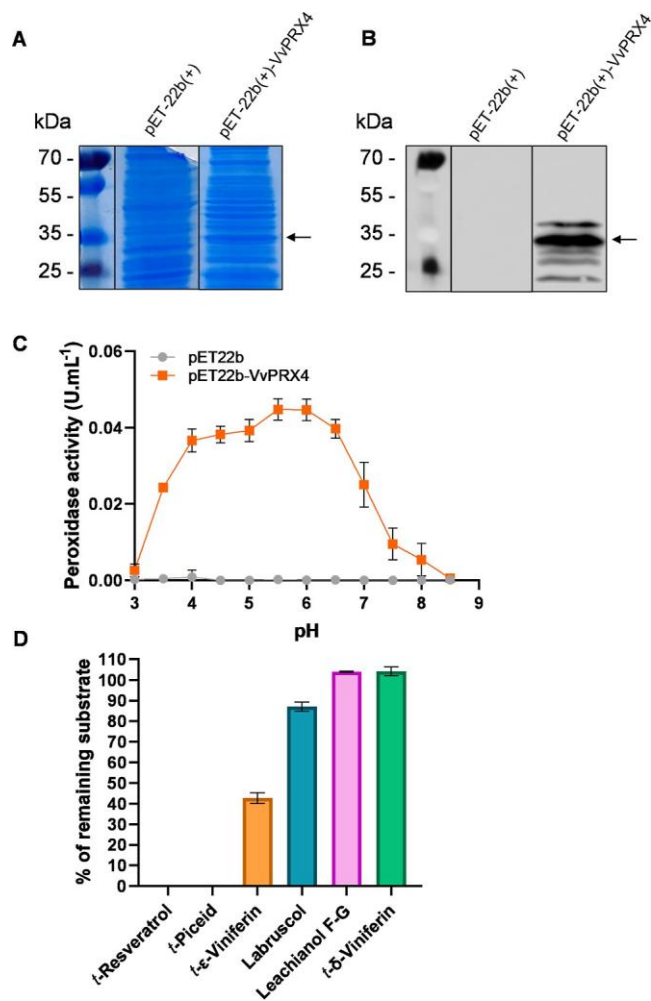


Figure 3. Electrophoretic profiling and peroxidase activity of crude enzyme extract from *E. coli* expressing histidine-tagged VvPRX4 regarding guaiacol and various substrates. (A) SDS-PAGE protein profiles revealed by Coomassie blue staining and (B) detection by Western blot of histidine-tagged VvPRX4 by 6 × -His epitope tag antibody. Culture of *E. coli* BL21 (DE3) star transformed with pET22b(+) (negative control) or with pET22b(+)-VvPRX4 was recovered 3 h postinduction with 0.5 mM IPTG. Bacteria were lysed using sonication to obtain crude extracts, from which 20 μ g of proteins was loaded on gel. Arrows indicate the position of VvPRX4 at 34 kDa (C) Peroxidase activity assays conducted at varying pH using bacterial crude extracts with or without VvPRX4. Enzyme activity was determined by measuring the oxidation of guaiacol to tetraguaiacol at 25 °C every 30 s for 5 min. Data are means of three technical repeats. Error bars represent the standard error of the mean (SEM). (D) Percentage of remaining substrates after 1 h of bioconversion by VvPRX4 (0.1 U.mL⁻¹) at 35.4 °C under magnetic stirring (600 rpm). *t*-Resveratrol, *t*-piceid, *t*- ϵ -viniferin, labruscol, leachianol F-G, and *t*- δ -viniferin were tested as substrates (4.38 mM), and their remaining quantities were evaluated using UPLC or HPLC analysis. Data are means of three technical repeats. Error bars represent the standard error of the mean (SEM).

DISCUSSION

Resveratrol oligomers are among the prominent phytoalexins produced in grapevines after biotic and abiotic stresses.² Previous studies reported that resveratrol could be oligomerized through oxidative dimerization catalyzed by plant peroxidases.^{2,26} Nevertheless, there is no evidence of specific peroxidases involved in resveratrol oligomerization in grapevine cell cultures. Few previous studies indicated that VvPRX4, belonging to the Class III peroxidase family, accumulated in the extracellular proteome of grapevine cell cultures after MeJA treatment,^{40,41} and the *PRX4* gene was upregulated in *V. vinifera* Monastrell MeJA-treated cells.³⁹ However, the relationship between *PRX4* expression and resveratrol oligomerization in grapevine cell cultures remained unclear. In this study, we reported that VvPRX4 produced in *E. coli* catalyzes the formation of various stilbene oligomers from monomers (*t*-resveratrol and *t*-piceid) and a dimer (*t*- ϵ -viniferin), highlighting the functional role of VvPRX4.

