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Heterologous production and secretion of active-form plant peroxidase in *Brevibacillus choshinensis* and their monolignol polymerization abilities analysis

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Abstract

Plant peroxidases are important for several processes, such as defense against pathogens, and auxin metabolism. In this study, we report the active production and secretion of plant peroxidase and mutated enzymes in the bacterium *Brevibacillus choshinensis* for the first time in the world. We introduced mutations into *prxA3a*, an anionic peroxidase gene of hybrid aspen, *Populus* × *kitakamiensis*, to substitute the amino acid residues at the surface of the protein and analyzed their modified substrate specificities. We have also reported heterologous expression of PrxA3a and mutated enzymes in yeast. Enzymes secreted in the culture medium by *B. chosinensis* were purified by Ni affinity chromatography, anion-exchange chromatography, and size-exclusion chromatography. The ability of the mutated enzymes to polymerize sinapyl alcohol, a monolignol, was higher than that of the wild-type enzyme. In particular, the FYAW-mutated enzyme produced by the bacterium showed higher polymerization activity, similar to that of the FYAW-mutated enzyme produced by yeast.

Keywords Peroxidase, *Brevibacillus chosinensis*, Oxidation, Polymerization, Sinapyl alcohol, PrxA3a

Introduction

Peroxidases (EC 1.11.1.7) catalyze the oxidation of various reductants using hydrogen peroxide [1, 2]. Plant peroxidases are involved in several physiological processes, *e.g.,* as the final step of monolignol oxidation in lignin biosynthesis [3, 4], polymerization of extensin [5], defense response to wounding [6, 7], defense against pathogens [7, 8], and auxin metabolism [9, 10]. Since plant peroxidase genes form a multigene family in a plantgenome, many peroxidase species are produced in plant cells. It is cumbersome to purify the specific peroxidase from others to investigate its properties. Therefore, it is important to express the isolated plant peroxidase gene in microorganisms and purify the product by recombinant technology.

Horseradish peroxidase C (HRP-C) is a typical plant peroxidase used to study the mechanism of lignin polymerization. However, HRP-C is not present to any extent in lignified tissues [11, 12]. HRP-C, horseradish peroxidase A2 (HRP-A2), and *Arabidopsis thaliana* peroxidase A2 (ATP-A2; 95% identity to HRP-A2) can oxidize coniferyl alcohol efficiently, but not sinapyl alcohol [13, 14], because sinapyl alcohol is prevented from entering the substrate binding site of plant peroxidases by interference between the proline residue in their substrate-binding site, *e. g.,* Pro-139 of ATP-A2, and the 5-methoxy moiety of sinapyl alcohol [15]. This assumption is generally applicable because proline residues in the corresponding

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positions are highly conserved in plant peroxidases (Fig. 1). For instance, 69 of 73 peroxidases in *A. thaliana* contain proline residues at their corresponding positions [13, 16]. Because HRP-C and ATP-A2 share high identity, including this proline residue, HRP-C, as a representative of plant peroxidases, cannot catalyze sinapyl alcohol as a preferred substrate. PrxA3a [protein_id = "BAA07240.1"], a peroxidase from hybrid aspen *Populus* × *kitakamiensis* (*P. sieboldii* × *P. grandidentata*), is involved in lignin biosynthesis [1]; it also contains the proline residue in the corresponding position (Fig. 1) and has similar substrate specificities as HRP-C. On the other hand, cationic cell wall-bound peroxidase (CWPO-C) [protein_id = "BAE16616.1"] from *Populus alba* could catalyze the oxidation of sinapyl alcohol as preferred substrate [17–21], though Pro-135 corresponding to Pro-139 of ATP-A2 is conserved in CWPO-C. Furthermore, CWPO-C can catalyze the oxidation of synthetic lignin polymers

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                                                          *   *
PrxA3a      1 -QLSPTFYDEACPNVNNIIRGVLVQALYTDPR--IGASLTRLHFHDCFVNG-CDGSILLDNTDTIESEKE
ATP-A2      1 -QLNATFYSGTCPNASAIVRSTIQQALQSDTR--IGASLIRLHFHDCFVNG-CDASILLDDTGSIQSEKN
HRP-C       1 -QLTPTFYDNSCPNVSNIVRDTIVNELRSDPR--IAASILRLHFHDCFVNG-CDASILLDNTTSFRTEKD
BGP-1       1 ---------QTCPRAESIVREFVQEAVRKDIG--LAAGLLRLHFHDCFVQG-CDASVLLDGSATGPGEQQ
SBP         1 -QLTPTFYRETCPNLFPIVFGVIFDASFTDPR--IGASLMRLHFHDCFVQG-CDGSVLLNNTDTIESEQD
CWPO-C      1 QGTRVGFYATTCRRAESIVRATVQSHFTSDSS--IAPGLLRMHFHDCFVNG-CDASILIDGANT---EKT
LiP-H8      1 ---GKTVGDASCCAWFDVLDDIQQNLFHGGQCGAEAHESIRLVFHDSIAISPAMEAQGKFGGGGADGSIM

PrxA3a     67 AAPNNNSVR-GFDVVDDMKAALENACPG-IVSCADILAIAAEQSVCLAGGPSWTVPLGRRDSLI-ANRSG
ATP-A2     67 AGPNVNSAR-GFNVVDNIKTALENACPG-VVSCSDVLALASEASVSLAGGPSWTVLLGRRDSLT-ANLAG
HRP-C      67 AFGNANSAR-GFPVIDRMKAAVESACPR-TVSCADLLTIAAQQSVTLAGGPSWRVPLGRRDSLQ-AFLDL
BGP-1      59 APPNLTLRPSAFKAVNDIRDRLERECRGAVVSCSDILALAARDSVVVSGGPDYRVPLGRRDSRSFASTQD
SBP        67 ALPNINSIR-GLDVVNDIKTAVENSCPD-TVSCADILAIAAEIASVLGGGPGWPVPLGRRDSLT-ANRTL
CWPO-C     65 AGPNLLLR--GYDVIADAKTQLEAECPG-VVSCADILALAARDSVVLTKGLTWPVPTGRRDGR--VSLAS
LiP-H8     68 IFDDIETAFHPNIGLDEIVKLQKPFVQKHGVTPGDFIAFAGRVALSNCPGAPQMNFFTGRAPAT---QPA

