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Original Paper

Isolation of polyphenol oxidase genes from *Portulaca oleracea* and evaluation of their ability to metabolize endocrine-disrupting chemicals

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Abstract Portulaca (*Portulaca oleracea* cv.), a garden plant, efficiently removes endocrine-disrupting chemicals (EDCs) including bisphenol A (BPA) from hydroponic solution. We hypothesized that polyphenol oxidase (PPO) was involved in the initial steps of detoxifying EDCs in portulaca roots. In order to elucidate the molecular basis of portulaca's ability to metabolize EDCs, we first isolated five *PPO* genes (*PoPPO1–5*) that were expressed mainly in portulaca roots. Among these genes, *PoPPO2*, *PoPPO4* and *PoPPO5* were introduced into cultured tobacco cells and expressed as active forms. We found that crude extracts from the cells expressing PoPPO2, PoPPO5, and to a lesser extent PoPPO4, could metabolize BPA. In addition, we found that the BPA metabolites from crude extracts of cells expressing PoPPO2, PoPPO4 and PoPPO5 were identical to those of portulaca. Moreover, PoPPO2 and PoPPO5 also caused hydroxylation of octylphenol, nonylphenol and 17β-estradiol. Therefore, these results strongly suggest that PoPPOs significantly contribute to the superior ability of portulaca to metabolize EDCs.

Key words: *Portulaca oleracea*, polyphenol oxidase, phytoremediation, endocrine-disrupting chemicals, *Nicotiana tabacum* L. cv BY-2

With increasing industrial activities, environmental pollutants have spread in some regions. These pollutants include heavy metals, organic chloride and nitrogen compounds, and phenolic compounds. Also among these pollutants are endocrine-disrupting chemicals (EDCs), compounds that act like hormones in the endocrine system and disrupt the physiological function of endogenous hormones (Elobeid and Allison 2008). EDCs can cause disorders including abnormal sexual differentiation or disrupted reproductive function. Hence, it is important to develop a method of removing EDCs from polluted waste water and soil.

The main methods currently utilized to remove EDCs from the environment are based on physicochemical treatment, including adsorption to activated carbon and ozonation (Liu et al. 2009), but these methods generally consume a great deal of energy and come at a relatively high cost. Other methods for the removal of EDCs include the use of living organisms, micro-organisms, and plants (Pilon-Smits 2005; Suresh and Ravishankar 2004). The use of plants and their associated microbes to remove environmental pollutants is called phytoremediation (Pilon-Smits 2005; Suresh and Ravishankar 2004). This method is thought to be cost-effective and requires relatively low energy input, but the removal efficiency is generally not as high as that of physicochemical methods. Therefore, to use phytoremediation practically, it is important to find (or generate) plants that have a high capacity to remove a target compound efficiently.

We have screened more than 100 kinds of plants for efficient removal of a typical EDC, bisphenol A

Abbreviations: BPA, bisphenol A; PPO, polyphenol oxidase; EDCs, endocrine-disrupting chemicals; OP, octylphenol; NP, nonylphenol; E2, 17β-estradiol; L-DOPA, L-3, 4-dihydroxyphenylalanine; LS, Linsmaier-Skoog; RACE, rapid amplification of cDNA ends; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; LC/PDA/MS/MS, liquid chromatography/photodiode array detection/tandem mass spectrometry.
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(2,2-bis(4-hydroxyphenyl)propane, BPA). We found that portulaca (*Portulaca oleracea* cv.) was superior to other plants in removing BPA; the plant almost completely removed 50 μM BPA from a hydroponic solution within 24 h (Imai et al. 2007). Immediately after soaking portulaca roots in a BPA solution, BPA is converted to metabolites that do not have estrogenic activity. Portulaca roots also removed other EDCs, including octylphenol (OP), nonylphenol (NP) and the female hormone 17β-estradiol (E2) (Imai et al. 2007). These observations indicate that portulaca holds great promise as a useful tool in the development of a practical phytoremediation system for EDCs.

