Factors regulating the differential uptake of persistent organic pollutants in cucurbits and non-cucurbits

Iwabuchi, Aya
Katte, Nonoka
Suwa, Mizuki
Goto, Junya
Inui, Hideyuki

(Citation)
Journal of Plant Physiology,245:153094

(Issue Date)
2019-12-17

(Resource Type)
journal article

(Version)
Accepted Manuscript

(URL)
https://hdl.handle.net/20.500.14094/0100476377

Title: Factors regulating the differential uptake of persistent organic pollutants in cucurbits and non-cucurbits

Authors: Aya Iwabuchi[a], Nonoka Katte[b], Mizuki Suwa[a], Junya Goto[a], Hideyuki Inui[a,c,*]

[a]Graduate School of Agricultural Science, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe, Hyogo 657-8501, Japan,

[b]Faculty of Agriculture, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe, Hyogo 657-8501, Japan,

[c]Biosignal Research Center, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe, Hyogo 657-8501, Japan

Corresponding author: Hideyuki Inui

Address: Biosignal Research Center, Kobe University, 1-1 Rokkodaicho, Nada-ku, Kobe, Hyogo 657-8501, Japan

Tel & Fax: +81-78-803-5863

E-mail: hinui@kobe-u.ac.jp

Abstract

Contamination with persistent organic pollutants (POPs) has become a worldwide concern owing to their the toxicity to humans and wildlife. Pumpkin, cucumber, and squash (Cucurbitaceae) accumulate POPs in their shoots in concentrations higher than those in non-cucurbits; to elucidate the underlying molecular mechanisms of this accumulation, POP transporters were analyzed in the xylem sap of cucurbits and non-cucurbits. The 17-kDa xylem sap proteins detected in all cucurbits but not in non-cucurbits readily bound polychlorinated biphenyl (PCB) in all tested cucurbits, except in cucumber and loofah, and to dieldrin in all tested cucurbits. Ten genes encoding major latex-like proteins (MLPs) responsible for the accumulation of PCBs in zucchini plants were cloned from cucurbits. Phylogenetic analysis using MLP sequences identified two separate clades, one containing Cucurbitaceae MLPs and the other containing those of non-cucurbit members. Recombinant MLPs bound PCB and dieldrin. Western blotting with anti-MLP antibodies identified translocatable and non-translocatable MLPs between root and stem xylem vessels. Translocation of MLPs from the root to stem xylem vessels and POP-binding ability of MLPs are important for selective accumulation of MLPs in cucurbits. This study provides basic knowledge about phytoremediation through overexpression of *MLP* genes and for breeding cucurbits that accumulate less contaminants.

Abbreviations: DDT, dichlorodiphenyltrichloroethane; DDE, *p*,*p*′-dichlorodiphenyltrichloroethylene; DTT, dithiothreitol; MLP, major latex-like protein; PR-10, pathogenesis-related proteins of class 10; CB106, 2',3,3',4',5'-pentachlorobiphenyl; POP, persistent organic pollutant; PMSF, phenyl-methylsulfonyl

Keywords: major latex-like protein, xylem sap protein, persistent organic pollutant, polychlorinated biphenyl, Cucurbitaceae, crop contamination.

fluoride; PCB, polychlorinated biphenyl; PCDF, polychlorinated dibenzofuran; PCDD, polychlorinated dibenzo-*p*-dioxin; UTR, untranslated region.

1. Introduction

The Cucurbitaceae family comprises popular vegetables cultivated worldwide, such as cucumber, pumpkin, watermelon, squash, and zucchini. However, fruits produced by these plant species easily accumulate toxic compounds, thereby adversely affecting human and wildlife health upon consumption. Toxic persistent organic pollutants (POPs), such as dichlorodiphenyltrichloroethane (DDT) and its metabolites dichlorodiphenyldichloroethylene (DDE) (White et al., 2003b), chlordane (Incorvia Mattina et al., 2000), dieldrin (Otani et al., 2007), polychlorinated dibenzo-*p*-dioxins (PCDD) and polychlorinated dibenzofurans (PCDFs) (Hülster et al., 1994), and polychlorinated biphenyls (PCBs) (Inui et al., 2008), accumulate in the shoots of cucurbits. The distinctive physicochemical properties shown by these POPs, such as high hydrophobicity and chemical stability, result in their accumulation in animal tissues ——including human tissues ——through the food chain. In animals, accumulated POPs damage the reproductive and immune systems, affect development, and promote carcinogenesis. Although the release of POPs has been restricted, if not altogether banned following the adoption of the Stockholm Convention, they are still present in the environment around the world.

Several possible processes may be involved in the accumulation of POPs to high levels in cucurbits: desorption and solubilization of POPs in the rhizosphere, adsorption and absorption of POPs into root cells, and translocation of POPs to shoots. Highly hydrophobic compounds show low bioavailability in the soil because they usually bind tightly to soil organic matter components. Organic acids excreted from plant roots solubilize nutrients necessary for growth (Neumann and Martinoia, 2002), but they also desorb hydrophobic pollutants, which are then absorbed by plant roots (White et al.,

2003a; Yoshihara et al., 2014). However, there is no clear evidence for the involvement of organic acids in the selective accumulation of POPs in high concentrations in cucurbits.