                 *                                 *
PrxA3a    134 ANSALPSPFASLDVLKSKFAAVGLDTSSDLVALSGAHTFGRAQCSSFNLRLYNFSGS-GNPDPTLNTTYL
ATP-A2    134 ANSSIPSPIESLSNITFKFSAVGLNTN-DLVALSGAHTFGRARCGVFNNRLFNFSGT-GNPDPTLNSTLL
HRP-C     134 ANANLPAPFFTLPQLKDSFRNVGLNRSSDLVALSGGHTFGKNQCRFIMDRLYNFSNT-GLPDPTLNTTYL
BGP-1     129 VLSDLPGPSSNVQSLLALLGRLGLDAT-DLVTISGGHTIGLAHCSSFEDRLF------PRPDPTISPTFL
SBP       134 ANQNLPAPFFNLTQLKASFAVQGLNTL-DLVTLSGGHTFGRARCSTFINRLYNFSNT-GNPDPTLNTTYL
CWPO-C    130 DTSNLPGFTDSVDVQKQKFAAFGLNAQ-DLVTLVGGHTIGTTACQFFRYRLYNFTTTGNGADPSINPSFV
LiP-H8    135 PDGLVPEPFHTVDQIINRVNDAGEFDELELVWMLSAHSVAAVNDVDPTVQGLPFDST---PGIFDSQFFV

PrxA3a    203 AELQQLCPQAGNESVVTNLDPTTPDTFDGNYFSNLQTNEGLLRSDQELFSTT-GADTIDIVNNFSSNQT-
ATP-A2    202 STLQQLCPQNGSASTITNLDLSTPDAFDNNYFANLQSNDGLLQSDQELFSTT-GSSTIAIVTSFASNQT-
HRP-C     203 QTLRGLCPLNGNLSALVDFDLRTPTIFDNKYYVNLEEQKGLIQSDQELFSSPNATDTIPLVRSFANSTQ-
BGP-1     192 SRLKRTCPAKG-TDRRTVLDVRTPNVFDNKYYIDLVNREGLFVSDQDLFTNA---ITRPIVERFAQSQQ-
SBP       202 EVLRARCPQNATGDNLTNLDLSTPDQFDNRYYSNLLQLNGLLQSDQELFSTP-GADTIPIVNSFSSNQN-
CWPO-C    199 SQLQTLCPQNGDGSRRIALDTGSQNSFDSSFFANLRSGQGILESDQKLWTDA---TTRTFVQRFLGVRGL
LiP-H8    202 ETQLRGTAFPGSGGNQGEVESPLPGEIR----------IQSDHTIARDS---RTACEWQSFVNNQS-

PrxA3a    271 ---AFFESFVVSMIRMGNISPLTGTDGEIRLNCRRVNDNSTGSNALLVSSI  318
ATP-A2    270 ---LFFQAFAQSMINMGNISPLTGSNGEIRLDCKKVNGS------------  305
HRP-C     272 ---TFFNAFVEAMDRMGNITPLTGTQGQIRLNCRVVNSN------------  307
BGP-1     257 ---DFFEQFGVSIGKMGQMRVRTSDQGEVRRNCSVRNPGPG----------  294
SBP       270 ---TFFSNFRVSMIKMGNIGVLTGDEGEIRLQCNFVNG------------  304
CWPO-C    266 AGLTFGVEFGRSMVKMSNIGVKTGTTGEIRRVCSAIN-------------  302
LiP-H8    255 KLVDDFQ-FIFLALTQLGQDPNAMTDCSDVIPQSKPIPG------------  292
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Fig. 1 Alignment of amino acid sequences for mature peroxidases. PrxA3a, ATP-A2 (PDB ID: 1PA2), HRP-C (PDB ID: 1HCH), soybean seed coat peroxidase (PDB ID: 1FHF), barley grain peroxidase 1 (PDB ID: 1BGP), CWPO-C, and LiP-H8 (PDB ID: 1B85). Sequence homology analysis was carried out using CLUSTALW (http://www.ebi.ac.uk). Conserved amino acid residues in the hem pocket and proline residue corresponding to Pro-139 of ATP-A2 are marked with a star. Trp-166 of LiP-H8, Tyr-74 and Tyr-177 of CWPO-C are yellow-highlighted. Putative *N*-glycosylation sites (N-X-S/T, X ≠ P) are underlined

and ferrocytochrome *c*, unlike other plant peroxidases [21]. Fungal lignin peroxidase (LiP) can also catalyze the oxidation of lignin polymers and ferrocytochrome *c* [22]. CWPO-C and LiP may have additional active sites other than heme pockets. These enzymes can also oxidize substrates that cannot enter the heme pocket according to physical size, etc. Therefore, amino acid residues located on the surface of the enzyme were predicted as catalytic sites other than the heme pocket and were investigated. As a result, Tyr-177 or Tyr-74 of CWPO-C were found to be catalytic sites (Fig. 1) [23–27]. It was also found that the Trp-166 residue located on the surface of LiP-H8 is a catalytic site [28–30].

In a previous study [31], we transplanted the putative additional catalytic sites of CWPO-C and LiP to PrxA3a for substrate specificity modifications and certifications of the surface amino acid residue hypothesis. The amino acid residues, corresponding to the surface catalytic sites circumferential amino acid residues, of catalytic sites in these enzymes were chosen for mutagenesis into *prxA3a* gene. Phe-77 and Ala-165 of PrxA3a corresponded to Tyr-74 of CWPO-C and Trp-166 of LiP-H8, respectively (Fig. 1).

Escherichia coli is usually used as a host for heterologous recombinant protein production, but the protein secretion ability of *E. coli* is so poor that secretion proteins produced heterologously in *E. coli* often form inactive inclusion bodies. Plant recombinant peroxidases were also produced as inclusion bodies in *E. coli*, and were converted to the active form by the complicated and laborious steps of solubilization and refolding [17–21]. In contrast, yeasts can produce secreted proteins with disulfide bonds and sugar chains. The yeast expression system in our previous study [31] was adopted for the production and secretion of the active form of PrxA3a and the mutated enzymes. Therefore, in wild-type PrxA3a, the mutated PrxA3a with Phe-77 was replaced with Tyr (F77Y: FY), and in addition, in a mutation with Ala-165 replaced with Trp (F77Y A165W: FYAW), other mutated PrxA3a were produced [31]. The FY and FYAW mutations in PrxA3a resulted in improved sinapyl alcohol oxidation ability. In addition, the enhanced oxidation of ferrocytochrome *c* and the ability to polymerize sinapyl alcohol indirectly confirmed that the substituted amino acid residues Trp-165 and Tyr-77 of PrxA3a functioned as substrate oxidation sites on the protein surface [31].