Increased understanding of the molecular mechanisms involved in the phytoremediation process will certainly lead to novel strategies for creating genetically engineered plants for improved phytoremediation. Previously, we analyzed the structure of BPA metabolites produced by portulaca roots and detected a hydroxylation product of BPA (Imai et al. 2007). Therefore, we hypothesized that polyphenol oxidase (PPO) is involved in BPA detoxification by portulaca roots.

In this study, we identified five PPO genes (*PoPPO1–5*) in portulaca roots and evaluated their metabolic activity toward EDCs. We found that crude extracts from tobacco cells (*Nicotiana tabacum* L. cv. BY-2) expressing PoPPO2, PoPPO4 and PoPPO5 oxidize BPA and that the same BPA metabolites are produced as when portulaca roots metabolize BPA. Moreover, PoPPO2 and PoPPO5 also caused hydroxylation of OP, NP and E2. These results strongly suggest that PoPPOs significantly contribute to the superior ability of portulaca to metabolize EDCs.

Materials and methods

Plant materials and culture conditions

Portulaca (*Portulaca oleracea* cv.) was purchased from a local market in Osaka, Japan. Sterile plants were obtained by successive transfers and cultivation of shoots cut from whole plants on Murashige-Skoog medium (including vitamins) containing 1% sucrose and 0.2% gellan-gum in a culture bottle at 25°C under continuous light (50–100 μmol m^{-2} s^{-1}), as described previously (Imai et al. 2007). Plants cultivated under the same conditions for 2 months were used for this study. Tobacco cells (*N. tabacum* L. cv. BY-2) were cultured in liquid Linsmaier-Skoog (LS) medium at 25°C with shaking (130 rpm) in the dark. Every 7 days, 1.5 ml of culture containing fully-grown cells was transferred to fresh media (30 ml) for maintenance. Transformation of BY-2 cells were performed as previously described (Nakayama et al. 2000) and the resulting transformants were cultured as described above. Seven-day-old cultured cells were used in this study.

Cloning of PoPPO genes by rapid amplification of cDNA ends (RACE)

All the primers used are listed in Supplemental Table 1. Cloning of *PoPPO1–4* genes was performed as follows: Total RNA was prepared from portulaca roots using an RNeasy Mini Kit (Qiagen, Huntsville, AL) with DNaseI treatment. For 3′ RACE, first-strand cDNA was synthesized with adaptor-tagged oligo(dT) primer (*Not*I-oligodT primer) using a Transcriptor Reverse Transcriptase Kit (Roche, Mannheim, Germany). A degenerate primer, CuA1, designed against the CuA copper-binding site that is highly conserved among plant PPO proteins was used in a PCR with a *Not*I-oligodT primer. The 5′-RACE was performed using a GeneRacer Kit (Invitrogen, Carlsbad, CA) and specific primers for each *PoPPO* gene, according to the manufacturer's instructions. PCR products were subcloned and sequenced. Based on the results of 3′- and 5′-RACE, primers were designed around the predicted start and termination codons and used in a PCR with root cDNA as the template.

Cloning of *PoPPO5* gene was performed as follows: Total RNA was prepared from portulaca roots using an RNAiso Plus Kit (Takara Bio Inc., Shiga, Japan) with DNaseI treatment, according to the manufacturer's protocol. First-stand cDNA was synthesized using a PrimeScript RT reagent Kit (Takara Bio Inc.) with oligo(dT) primer. Degenerate primary and nested primers were designed against the CuA and against another copper-binding site (CuB) region and used in a PCR. PCR products were subcloned and sequenced. The 5′- and 3′-RACE were performed using ExactSTART Eukaryotic mRNA 5′- & 3′-RACE Kits (Epicentre), and the full-length *PoPPO5* gene was amplified by PCR using a root cDNA as template and the primers corresponding to 5′- and 3′-UTRs. The PCR products were cloned into T-Vector PMD20 (Takara Bio Inc.) and sequenced, resulting in the pMD20::PoPPO5 vector.

Sequence data can be found in the DDBJ databases under the accession numbers AB689856–60 for PoPPO1–5, respectively.