The adsorption of the hydrophobic compound perylene reportedly occurs on root epidermal cells, and it is preferentially accumulated in endodermal and pericycle cells of root tissues (Yamazaki et al., 2015). However, high accumulation of perylene in these cells was observed in both low and high accumulators. Further, several studies have shown that the accumulation of POPs in roots did not differ between low and high accumulators (Gent et al., 2007; Inui et al., 2011). These results suggest that the first two processes involved in POP accumulation are not major branching points between cucurbits and non-cucurbits.

Xylem sap is important for the transport of chlordane from roots to shoots (Mattina et al., 2004), and xylem sap proteins with molecular weight of approximately 13.7 kDa enhance the solubilization of dieldrin (Murano et al., 2010). These results suggest that xylem sap proteins produced in root cells determine the capacity for POP accumulation in the shoot. Previously, we proposed that major latex-like proteins (MLPs) detected in xylem sap played a key role in the contamination of zucchini (*Cucurbita pepo* L. ssp. *pepo*) with PCBs (Inui et al., 2013). MLPs were detected in the xylem sap, and their level was dependent on accumulated POPs in the shoots of *C. pepo* cultivars (Goto et al., 2019). Furthermore, xylem sap proteins and the recombinant MLPs of *C. pepo* bind PCB and other environmental contaminants, such as 17β-estradiol, dieldrin, and 4-*t*-octylphenol. All these results suggest that MLPs produced in root cells bind contaminants to solubilize them, upon which, they then move as MLP-PCB complexes into the xylem vessels for translocation to the shoot as components of the xylem sap.

MLPs belong to a family of pathogenesis-related proteins of class 10 (PR-10) or Bet v 1-like super family (Radauer et al., 2008; Fernandes et al., 2013) owing to the strong similarity of their 3D structures, especially in the inner hydrophobic pocket (Lytle et al., 2009). Various structures of ligands, such as flavonoids, cytokinins, and brassinosteroids, bind MLPs in this pocket (Fernandes et al., 2013). These results suggest that the affinity of MLPs for POPs affects the accumulation of POPs in cucurbits. However, the mechanisms underlying this phenomenon are yet to be clarified.

In this study, we focused on translocation of MLPs from roots to shoots and binding of MLPs by POPs to elucidate the mechanism underlying the distinctive capacity of cucurbits for high POP-accumulation. Thus, xylem sap proteins from several plant species within Cucurbitaceae and other families were detected and subjected to binding experiments with PCB and dieldrin. Further, we isolated cDNAs encoding MLPs from Cucurbitaceae and confirmed the binding capacity of these MLPs.

2. Materials and methods

2.1. Plant materials

The seeds used in this study were purchased from the following companies: pumpkin (*Cucurbita moschata* L. 'Hayato') from Takii Co., Ltd. (Kyoto, Japan), fig leaf squash (*Cucurbita ficifolia* L. 'Kurodane') from Nemotonouen (Chiba, Japan), loofah (*Luffa cylindrica* L. 'Futohechima') from Noguchiseed (Saitama, Japan), white-flowered gourd (*Lagenaria siceraria* L. 'Renshi') and watermelon (*Citrullus lanatus* L. 'Crimson108') from Kanda Seed Co., Ltd. (Nara, Japan), cucumber (*Cucumis sativus* L.

‘Sharp-1’) from Saitama Gensyu Ikuseikai Co., Ltd. (Saitama, Japan), and soybean (*Glycine max* L. ‘Tachinagaha’) from Tsurushinsyubyou (Nagano, Japan). Tomato (*Solanum lycopersicum* L. ‘Micro-Tom’) seeds (TOMJPF0001) were purchased from the University of Tsukuba, Gene Research Center, through the National Bio-Resource Project of the Japan Agency for Research and Development, Japan.

2.2. Collection of xylem sap and roots

Seeds were sterilized with 70% (v/v) ethanol and immersed in tap water for 1 d at 4°C and then sown and cultivated in glass jars (400 mL) filled with soil (Hyponex Japan Corp., Ltd., Osaka, Japan), and kept in a plant incubator at 25°C under a 16/8 h light/dark cycle. After approximately 10 d, seedlings were transferred to larger glass jars (900 mL) filled with soil and cultivated for another 35 d at 25°C under the same conditions. To collect xylem sap, the area just below the cotyledons was cut and washed with sterilized water, and the sap was collected into 1.5 mL plastic tubes after confirmation of pH change of exudates from neutral to acidic. Collected xylem sap was stored at 4°C until use. After collecting the xylem sap, the roots were washed with tap water to remove all the soil, and then frozen with liquid nitrogen. Xylem sap and root samples were separately combined prior to their use (n = 2–3). The roots were stored at −80°C until use.