In this study, *Brevibacillus choshinensis* was used as a host for the expression system. *B. choshinensis* is a gram-positive bacterium with strong protein secretion activity, but it cannot add sugar chains to secreted proteins. Therefore, the *B. choshinensis* expression system is useful for producing recombinant secreted proteins with disulfide bonds and no sugar chains. Plant peroxidases are secreted proteins that contain disulfide bonds and sugar chains. Yeast adds sugar chains to secreted proteins, whereas *B. choshinensis* does not. Sugar chains of peroxidases may interact to polysaccharide components in plant cell wall during lignin deposition. With our study, we aimed to examine various effects of peroxidases with and without sugar chains on lignin deposition, stability, and substrate specificity, using yeast and *B. chosinensis* expression systems. We attempted to verify the oxidation and polymerization abilities of purified enzymes without sugar chains produced by *B. choshinensis*, to coniferyl alcohol and sinapyl alcohol.

Materials and methods

Strains

Brevibacillus choshinensis strain HPD31-SP3 was used as a host to produce PrxA3a and other mutated peroxidases. The *Escherichia coli* strain used for the construction and propagation of plasmids was XL10-gold (Δ(*mcrA*)*183* Δ(*mcrCB-hsdSMR-mrr*)*173 endA1 supE44 thi-1 recA1 gyrA96 relA1 lac* Hte) [F′ *proAB lacI*^{q} *Z*ΔM15 Tn*10* (Tet^r) Amy Cam^r].

Media and growth conditions

E. coli transformants were grown at 37 °C in Luria–Bertani (LB) broth supplemented with antibiotics. Selected *B. choshinensis* transformants were inoculated in MT liquid medium (1.0% Phytone peptone, 0.575% Ehrlich bonito extract, 0.2% Bacto yeast extract, 1.0% glucose, 10 mg/L $FeSO_4$•$7H_2O$, 10 mg/L $MnSO_4$•$4H_2O$, 1 mg/L $ZnSO_4$•$7H_2O$, 20 mg/L $MgCl_2$) supplemented with kanamycin (50 μg/mL) and grown aerobically at 37 °C. *B. choshinensis* transformants were aliquoted into 0.5 mL microtubes and stored at −80 °C. The medium 2SY (4.0% Bacto soyton, 0.5% Bacto yeast extract, 2.0% glucose, 0.015% $CaCl_2$•$2H_2O$, 150 μg/mL kanamycin) was used for the pre-culture and protein expression culture.

Plasmids construction and transformation of *B. chosinensis* and *E. coli*

Polymerase chain reaction (PCR) was performed on wild-type *prxA3a* [DNA database accession number: D38050] with deleted introns as the template with KOD polymerase (ToYoBo, Osaka, Japan) using primers B and C to introduce the FY mutation and primers A and D for amplification of full-length *prxA3a* CDS (Fig. 2A, B). First, PCR was performed with primer sets A and C, and with primer sets B and C. Both PCR products were purified by agarose gel electrophoresis, and the target DNA fragments were rescued. Second, both recovered DNA fragments were mixed, and the full-length FY-mutated *prxA3a* CDS was amplified using primers A and D. The amplified fragment was digested with *Hin*dIII and

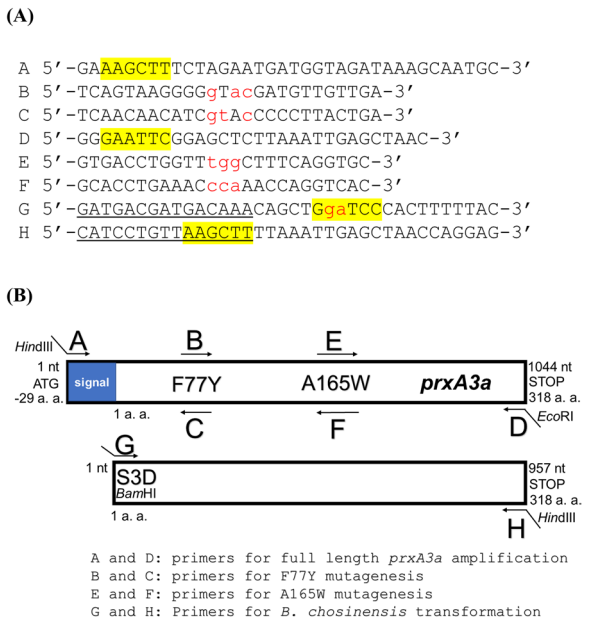

Fig. 2 Primer sequences and their positions. **A** Primer sequences used for mutagenesis and vector construction. Red lower-case letters represent substituted nucleotides. The flanking sequences of the *Hin*dIII recognition sites in pBIC1–4 are underlined. Yellow-highlighted sequences are *Hin*dIII, *Eco*RI or *Bam*HI recognition sites. Primer **A** and **D**: primers for full length *prxA3a* amplification. Primer **B** and **C**: primers for F77Y mutagenesis. Primer **E** and **F**: primers for A165W mutagenesis. **G** and **H**: Primers for creation of DNA fragments for *B. chosinensis* transformation. Primer **G** contains a *Bam*HI site and causes an amino acid residue substitution of Ser to Asp at the third position of the native mature PrxA3a. **B** Positions of the primers for mutagenesis and vector construction on *prxA3a*.

inserted into pUC119 to confirm its sequence. Similarly, FYAW-mutated *prxA3a* was generated using FY-mutated *prxA3a* CDS and primers E and F for the introduction of the AW mutation into FY-mutated *prxA3a*. The full-length DNA fragment contained the nucleotide sequence of the native plant extracellular secretion signal. For insertion into *Brevibacillus* expression plasmids, pBIC1–4 (Takara Bio, Shiga, Japan), containing the nucleotide sequences encoding the bacterial signal sequences, the truncated DNA fragments for the mature peroxidase deleted the nucleotide sequence for the authentic plant signal sequence and added short homologous sequences flanking the *Hin*dIII cloning site of the plasmids. The DNA fragments were then amplified with primers G and H using the full-length peroxidase CDSs in pUC119 as the templates, and the amplified fragments were used as insert fragments for *B. choshinensis* transformation. Primer G caused the substitution of serine with aspartic acid at the third amino acid residue in mature PrxA3a.