Extraction of total RNA from a range of tissues and RT-PCR

Roots, stems, leaves and flowers from portulaca were flash-frozen in liquid nitrogen and homogenized with a ball mill. For *PoPPO1–4*, total RNA was extracted with an RNeasy Mini Kit (Qiagen) with DNaseI treatment. cDNAs were synthesized with oligo(dT) primer using AMV Reverse Transcriptase XL (Takara Bio Inc.) and subjected to PCR using gene-specific primers (Supplemental Table 1). For *PoPPO5*, total RNA was extracted with an RNAiso Plus Kit (Takara Bio Inc.) and an RNeasy Mini Kit (Qiagen) with DNaseI treatment was applied to RT-PCR using Qiagen OneStep RT-PCR kit (Qiagen) with gene-specific primer pairs (Supplemental Table 1). PCR products were subjected to agarose gel electrophoresis and visualized with ethidium bromide staining. All kits were used according to the manufacture's protocol.

Construction of expression vectors for PoPPO genes

All the primers used are listed in Supplemental Table 1. *PoPPO1–4* genes were amplified by PCR using gene-specific primers designed against the 5′- and 3′-UTR. A *Xba*I site was added to each forward primer. The PCR products were introduced into the *Hin*cII gap of pUC118 using a DNA Blunting Kit (Takara Bio Inc.). Then, *PoPPO1* and *PoPPO3* genes were spliced out with *Xba*I and *Sal*I in the 3′-UTR and inserted into the ADH 5′-UTR:: PoPRX2::HA:strep vectors (Matsui et al. 2011) using the same restriction sites. *PoPPO2* and *PoPPO4* genes were spliced out with *Xba*I and *Sac*I from pUC118 and inserted into the pBI121 vector (Clontech, Palo Alto, CA) using the same restriction sites. The *PoPPO5* gene was spliced out from the pMD20::PoPPO5 vector with *Spe*I and *Sac*I, and inserted into the *Xba*I/*Sac*I gap of pBI121.

Extraction of total RNA from BY-2 cells and RT-PCR

BY-2 cells were collected by filtration, flash-frozen in liquid nitrogen and homogenized with a ball mill. Total RNA was extracted with an RNAiso Plus Kit (Takara Bio Inc.) with DNaseI treatment, according to the manufacturer's protocol. cDNAs were synthesized with oligo(dT) primer using SuperScript III (Invitrogen). *PoPPO* isoforms and *cytosolic glyceraldehyde-3-phosphate dehydrogenase* as a reference gene were amplified by PCR with gene-specific primers (Supplemental Table 1). PCR products were subjected to agarose gel electrophoresis and visualized with ethidium bromide staining.

Preparation of crude protein extracts from BY-2 cells

BY-2 cells were collected by filtration, and extraction buffer (50 mM HEPES-KOH pH 7.5, 0.33 M D-sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 2 μM EDTA and 1.14 mM ascorbic acid) was added at a ratio of 5 ml per 1 g of fresh weight. Then, the cells were homogenized on ice by sonication. The resulting cell lysate was centrifuged at 9,000×g for 10 min at 4°C, and the supernatant was collected and adjusted to a concentration of 700 μg protein ml^{-1} for assays. The protein content was determined according to the Bradford dye binding method, using bovine serum albumin as a standard (Bradford 1976).

PPO activity assay

The composition of the enzyme assay reaction mixture was the following: 1 mM $CuCl_2$ as a cofactor, 0.02% sodium dodecyl sulfate (SDS) as an activator (Angleton and Flurkey 1984), 637 μg protein ml^{-1} PoPPO enzyme solution, and 1 mM L-3,4-dihydroxy phenylalanine (L-DOPA) (in 10 mM Tris-HCl, pH 6.0) or 50 μM BPA (in extraction buffer) as a substrate. For the L-DOPA oxidization assay, an increase of *o*-quinone was measured for PPO activity (Garcia-Molina et al. 2007). In detail, we monitored the increase in absorbance at 490 nm over time at room temperature using a spectrophotometer. The rate of increase in absorbance at 490 nm per min (ΔA_{490}) was calculated. The specific activity of PPO was calculated as ΔA_{490} per mg protein per min. For assay of BPA metabolism, the reaction mixture was incubated at 25°C, and the BPA level was examined over time. Reaction mixtures were extracted with ethyl acetate. The extracts were dried with nitrogen, dissolved in 65% methanol, and separated with high performance liquid chromatography (HPLC) (Inertsil ODS-3, 4.6×250 mm; GL sciences, Inc., Tokyo, Japan). The injection volume was 80 μl, and 65% aqueous methanol was the eluent. The flow rate was 1.0 ml min^{-1}, and the absorption of the eluent was monitored at 280 nm to calculate the concentration of BPA.