We identified five distinct dimers (*t*- ϵ -viniferin, *t*- δ -viniferin, labruscol, leachianol F-G, and pallidol) and several trimers in *V. labrusca* cell culture medium in response to MeJA treatment (Figure 1). *t*- δ -Viniferin and labruscol were detected earlier and at a higher level than *t*- ϵ -viniferin (Figure 1A), suggesting that this dimer is more reactive and may act as a substrate for further oligomerization, which is consistent with the presence of resveratrol trimers (m/z 679) (Figure 1B). Other predicted resveratrol dimers of m/z 471 were detected by UPLC-MS, requiring further structural investigations (Figure 1B). This is in line with other studies showing a low amount (or none) of *t*- ϵ -viniferin in MeJA-elicited grapevine cells,^{12,33} unlike other dimers such as *t*- δ -viniferin. Indeed, resveratrol is synthesized within the cells before being excreted into the extracellular environment. Once outside the cell, resveratrol is mostly converted by peroxidase 4 to oligomers within the cell wall and partially converted in the extracellular medium. This process explains why these oligomers, such as *t*- ϵ -viniferin, *t*- δ -viniferin, and labruscol, are found in large quantities in the culture medium as well as at the cellular level (Figure S5).

Our results also showed a significant upregulation of *VvPAL15* and *VvSTS3* genes in MeJA-elicited grapevine cells at 12 h post-treatment, which is consistent with the production of resveratrol as the primary stilbene synthesized in grapevines. However, the low amount of resveratrol detected at 12 and 24 h could be attributed not only to its biosynthetic pathway being activated via *PAL* and *STS* genes but also to its oligomerization mediated by peroxidases, including VvPRX4 (Figure 2). Indeed, the significant upregulation of *VvPRX4* by MeJA at 24 h post-treatment preceded the accumulation of resveratrol oligomers (Figure 1A) and was previously reported through transcriptomic and RT-qPCR analyses in *V. vinifera* Monastrell cell cultures.^{39,47} This temporal correlation suggests that VvPRX4 plays a role in resveratrol oligomerization in grapevine cells.

To evaluate the capacity of VvPRX4 to oligomerize stilbenes, the *E. coli* heterologous system was used to produce VvPRX4 (Figure 3A,B). Among the different stilbene substrates tested, VvPRX4 exhibited peroxidase activity (Figure 3C) and a strong affinity for stilbene monomers *t*-resveratrol and *t*-piceid and dimer *t*- ϵ -viniferin, but also for combined stilbenes (*t*-resveratrol and *t*- ϵ -viniferin and *t*-resveratrol and *t*-piceid). However, the enzyme showed minimal or no activity toward other dimers such as leachianol F-G and *t*- δ -viniferin and only slight activity with labruscol (10%) (Figure 3D). The reactivity of *t*- ϵ -viniferin could be due to its *para*-conjugated moiety, which is lacking in the other stilbene dimers.^{16,48} This is consistent with the low amount of *t*- ϵ -viniferin in extracellular media of MeJA-treated *V. labrusca* cell cultures and the production of resveratrol trimers, consisting of resveratrol coupled with *t*- ϵ -viniferin (Figure 1). This is also in line with enzymatic assays pointing to a significant VvPRX4 affinity for *t*-resveratrol, *t*-piceid, and to a lesser extent for *t*- ϵ -viniferin (Figure 3D). The glycosylated backbone in *t*-piceid did not hinder VvPRX4 activity, as evidenced by the production of glycosylated dimers and oligomers.