Transformation of *B. chosinensis* was performed using the BIC method, according to the instructions for the *Brevibacillus* Expression System (Takara Bio, Shiga, Japan). A mixture of linearized pBIC1–4 by digestion with *Hin*dIII and the amplified DNA fragment with primers G and H was used for the transformation of *B. chosinensis*. We used pBIC3 as the expression vector in further experiments because it produced the most efficient PrxA3a among pBIC1–4 (Additional file 1: Fig. S1).

Ligation reactions of *E. coli* plasmids and PCR products were performed using the Seamless Ligation Cloning Extract (SLiCE) method [32], and SLiCE ligation mixtures were used for transformation of *E. coli*.

Production of recombinant enzymes by *B. choshinensis*

After thawing the frozen stocks of *B. choshinensis* transformants at 37 °C for 1 min, they were cultured in 10 mL of 2SY pre-culture medium with 150 μg/mL kanamycin in L-tubes at 30 ℃ for 24 h with shaking (80 rpm). Each *B. choshinensis* pre-culture medium was aseptically transferred to 100 mL of 2SY expression culture medium with 150 μg/mL kanamycin and 50 μg/mL hemin in Sakaguchi flasks and cultured aerobically at 30 ℃ for 96 h (140 rpm).

Protein purification

Protein purification was performed in the following three steps.

1. The supernatant of the culture medium was collected by centrifugation and used for further purification. Ni affinity purification using the batch method was performed with beads of Ni Sepharose^{TM} High Performance (Cytiva, Tokyo, Japan) according to the protocol provided by the manufacturer, and wild-type and mutated PrxA3a enzymes were purified from the resulting filtrate. The crudely purified proteins obtained in this study were used to examine the enzyme activity and polymerization ability of monolignols, as described below, as well as for protein quantification.
2. Anion exchange chromatography was performed according to the following procedure. The buffer of the crudely purified protein solutions in step 1 was replaced with 20 mM Tris-HCl (pH 8.0), and the solutions were concentrated using a 10 kDa ultrafiltration unit (Amicon Ultra; MILLIPORE, Merck, Tokyo, Japan). Sample solutions were passed through a 0.45-μm pore size filter (Millex-HV; MILLIPORE, Merck, Tokyo, Japan). An appropriate amount of sample was injected into a high-performance pulseless constant flow pump (SSC), and the eluate was aliquoted every minute with a fraction collector. The fractions in which enzyme activity was confirmed were collected. The purification was conducted using a Gradient Controller, SSC-3461, and SSC-5410

(Senshu Scientific Co., Ltd., Tokyo, Japan) with an IEC DEAE-825 column (8.0 × 75 mm, 8 μm; Showa Denko, Tokyo, Japan). Mobile phase A (20 mM Tris-HCl (pH 8.0)) and mobile phase B (20 mM Tris-HCl (pH 8.0) with 0.5 M NaCl) were used to elute the crudely purified protein solution at a flow rate of 1.0 mL/min. The gradient elution was held at 100% A for 5 min, stepped to 80% A/20% B for 5 min, held for 12 min, stepped to 65% A/35% B for 6 min, to 60% A/40% B for 12 min, and 100% B for 1 min, after which it returned to 100% A for 1 min.

3. Size-exclusion chromatography was performed using the following procedure. PROTEIN KW-G 6 B and PROTEIN KW-802.5 columns (Showa Denko, Tokyo, Japan) were used, and 50 mM sodium phosphate buffer (pH 7.0) with 0.3 M NaCl was used as the mobile phase. The buffer of PrxA3a purified in step 2 was replaced with 50 mM sodium phosphate buffer (pH 7.0) with 0.3 M NaCl using a 10 kDa ultrafiltration unit and concentrated. The sample collected in step 2 was passed through a 0.45-μm pore diameter filter. An appropriate amount of the sample was injected into the device, and the eluate was aliquoted every 20 s with a fraction collector. The fractions were collected and peroxidase activity was detected using the simple activity assay described below.

Measurement of enzyme activities

The guaiacol-oxidizing activities of wild-type and mutated PrxA3a were measured. The buffer used for the activity assay consisted of 50 mM sodium phosphate buffer (pH 6.0), 0.1% (w/v) guaiacol, and 0.003% hydrogen peroxide. Appropriate amounts (2 μL or 5 μL) of the enzyme solutions were added to 1 mL of buffer, and the activity assay was performed. The oxidized guaiacol was measured using a spectrophotometer at a wavelength of 470 nm. One unit of enzyme activity was defined as the amount of enzyme that increased one unit of absorbance at 470 nm (A_{470}) per minute in a 1-mL reaction mixture at about 20 °C.

Protein quantification

The protein quantification reagent was composed of Bio-Rad Protein Assay Dye Reagent Concentrate and Milli-Q water (1:4). Protein concentration standards (BSA 0.1 mg/mL, 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1.0 mg/mL) and each enzyme were prepared according to the instructions, and 10 μL of each was dispensed into the disposable cell. One milliliter of well-stirred protein quantification reagent was added to each disposable cell and allowed to stand at room temperature for at least 5 min. Within 60 min, the absorbance was measured at 595 nm to determine the protein concentration.

Protein analyses by SDS-PAGE and native-PAGE, and peroxidase activity staining

In this study, we used both of SDS-PAGE and native-PAGE methods developed by Laemmli [33, 34]. In SDS-PAGE, usually proteins are separated in the presence of a detergent and under heat-denaturing and reducing conditions. Sodium dodecyl sulfate (SDS) is used as a detergent, and 2-mercaptoethanol or dithiothreitol is used as a reducing agent. In this study, SDS-PAGE was performed with final concentration of 37.5 mM dithiothreitol for flow-through and eluted fractions at each purification step for the wild-type, FY-mutated, and FYAW-mutated enzymes. The resulting gels were stained with Coomassie Brilliant Blue R-250 (CBB).

On the other hand, proteins in native-PAGE are separated without a detergent and under non-denaturing and non-reducing conditions. Under such conditions, the charge, molecular weight, and shape of each of the proteins and the pH during electrophoresis, mainly affect the mobility of each protein during electrophoresis. And many proteins can retain their activity after native-PAGE. Native-PAGE was also performed on the same SDS-PAGE samples. The resulting gels were stained with CBB and/or analyzed by peroxidase activity staining, and photographed. The buffer, containing 0.1% (w/v) guaiacol and 0.003% hydrogen peroxide, used for peroxidase activity staining of the gels was the same as that used for measurement of enzyme activity. The gels after native-PAGE were soaked in the buffer for 3 min.