Structural characterization of EDC metabolites by mass spectrometry

Liquid chromatography/photodiode array detection/tandem mass spectrometry (LC/PDA/MS/MS) was performed using a Waters Acquity UPLC system (Waters, Milford, MA, USA) coupled to a Waters Quattro Premier XE tandem quadrupole mass spectrometer (Waters). LC separations were carried out at 40°C with an Acquity UPLC BEH C18 column, 1.7 μm, 2.1×50 mm (Waters). A flow rate was set at 0.5 ml min^{-1}, and 5.0 μl of sample solution was applied to the column for each analysis. A photodiode array detector was used for monitoring between 200 to 400 nm. For the MS and MS/MS experiments, the instrument was set to collect data using an electrospray interface and the negative-ion mode. An MS scan was performed to detect m/z of intact deprotonated ions. The scan range was set at m/z 50 to 500. Product ion scan was used to acquire fragmentation patterns of EDC metabolites. The collision gas was argon. Other instrumental conditions included a capillary voltage of 4.5 kV, a desolvation temperature of 350°C, a source temperature of 120°C, a desolvation gas flow of 800 l h^{-1} (N_2), and a cone gas flow of 50 l h^{-1} (N_2). Data acquisition and processing were performed using MassLynx 4.1 (Waters).

The following parameters were set for each EDC species. BPA: the eluent was a mixture of distilled water and acetonitrile (67 : 37 v/v); the m/z of the target precursor ion for product ion scan was set at 241.0, 243.1, 255.0, 257.0 and 259.0; cone voltage and collision energy of the product ion scan were set at 40 V and 40 eV, respectively. OP: the eluent was mixture of distilled water and acetonitrile (36 : 64 v/v); the m/z of the target precursor ion for product ion scan was set at 221.0; cone voltage and collision energy of the product ion scan were set at 40 V and 20 eV, respectively. NP: the eluent was mixture of distilled water and acetonitrile (27 : 73 v/v); the m/z of the target precursor ion for product ion scan was set at 235.0; cone voltage and collision energy of product the ion scan were set at 40 V and 30 eV, respectively. E2: the eluent was a mixture of distilled water and acetonitrile (58 : 42 v/v); the m/z of the target precursor ion for the product ion scan was set at 287.0; cone voltage and collision energy of the product ion scan were set at 40 V and 40 eV, respectively.

Results and discussion

Cloning and expression analysis of PoPPOs

As described in the introduction, we hypothesized that PPO was involved in BPA detoxification by portulaca roots. To verify this hypothesis, we first isolated genes for the PPO isozymes from portulaca roots. The 5′- and 3′-RACE methods were employed for cloning with the use of degenerate primers that corresponded to DNA regions highly conserved among known PPO genes. The entire nucleotide sequences of five types of cDNA fragments we obtained are shown in the DDBJ databases under the accession numbers AB689856–60 for PoPPO1–5, respectively. We analyzed the aligned sequences of deduced PoPPO1–5 and sweet potato (*Ipomoea batatas*) PPO (GeneBank accession number: AY822711) by Geneious Pro software (ver. Pro 5.0.3, Biomatters, Auckland, New Zealand). IbPPO is a PPO enzyme that has been extensively studied as a model PPO enzyme, and its crystal structure has been solved (Klabunde et al. 1998). PoPPO1–5 showed 56.8–71.7% and 41.7–67.0% identity with each other in their nucleotide sequences and amino acid sequences, respectively. PoPPO1–5 showed 56.5–58.8% and 41.8–51.4% identity with IbPPO in their nucleotide sequences and amino acid sequences, respectively. In general, all PPO enzymes possess two highly conserved copper-binding domains, named CuA and CuB, and each domain has three histidine residues that are necessary for PPO activity (Klabunde et al. 1998). All of the deduced PoPPO1–5 contained putative CuA and CuB domains and the conserved histidine residues in those domains, suggesting that *PoPPO1–5* are members of the *PPO* gene family (Supplemental Figure 1).