UPLC-MS analysis confirmed that VvPRX4 catalyzes the formation of various stilbene oligomers, including dimers (e.g., *t*- δ -viniferin, pallidol, and leachianol F-G), monoglycosylated dimers, trimers, diglycosylated *t*- δ -viniferin, and tetramers (e.g., hopeaphenol). Interestingly, the dimers obtained from resveratrol conversion by VvPRX4 correspond to the dimers detected in *V. labrusca* cell culture media (Figures 1 and 4A). Similar reaction products were also obtained using HRP at a similar pH range.⁴⁹ Detection of these compounds suggests that VvPRX4 functions in the oxidative oligomerization of stilbenes in MeJA-elicited grapevine cell cultures. However, some products observed in grapevine cell cultures, such as labruscol,⁵⁶ were not detected in VvPRX4-catalyzed reactions (Figures 1 and 4A). This suggests that other specific peroxidases or enzymes might likely be involved rather than VvPRX4. For instance, plant laccases have been shown to polymerize various secondary metabolites including flavonoids.⁵⁰ It cannot be excluded that some plant laccases could contribute to the formation of labruscol, as it was shown with fungal laccases.^{23,24,51} Similarly, *t*- ϵ -viniferin was not detected from the resveratrol bioconversion reaction through VvPRX4, as it was previously observed through HRP activity.^{31,38}

The detection of tetramers (e.g., hopeaphenol) from *t*- ϵ -viniferin oligomerization (Figure 4B) supports previous studies where such compounds, including vitisin B, hopeaphenol, isohopeaphenol, and vitisin C, were produced by other peroxidases like HRP.^{2,48} Moreover, the formation of resveratrol dimers, trimers, and tetramers in reactions involving *t*-resveratrol and *t*- ϵ -viniferin (Figure 4D) suggests that VvPRX4 plays a significant role in their biosynthesis, contributing to the lower abundance of *t*- ϵ -viniferin in culture media compared to *t*- δ -viniferin (Figures 1 and 4). Similar results have been reported in bioconversion assays with HRP.⁵²

The dimerization of *t*-piceid under VvPRX4 activity resulted in a unique glycosylated dimer (m/z 777), contrasting with the formation of three distinct dimers when *t*-resveratrol was further used as a substrate (Figures 4C and 4A). This suggests that the sugar backbone in *t*-piceid seems to influence the enzymatic reaction toward a single dimer. Moreover, the combination of *t*-piceid and *t*-resveratrol in the reaction medium results in the production of two monoglycosylated dimers with m/z = 615 (Figure 4E). This might result from the cross-link of *t*-resveratrol and *t*-piceid through VvPRX4 activity. Resveratrol glucoside dimers have already been identified in grapevine cell cultures producing piceid.⁵³ A large diversity of oligomers was also identified in vegetative organs of grapevine, including 30 resveratrol dimers and 8 resveratrol trimers.³ This is consistent with the expression of the *VvPRX4* gene in grapevine roots,⁵⁴ in stems following drought stress,⁵⁵ and in brown striped trunk wood of grapevine plants, which also accumulate stilbene oligomers.⁵⁶

Overall, our study demonstrated that VvPRX4 is involved in stilbene oligomerization and that this reaction depends on the stilbene backbone (Figure 5). The VvPRX4 affinity was very high toward monomers, while resveratrol dimers were partly or not oligomerized. However, it is not excluded that other peroxidases such as those previously identified in transcriptomic analysis³⁹ could also be

involved in the stilbene oligomerization process. This hypothesis is supported by another study, indicating that several peroxidases seem to be involved in δ -viniferin production³⁸. Further studies are needed to understand the functional role of peroxidase 4 and other enzymes with oxidative oligomerization activity in different grapevine organs. Such studies could provide a better understanding of the contribution of these enzymes to stilbene oligomer production under natural conditions. This would complement the findings obtained with cell cultures and help to determine their ultimate role in enhancing plant tolerance to various environmental stresses. Overall, this study opens new ways toward the bioproduction of bioactive resveratrol oligomers.