In all of SDS-PAGE and native-PAGE, we used Blue Prestained Protein Standard, Broad Range (11–250 kDa) (New England BioLabs Japan, Tokyo, Japan) as the molecular weight marker.

Measurement of monolignols polymerization ability of wild-type and mutated PrxA3a enzymes

An Asahipak GF-310 column (Showa Denko, Tokyo, Japan) was used, and *N*,*N*-dimethylformamide (DMF; HPLC grade) was used as the mobile phase. The substrate specificities of PrxA3a and the mutated enzymes were examined using coniferyl alcohol and sinapyl alcohol as substrates, respectively. The reaction buffer consisted of 50 mM sodium phosphate buffer (pH 6.0), 0.2 mM coniferyl or sinapyl alcohol, and 0.003% hydrogen peroxide. Each 0.2 unit of enzyme, based on their specific activities to guaiacol, were added to 50 μL of the reaction buffer. Coniferyl alcohol was reacted with each enzyme for 1 h and 24 h. Sinapyl alcohol was reacted with each

enzyme for 24 h and 72 h. The reaction was stopped by placing sample tubes in boiling water for 5 min. The reaction mixtures were dried under vacuum for 1 h using a centrifugal evaporator (IWAKI VEC-260) connected to an aspirator. The residues in the reaction tubes were dissolved in 100 µL of DMF and passed through 0.45-µm pore size filters (Millex-HV). A 500 µL glass syringe was used as the injector for HPLC. The samples were injected into a column that had been previously equilibrated with degassed DMF, the flow rate was set at 0.4 mL/min, and the absorbance at 278 nm was measured. ChromatoPRO Ver. 5.0.0 (Runtime Instruments, Tokyo, Japan) was used for the analysis. We used polystyrene STANDARD SL-105 (Showa Denko, Kanagawa, Japan) composed of S-20, S-13, S-9.6, S-6.3, S-4.7, S-3.0, S-1.9, S-1.2, S-0.9, and S-0.6, with peak molecular weights (*Mp*) of 19,500, 13,000, 9,590, 6,320, 4,730, 2,970, 1,920, 1,150, 860, and 580, respectively, as the molecular weight standard.

Results and discussion

Purification and specific enzyme activities of wild-type and mutated enzymes produced by *Brevibacillus*

Enzyme activity in the supernatants from the culture medium of the transformant of wild-type *prxA3a* in pBIC3 was higher than that in the other vectors (Additional file 1: Fig. S1). Therefore, pBIC3 was used as the expression vector for the subsequent protein expression experiments.

The mature recombinant PrxA3a enzymes secreted by *Brevibacillus* had an amino acid residue substitution from serine to aspartic acid at the third position of the native mature PrxA3a and, furthermore, an additional amino acid sequence, ADHHHHHHDDDDK, at the N-terminus. The additional sequence was composed of a His-tag and enterokinase site. Therefore, Ni affinity purification using the His-tag was performed prior to the other protein purification steps.

The protein concentration of each enzyme was then calculated (Table 1-iv). The protein concentration of the wild-type, FY-mutated, and FYAW-mutated enzymes were 0.574, 2.24, and 0.455 mg/mL, respectively. The specific activity of each enzyme toward guaiacol was calculated in the same manner (Table 1-v). The individual specific activities of the wild type, FY-mutated, and FYAW-mutated enzymes were 95.8, 101, 114 U/mg.

SDS- and native-PAGE and peroxidase activity staining

As shown in Fig. 3, two bands are observed. Their molecular weights are estimated to be 51.2 kDa and 37.5 kDa.

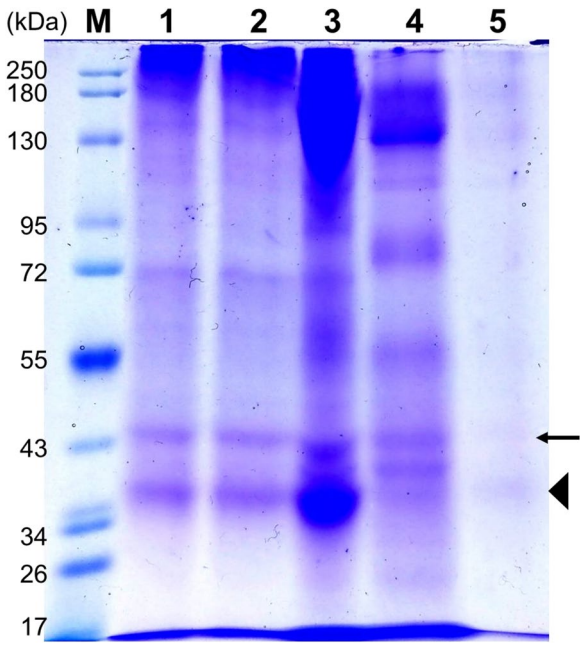

Fig. 3 Wild-type recombinant PrxA3a in fractions during purification in SDS-PAGE. The bands of 51.2 kDa and 37.5 kDa are shown with an arrow and an arrowhead, respectively. M: Molecular weight marker; 1: elution fraction by 10 mM imidazole; 2: elution fraction by 20 mM imidazole; 3: elution fraction by 50 mM imidazole; 4: collected fraction after anion exchange chromatography of the elution fraction by 50 mM imidazole; 5: collected fraction after both size-exclusive and anion-exchange chromatography of the elution fraction by 50 mM imidazole

Table 1 The activity and concentration of wild-type and mutated PrxA3a were measured

	(i) Enzyme activity	(ii) 1 mL equivalent of enzyme activity [U/mL]	(iii) Protein quantification	(iv) Protein concentration [mg/mL]	(v) Specific activity [U/mg]
Wild type (Sample volume)	0.275 ± 0.0155 (5 µL)	55.0	0.293 ± 0.0273 (10 µL)	0.574	95.8
FY mutanted (Sample volume)	0.454 ± 0.0218 (2 µL)	227.0	0.468 ± 0.0188 (4 µL)	2.24	101
FYAW mutanted (Sample volume)	0.260 ± 0.0145 (5 µL)	52.0	0.228 ± 0.0160 (10 µL)	0.455	114

(i)The guaiacol oxidizing activities of wild-type and mutated PrxA3a were measured. (ii) Enzyme activity per 1 mL was calculated based on the measured guaiacol oxidation activity. (iii) and (iv) Protein concentrations were determined based on the Bradford method. (v) Specific activity was calculated using enzyme activity per 1 mL and protein concentration. The SD were calculated from the triplicate experiments.