The expression pattern of *PoPPO1–5* were examined by RT-PCR using gene-specific primers. Total RNAs from leaves, stems, roots and flowers were subjected to RT-PCR analysis (Figure 1). All of the *PoPPO1–4* mRNAs were mainly expressed in roots and to a lesser extent in the other organs. *PoPPO5* mRNA was expressed in roots, and was not accumulated in any other organ. We have previously shown that portulaca roots are able to metabolize BPA better than shoots (Imai et al. 2007). PoPPOs that we suggested to be involved in BPA detoxification are certainly expressed mainly in portulaca roots.

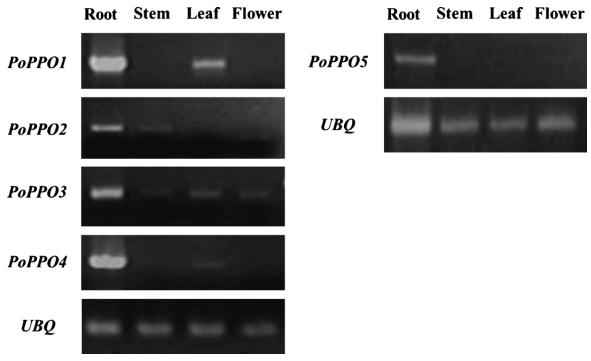

Figure 1. *PoPPO1*, *PoPPO2*, *PoPPO3*, *PoPPO4* and *PoPPO5* are differentially expressed. Total RNA isolated from roots, stems, leaves and flowers of portulaca were used for RT-PCR analysis with gene-specific primers. *Ubiquitin* (*UBQ*) was used as a reference gene.

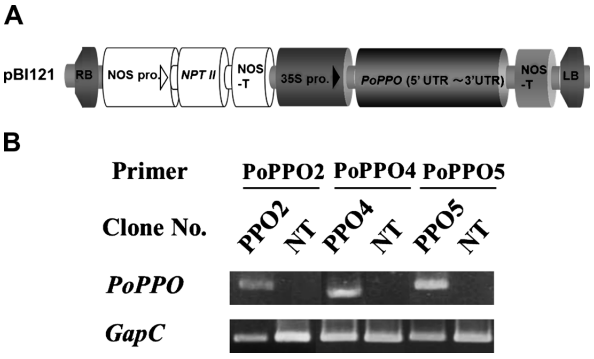

Figure 2. Generation of BY-2 cultured cells expressing *PoPPO*. (A) Expression construct of *PoPPO* in BY-2 cultured cells. (B) Expression of *PoPPO* in BY-2 cultured cells expressing *PoPPO2*, *PoPPO4* or *PoPPO5* as indicated (PPO2, PPO4 or PPO5). Non-transformed BY-2 cells were used as a control (NT). *Cytosolic glyceraldehyde-3-phosphate dehydrogenase* (*GapC*) was used as a reference gene.

Recombinant PoPPO proteins are expressed as active forms in transgenic BY-2 cells

To evaluate the metabolic activity of five PoPPO enzymes for BPA, the *PoPPO* genes were introduced and expressed in cultured BY-2 tobacco cells. Each *PoPPO1–5* gene was introduced between the CaMV 35S promoter and the NOS terminator in the pBI121 vector (Figure 2A). Transgenic BY-2 cells transformed with the pBI121 vector for the expression of *PoPPO* genes were obtained for all isoforms except PoPPO1 and PoPPO3. In detail, we weren't able to express PoPPO1 stably into BY-2 cells (data not shown). In the case of PoPPO3, we weren't able to get transgenic BY-2 cells which were tolerant to carbenicillin. The reason was not clear. We designated the transgenic BY-2 cells expressing *PoPPO2*, *PoPPO4* and *PoPPO5* as PPO2, PPO4 and PPO5, respectively. The expression of each *PoPPO* mRNA in the transgenic cell line was confirmed by RT-PCR using gene-specific primers (Figure 2B). The transgenic BY-2 cells were cultured in liquid LS medium and showed no apparent difference in their growth (data not shown).