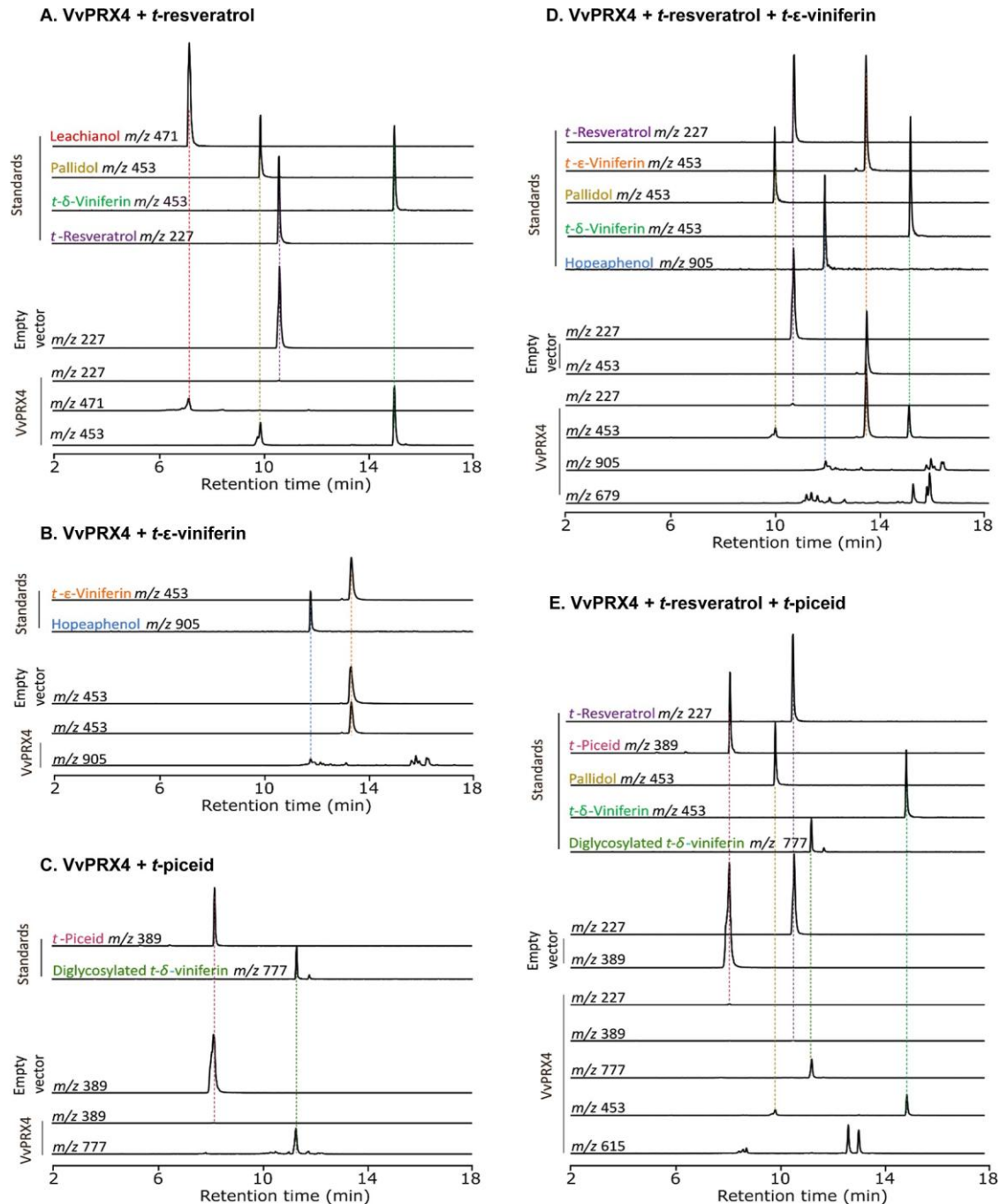


Figure 4. *t*-Resveratrol, *t*-piceid, and *t*- ϵ -viniferin are oligomerized by VvPRX4 to form stilbenoids. UPLC-MS analysis of stilbenoids produced following bioconversion of various stilbenes by crude extract of *E. coli* BL21 (DE3) star bacteria transformed with pET-22b(+)-VvPRX4. (A) *t*-Resveratrol, (B) *t*- ϵ -viniferin, (C) *t*-piceid, (D) *t*-resveratrol and *t*- ϵ -viniferin, and (E) *t*-resveratrol and *t*-piceid were used as substrates. They were incubated at 4.38 mM with VvPRX4 (0.1 U.mL⁻¹) for 1 h at 35.4 °C under magnetic stirring (600 rpm). For each experiment, crude extracts of *E. coli* BL21 (DE3) star lysate transformed with the pET-22b(+) empty vector were used as a negative control. Upper SIM chromatograms correspond to the standards of leachianol F-G, pallidol, *t*- δ -viniferin, *t*-resveratrol, *t*- ϵ -viniferin, hopeaphenol, *t*-piceid, and diglycosylated *t*- δ -viniferin. Lower SIM chromatograms correspond to stilbenoids formed by VvPRX4 oligomerization.