The estimated molecular weight of mature PrxA3a with heme and the additional amino acid sequence without a sugar chain is approximately 36 kDa. Therefore, it is thought that the lower bands with an arrowhead in Fig. 3 were derived from the denatured monomer of PrxA3a, while the upper bands represented by an arrow were resulted from the insufficiently denatured PrxA3a. Some plant peroxidases are known to exist in the homodimer form [35–39]. Insufficient denaturation of the dimer might result in the difference between calculated and measured mobility in SDS-PAGE. Similar results for SDS-PAGE, according to the homodimer, were also observed in our previous report on PrxA3a produced by yeast [31].

Figure 4 shows that the bands of the wild-type and mutated enzymes are represented at the same positions. The molecular weights of the bands indicated by the arrow are estimated to be 38.1 kD, which is considered as a monomer. In Fig. 5, reddish-brown bands are observed in all lanes of the samples. This indicates that all enzymes were secreted in the active form. No reddish-brown staining by other peroxidases than wild-type and mutated PrxA3a in Fig. 5 was detected, suggesting that the peroxidase activity was solely derived from *prxA3*. Therefore, these crude enzymes could be examined in a test for monolignol polymerization ability, despite the presence of many other proteins in these solutions (Table 2).

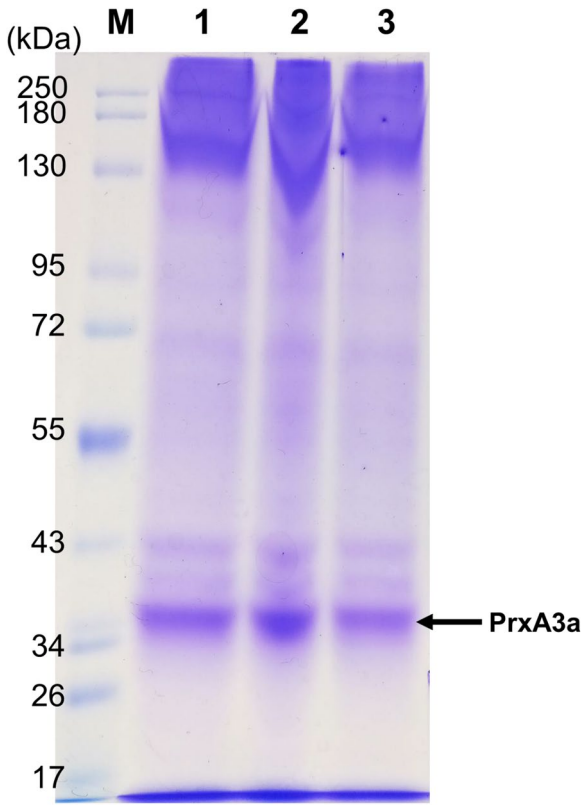

Fig. 4 Wild-type and mutated PrxA3a in fractions after the purification step 1 in SDS-PAGE. M: Molecular weight marker; 1: Wild-type enzyme; 2: FY mutated enzyme; 3: FYAW mutated enzyme

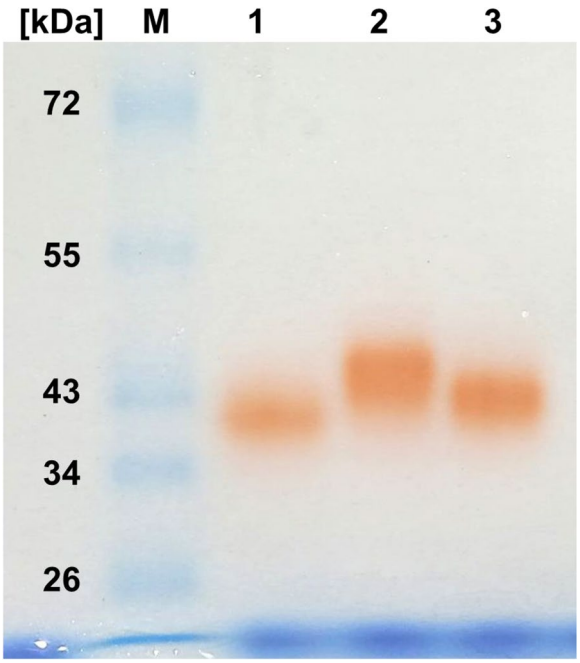

Fig. 5 Peroxidase activity staining of wild-type and mutated PrxA3a in fractions after the purification step 1. M, molecular weight marker; 1, wild-type enzyme; 2, FY-mutated enzyme; 3, FYAW-mutated enzyme. In native-PAGE, protein mobility is affected by many factors, not just the molecular weight. Therefore, there is no exact correlation between the molecular weight marker values and the molecular weights of other proteins

Monolignols polymerization abilities of the wild-type and mutated PrxA3a

The amount of enzyme was set to 0.2 units per volume in each reaction mixture.

We determined the molecular weights of the peaks in the size-exclusion chromatograms according to the molecular weights of polystyrene STANDARD SL-105 (Additional file 1: Fig. S2). The retention times for both sinapyl alcohol with a molecular weight of 210.226 and coniferyl alcohol with a molecular weight of 180.20 were approximately 22 min (Figs. 6 and 7), suggesting that this is the lower limit of the column's detection value. The molecular weight at approximately 16.5 min in the broad band of coniferyl alcohol reactant by wild-type PrxA3a in Fig. 6A was nearly 1920 Da. Therefore, the substance detected around this time was considered to be approximately 10-mer. Furthermore, multiple peaks

Table 2 Ratios of coniferyl alcohol polymerization products

						[%]	
	Monomer	**Dimer**	**Trimer**	**Oligomer**	**Polymer**	**Before 9 min**	**After 23.5 min**
(A) After 1 h reaction							
WT	2.83	45	5.98	38.6	7.66	0	0.0121
FY	5.87	21.7	8.43	30.7	33.1	0	0.189
FYAW	5.67	15.4	6.16	29.9	37.7	3.46	1.68
(B) After 24 h reaction							
WT	3.7	31.5	6.76	29.6	25.4	0.452	2.57
FY	3.04	10.9	7.27	37.3	40.4	0.687	0.493
FYAW	3.55	10.8	6.14	35.5	42.4	0	1.59

The area ratios of the peaks obtained from size exclusion chromatography were determined for coniferyl alcohol polymerization products. (a) after 1 h reaction, (b) after 24 h reaction. Monomer contains coniferyl alcohol and aldehyde. (monomer: from 21 min to 23.5 min, dimer: from 19.5 min to 21 min, trimer: from 18 min to 19.5 min, oligomer: from 13 to 18 min, and polymer: from 9 to 13 min)

observed between 18 and 21 min were attributed to tetramers, trimers, and dimers, respectively. Similarly, the band detected at approximately 15 min was thought to be about 30-mer, and those detected prior to 13 min were considered to be complete polymers because their molecular weight was more than 20 kDa. Polymerization capacity tests were conducted on the basis of these evaluations.