To confirm the expression of recombinant PoPPO in their active forms, PPO activity was measured in crude extracts of PPO2, PPO4 and PPO5. In these assays, PPO activity was determined by monitoring the production of *o*-quinone from L-DOPA. Production of *o*-quinone was detected even in crude extracts of non-transformed BY-2 cells (NT), probably caused by endogenous enzymes in BY-2 cells. The PPO activities of PPO2, PPO4 and

PPO5 were 2.7, 1.5, and 4.1-fold higher compared with NT, respectively (Figure 3). These results indicated that PoPPO2, PoPPO4, and PoPPO5 proteins are expressed as active forms in transformants. It remains unknown whether the difference in PPO activity among the crude extracts from individual PoPPOs is due to a difference in protein expression levels and/or in inherent enzymatic properties.

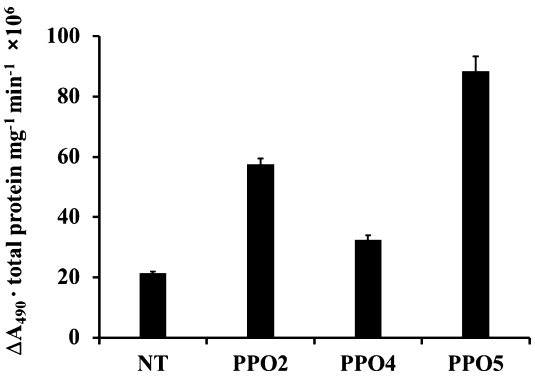

Figure 3. Activity of PoPPO proteins in transgenic BY-2 cultured cells. Extracts of BY-2 cultured cells transformed with *PoPPO2*, *PoPPO4* or *PoPPO5* as indicated (PPO2, PPO4 or PPO5) were incubated at room temperature with exogenously added PPO substrate (L-DOPA 1 mM final concentration). Extracts of non-transformed BY-2 cells (NT) served as the negative control. Data represent the mean±SD (n=3).

Recombinant PoPPOs remove BPA

We analyzed whether the recombinant PoPPO2, PoPPO4 and PoPPO5 expressed in BY-2 cells could metabolize BPA. Figure 4 shows the time-course of the BPA levels when 50 μM BPA was added to extracts of BY-2 cells expressing the PoPPO transgenes. About 76% of the added BPA was removed within 180 min by extracts of PPO4. Almost all the added BPA was removed within 180 min by extracts of PPO2 and PPO5. No decrease in BPA levels was observed in extracts of NT, even after a 12-h incubation period. These results suggested that recombinant PoPPO2, PoPPO4, and PoPPO5 metabolize BPA. The slower rate of BPA removal for PPO4 than PPO2 and PPO5 might be attributed to a lower expression level, lower activity, and/or distinct substrate specificity of PoPPO4. In the L-DOPA oxidization assay, the activity of PPO5 was higher than that of PPO2 (Figure 3). This was not the case for BPA metabolic activity, probably due to differences in substrate specificity between PoPPO2 and PoPPO5. Further characterization of the PoPPOs should be performed in a future study.

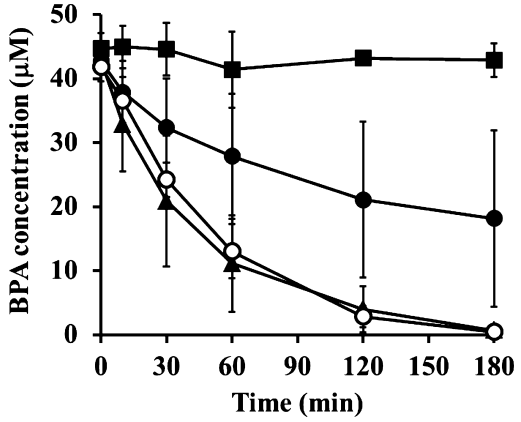

Figure 4. Time-course analysis of BPA removal by recombinant PoPPO proteins. BPA (50 μM) was added to extracts of BY-2 cells containing 0.02% SDS and 1 mM $CuCl_2$ and incubated at 25°C. The amounts of BPA were quantified by HPLC over time. Non-transformed BY-2 cells (closed squares), PPO2 (closed triangles), PPO4 (closed circles) or PPO5 (open circles). Data represent the mean±SD (n=3).