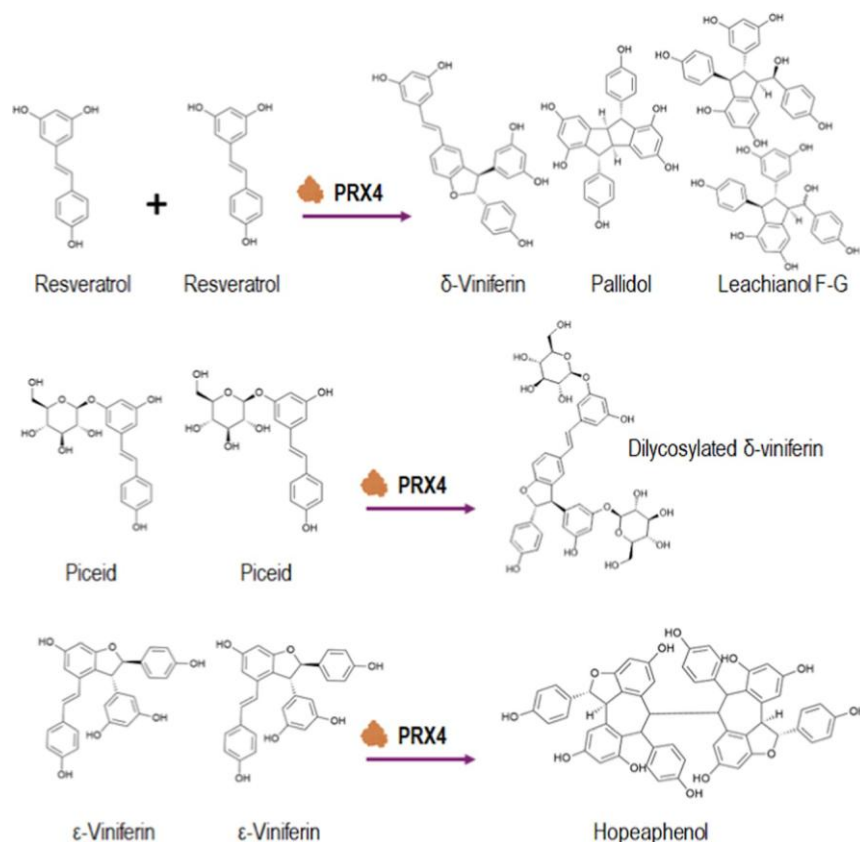


Figure 5. Representative stilbene oligomer structures produced by VvPRX4. The figure depicts the structure of the substrates (*t*-resveratrol, *t*-piceid, and *t*- ϵ -viniferin) and their oligomers (*t*- δ -viniferin, pallidol, leachianol F-G, diglycosylated *t*- δ -viniferin, and hopeaphenol) produced by VvPRX4.

Supporting Information

The Supporting Information is available free of charge at <https://pubs.acs.org/doi/10.1021/acs.jafc.4c09502>.

Optimal conditions for resveratrol bioconversion using bacterial crude extracts containing VvPRX4 determined by design of experiment; effect of MeJA concentration on stilbene accumulation in culture media at 72 h post-treatment; effect of MeJA treatment on *V. labrusca* cell growth; effect of MeJA treatment on *V. labrusca* cell viability; accumulation of stilbenes at the cellular level after 72 h of MeJA treatment; purification assay of heterologously expressed VvPRX4 by nickel resin; and specific primers and their efficiencies used for RT-qPCR analysis (PDF)

Author Contributions

A.A. and J.C. are co-last authors. Conceptualization, B.M., E.C., A.A., and J.C.; methodology, validation, analysis, and visualization, B.M., A.L.F., A.L., F.M., D.A., A.A., and J.C.; investigation, B.M., A.L.F., A.L., F.M., G.G., L.P., V.L., and D.A.; writing original draft preparation, B.M.; writing review and editing, B.M., A.A., and J.C. All authors have read and agreed to the published version of the manuscript.

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