Using coniferyl alcohol alone as the standard, a peak appeared at 22 min (Fig. 6A, B). In the chromatograms of coniferyl alcohol oxidized by wild-type, FY, and FYAW-mutated PrxA3a, the peaks at 20 min were inferred to be coniferyl alcohol dimers (Fig. 6A, B). In the non-enzyme control, a shoulder appeared at 23 min. This was thought to be coniferyl aldehyde, which is an oxidation product of coniferyl alcohol. A broad band was observed between 9 and 13 min (Fig. 6A, B). Compared to the molecular weight standard, this corresponds to a molecular weight of more than 20 kDa. Therefore, the broad band between 9 and 13 min was estimated to be that of coniferyl alcohol polymer, known as the guaiacyl dehydrogenative polymer (DHP). The results of the 1-h reaction of the enzyme with coniferyl alcohol and the 1-d reaction showed that the polymer increased with time in both the wild-type and mutated enzymes. The area ratios of the polymer bands after 24 h of reaction were 25.4% for wild-type PrxA3a, 40.4% for FY-mutated PrxA3a, and 42.4% for FYAW-mutated PrxA3a, suggesting that FYAW-mutated PrxA3a had the highest coniferyl alcohol polymerization ability.

As a result of flowing sinapyl alcohol alone, a peak appeared at 22 min, similar to that of coniferyl alcohol (Fig. 7A, B). In the non-enzyme control, the shoulder at 23 min appeared to be similar to that in the non-enzyme control of coniferyl alcohol. This is thought to be sinapyl aldehyde. After 24 h of enzyme reaction, in the samples of FY- and FYAW-mutated PrxA3a, the dimer peaks were the most intense, while the monomer peaks decreased (Fig. 7A). Compared to the wild-type enzyme, the mutated enzymes showed improved polymerization abilities. In particular, only the FYAW-mutated enzyme represented the syringyl DHP polymer band. The polymer band was obtained when the peroxidase enzymatic reaction was stopped in boiling water. Addition of hydrochloric acid resulted in the emergence of the oligomer bands of coniferyl and sinapyl alcohols even though no peroxidase was added (Additional file 1: Fig. S3). However, oligomer bands were not obtained when they were boiled to stop the enzymatic reaction. Therefore, the reaction mixtures were placed in microtest tubes in boiling water for 5 min to stop the enzymatic reaction. When sinapyl alcohol was reacted with the FY mutant and the FYAW mutant for 72 h, the polymerization was more advanced and the trimmer peak at around 19 min was larger (Fig. 7B). The polymer band area of the FYAW-mutated enzyme accounted for 15.2% of the total peak area, indicating that it has a higher sinapyl alcohol polymerization ability than the FY-mutated enzyme (Table 3B). These results suggest that the introduction of the FY and FYAW mutations into PrxA3a altered substrate specificities and enabled the surface substrate oxidation sites to function. In particular, the FYAW-mutated enzyme showed an improved polymerization ability for both coniferyl alcohol and sinapyl alcohol.

These results were similar to those of wild-type and mutated PrxA3a produced by yeast [31]. This indicates that the enhanced reactivities of mutated peroxidases with FY and FYAW substitutions towards sinapyl alcohol were not significantly affected by the presence or absence of sugar chains in these enzymes under the reaction conditions used in this study. However, further analyses for

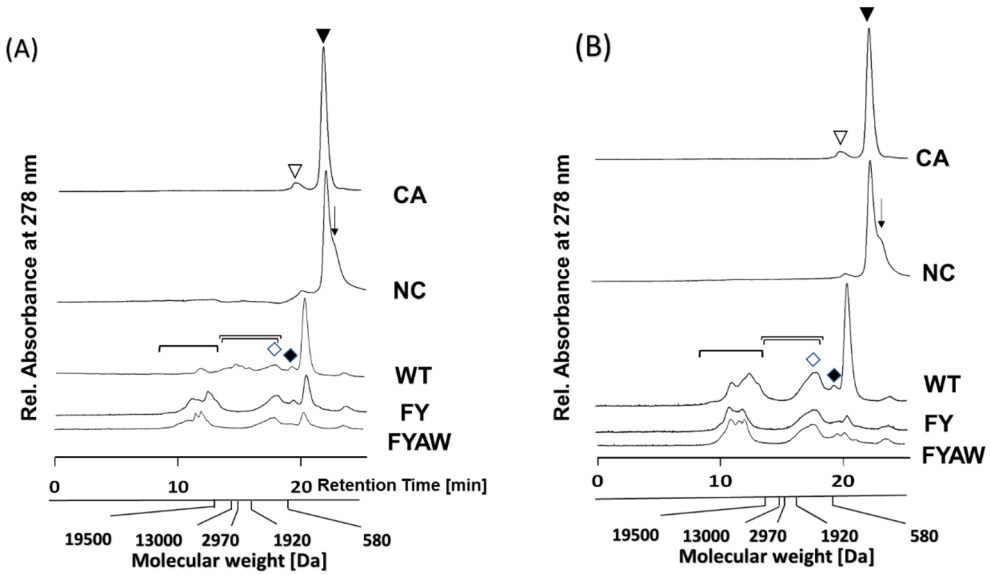

Fig. 6 In vitro analysis of coniferyl alcohol polymerization abilities of peroxidases by size exclusion chromatography. UV chromatograms at 278 nm are shown. Closed triangles, open triangles, closed diamonds, open diamonds, double parentheses, parentheses, and arrows represent monomer, dimer, trimer, tetramer, oligomer (13–18 min), polymer (9–13 min), and coniferyl aldehyde, respectively. *CA* coniferyl alcohol, *NC* non-enzyme control; WT, FY, and FYAW: reactants of wild-type, FY-mutated, and FYAW-mutated enzymes are shown at the top. **A**Each enzyme and coniferyl alcohol were allowed to react for 1 h. **B** Each enzyme and coniferyl alcohol were allowed to react for 24 h. The area ratios of the polymer bands after 24 h of reaction were 25.4, 40.4, and 42.4% for the wild-type, FY-mutated, and FYAW-mutated enzymes, respectively