PoPPOs metabolize EDCs to same metabolites as portulaca roots

To characterize BPA metabolites produced by extracts of PPO2, PPO4 and PPO5, we analyzed the reaction solutions by LC/PDA/MS/MS. The UV chromatogram at 280 nm of the BPA solution after incubation with extracts of portulaca, NT, PPO2, PPO4 or PPO5 for 120 min under optimal conditions is shown in Figure 5A. The peak at retention time (Rt) 1.38 min was consistent with that of BPA. Three peaks (a, b, c in Figure 5A) were observed at Rt of 0.55, 0.82, and 0.89 min on the chromatograms from PPO2, PPO4 or PPO5, identical to that of portulaca. An MS scan was performed to detect deprotonated ions of those peaks. Intact BPA was observed as an ion possessing m/z 227 by the MS scan. Negative ions of m/z 255, 257, 259 were detected at a Rt 0.55 min. Those of m/z 243 and 241 were observed at Rt 0.82 and 0.89 min, respectively. The m/z of peak b, 243, is 16 units larger than that of BPA, corresponding to the addition of an OH group to BPA. The m/z 241 of peak c is 2 units smaller than that of peak b, or a quinone of peak b. The m/z 259 is 16 units lager than that of peak b, thus a hydroxylated form of peak b. The m/z 257 and 255 became smaller by 2 units, corresponding to oxidized-mono- and bis-quinones of the metabolite with a m/z 259. These peaks, except for intact BPA, were not detected in a chromatogram of an aliquot of the solution removed just after the start of the reaction. Moreover, these peaks were not observed in a chromatogram of NT cells either. Therefore, the corresponding fractions should contain BPA metabolites produced by recombinant PoPPO. The fragmentation patterns in peaks of m/z 241, 243, 255, 257, 259 of PoPPO2, PoPPO4 and PoPPO5 are almost identical to those of portulaca (Figure 5B–F). Furthermore, we analyzed other EDC metabolites produced by PoPPOs. The metabolites of other EDCs were generated by PPO2 and PPO5. The metabolites of OP, NP and E2 were detected at m/z 221, 235 and 287, respectively. Because the m/z of OP, NP and E2 were

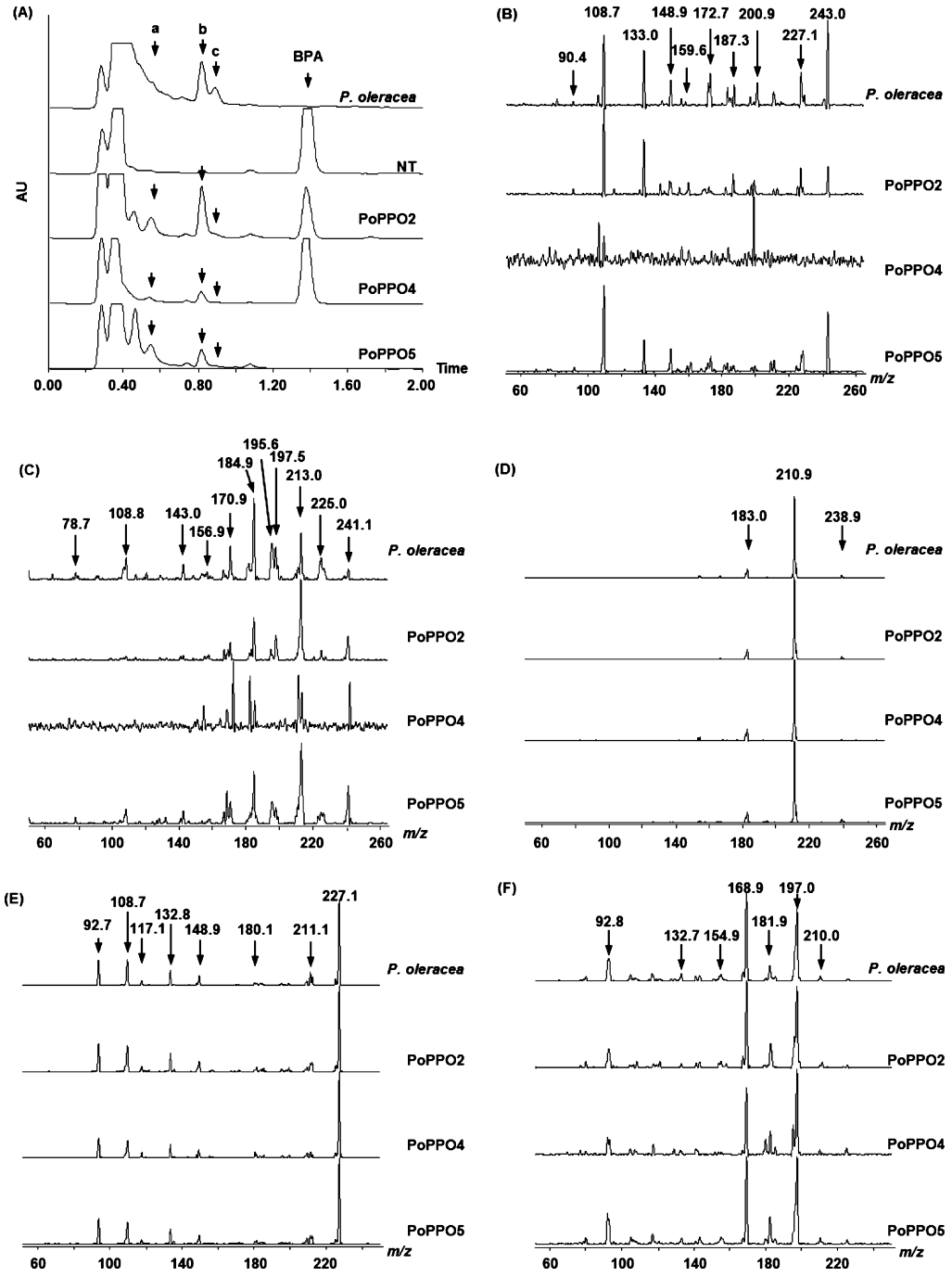

Figure 5. Analysis of BPA metabolites by LC/PDA/MS/MS. (A) UV chromatogram at wavelength 280 nm. Peaks a, b, and c for which retention times are 0.55, 0.82, and 0.89 min, respectively, are BPA metabolites. (B)–(F) Product ion spectra of BPA metabolites. Precursor ion; (B) *m*/*z* 259 (peak a), (C) *m*/*z* 257 (peak a), (D) *m*/*z* 255 (peak a), (E) *m*/*z* 243 (peak b), (F) *m*/*z* 241 (peak c). Expression of *PoPPO* in BY-2 cultured cells expressing *PoPPO2* or *PoPPO5* as indicated (PPO2 or PPO5). Non-transformed BY-2 cells were used as a control (NT).

205, 219 and 271, these metabolites were suggested to be hydroxylated forms of the substrates. The fragmentation patterns in these peaks are also identical to those of portulaca (Supplemental Figure 2). Therefore, these results showed that PoPPOs metabolize EDCs to same metabolites as portulaca roots.

We have demonstrated that portulaca roots have a superior ability to metabolize EDCs. In this study, we showed that PoPPO2, PoPPO4 and PoPPO5 are expressed mainly in portulaca roots, and that crude extracts of BY-2 cells expressing the genes for these proteins can metabolize EDCs to same metabolites as portulaca. The identities of the enzymes reported in this study will be useful for using portulaca as a practical and efficient EDCs phytoremediation system. In addition, the *PoPPO2*, *PoPPO4* and *PoPPO5* genes should be a

useful tool for producing engineered plants with high ability to remove EDCs. For example, we will introduce these genes to plants which grow easily on various environments. In the future, these engineered plants will be used to remove EDCs on various polluted soil and waters.

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