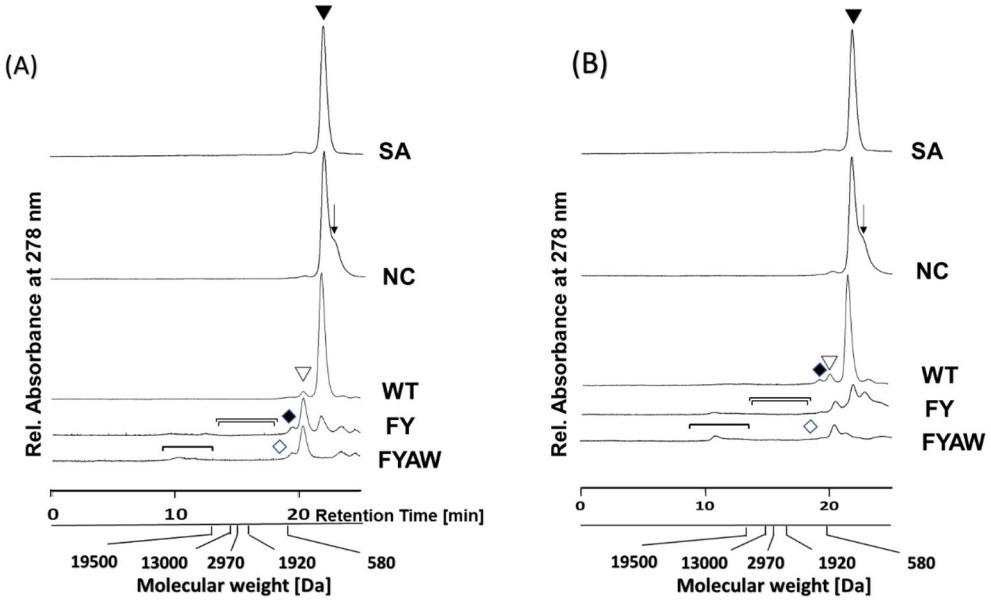

Fig. 7 In vitro analysis of sinapyl alcohol polymerization abilities of peroxidases by size exclusion chromatography. UV chromatograms at 278 nm are shown. Closed triangles, open triangles, closed diamonds, open diamonds, double parentheses, parentheses, and arrow represent monomer, dimer, trimer, tetramer, oligomer (13–18 min), polymer (9–13 min), and sinapyl aldehyde, respectively. *SA* sinapyl alcohol, *NC* non-enzyme control; WT, FY, and FYAW: reactants of wild-type, FY-mutated, and FYAW-mutated enzymes are shown at the top. **A** Each enzyme and sinapyl alcohol were allowed to react for 24 h. **B** Each enzyme and sinapyl alcohol were allowed to react for 72 h

their substrate specificities, stabilities, and specific activities of with or without cell wall components are required using wild and mutated peroxidases derived from gene expression systems of yeast and *Brevibacillus*.

Table 3 Ratios of sinapyl alcohol polymerization products

							[%]
	Monomer	**Dimer**	**Trimer**	**Oligomer**	**Polymer**	**Before 9 min**	**After 23.5 min**
(A) After 24 h reaction							
WT	82.5	6.40	2.5	2.49	1.60	0.436	4.09
FY	31.7	41.2	10	2.71	5.36	0	9.00
FYAW	14.2	43.6	5.6	5.56	11.5	0.556	19.0
(B) After 72 h reaction							
WT	66.5	8.40	4.7	7.84	5.17	2.17	5.23
FY	40.4	15.5	2.5	7.99	6.19	1.76	25.7
FYAW	18.4	24.4	6.5	17.1	15.2	1.21	17.3

The area ratios of the peaks obtained from size exclusion chromatography were determined for sinapyl alcohol polymerization products. (A) after 24 h reaction, (B) after 72 h reaction. Monomer contains sinapyl alcohol and aldehyde. (monomer: from 21 min to 23.5 min, dimer: from 19.5 min to 21 min, trimer: from 18 min to 19.5 min, oligomer: from 13 to 18 min, and polymer: from 9 to 13 min)

Conclusions

This experiment revealed the successful production and secretion of active form of wild-type and mutated plant peroxidases by *B. choshinensis*. The expression of the enzyme with these mutations in plants and alterations in its traits will contribute to the search for mutated peroxidases in the breeding of highly useful plants.

It is also true that in experiments on the ability of the mutant enzymes to polymerize sinapyl alcohol, the considerable amount of the sinapyl alcohol remained as a dimer, although the polymerization abilities were better than that of the wild-type enzyme. At present, the sinapyl alcohol polymerization ability of the mutant enzyme cannot be accurately compared to that of CWPO-C, because of differences of substrates and reaction conditions [20]. By the way, CWPO-C overexpression in *Arabidopsis* is known to cause dwarfing [39]. Therefore, it is beneficial to consider utilizing PrxA3a, at least in tobacco, in which wild-type overexpression does not result in morphological abnormalities (data not shown).

In the near future, we plan to analyze the differences in the effect of peroxidases on lignin deposition, stability, and specific activities of peroxidases with and without sugar chains using the enzymes produced by yeast and *Brevibacillus*. The *B. choshinensis* expression system is a new useful tool for functional analyses of plant peroxidase.

Abbreviations

F77Y (FY)	F77Y A165W FYAW
HRP-C	Horseradish peroxidase C
HRP-A2	Horseradish peroxidase A2
ATP-A2	Arabidopsis thaliana peroxidase A2
CWPO-C	Cationic cell wall-bound peroxidase
LiP	Fungal lignin peroxidase
LB	Luria–Bertani
PCR	Polymerase chain reaction
CBB	Coomassie Brilliant Blue R-250
DHP	Dehydrogenative polymer
SLiCE	Seamless Ligation Cloning Extract

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s10086-023-02099-w.

Additional file 1: Figure S1. Peroxidase activities from culture media. **Figure S2.** The relationship between retention times and molecular weights. **Figure S3.** Oligomerization of coniferyl and sinapyl alcohols by addition of HCl.

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Author contributions

SK designed this study and the methodology; HI, YK, JM, and MD, conducted the lab work; HI, YK and SK led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication. All the authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing interests

The authors declare that they have no competing interests.

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