2.3. SDS-PAGE of xylem sap and root extracts

Roots were homogenized in extraction buffer (50 mM phosphate buffer [pH 7.0],

10 mM EDTA [pH 8.0], 0.1% [v/v] Triton X-100, 0.1% [v/v] *N*-lauroyl sarcosine sodium salt, and 0.072% [v/v] 2-mercaptoethanol) with mortar and pestle. Homogenates were centrifuged at 22,000×*g* for 15 min at 4°C and the supernatants were collected. Protein concentrations in roots and xylem sap were measured using the Bradford method (Bradford, 1976). Samples containing 0.2 μg of protein from root extracts and 0.1 μg of protein from xylem sap were mixed with a sample buffer solution containing reducing reagent (Nacalai Tesque, Inc., Kyoto, Japan) and subjected to SDS-PAGE on 15% acrylamide gels. Protein bands were detected using a Silver Stain Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and antibodies against CpMLP-PG1 and CpMLP-GR3 derived from *C. pepo* (Medical & Biological Laboratories Co., Ltd. Aichi, Japan).

2.4. Cloning of MLP cDNAs in the Cucurbitaceae family

Total RNA was extracted from the roots using TRIzol Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA), and cDNA was synthesized using a ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd., Osaka, Japan) according to manufacturer instructions. The *MLP* cDNAs were amplified with primers 5′-UTR and 3′-UTR, containing 5′-untranslated region (UTR) and 3′-UTR of the *MLP* genes in *C. pepo* (Table S1). Amplified fragments including *MLP* cDNAs were subcloned into the pUC19 vector using In-Fusion Cloning Kit (Takara Bio Inc., Shiga, Japan). DNA sequences of *MLP* genes were deposited in the DNA Data Bank of Japan (Table S1). A phylogenetic tree using MLPs translated from the corresponding genes of cucurbits and non-cucurbits was constructed using the UPGMA method in CLUSTALW

(http://genome.jp/tools/clustalw/).

2.5. *MLP*-gene expression in recombinant *Escherichia coli*

MLP gene sequences including start and stop codons were amplified with the corresponding primers described in Table S1, using *MLP* cDNAs subcloned in the pUC19 as templates. Amplified fragments were inserted into the pET-28b(+) vector (Merck KGaA, Darmstadt, Germany) digested with *Nco*I and *Xho*I using the In-Fusion Cloning Kit. The DNA sequences of *MLP* genes were confirmed by sequencing. *E. coli* Rosetta-gami 2 (Merck KGaA) was used as a host for the expression of *MLP* genes by plasmid introduction, and MLPs were produced as hexa-His-tagged proteins.

2.6. *Purification of MLPs produced in recombinant E. coli*

Recombinant *E. coli* cells harboring each expression plasmid were precultured overnight at 37°C in Luria-Bertani medium containing 50 μg mL^{-1} kanamycin and 34 μg mL^{-1} chloramphenicol. Bacterial suspensions were added to 2× YT medium containing the above antibiotics and cultured at 37°C for 2 h. Isopropyl-β-D-thiogalactopyranoside was added to the culture medium to a final concentration of 0.1 mM to trigger transcription, and the culture was incubated for an additional 20 to 24 h at 20°C. Cells containing the expressed proteins were harvested and washed with a phosphate buffer (50 mM phosphate buffer [pH 7.0] and 200 mM NaCl). Next, phosphate buffer containing 1% (v/v) Triton X-100 was added to the cells, and then cells were disrupted by sonication. Insoluble components were removed by

centrifugation at 22,000×*g* for 20 min at 4°C. Supernatants were mixed with TALON Metal Affinity Resin (Clontech Laboratories, Inc., Mountain View, CA, USA) and incubated for 20 min at 4°C with continuous rotation. The solutions were centrifuged at 400×*g* for 5 min at 4°C; supernatants were removed and phosphate buffer was added to wash the affinity resin. Finally, phosphate buffer was added and the mixture was filtered through a glass filter paper. The affinity resin was washed with phosphate buffer containing 5 mM imidazole and proteins were eluted with phosphate buffer containing 150 mM imidazole.

2.7. Measurement of PCB- and dieldrin-binding activities of xylem sap proteins and recombinant MLPs

4-Hydroxy-2′,3,3′,4′,5′-pentachlorobiphenyl (AccuStandard, New Haven, CT, USA) and dieldrin (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) were bound to magnetic beads (Tamagawa Seiki Co., Ltd., Nagano, Japan) in 12.5 and 25 mM PCB solutions and in a 100 mM dieldrin solution according to manufacturer instructions. A 200-μL volume of 100 mM KCl buffer (20 mM HEPES-NaOH [pH 7.9] for xylem sap or 50 mM sodium phosphate [pH 5.6] for recombinant MLPs, 100 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM EDTA, 10% [v/v] glycerol, and 0.1% [v/v] Nonidet P-40) supplemented with 1 mM dithiothreitol (DTT) and 0.2 mM phenyl-methylsulfonyl fluoride (PMSF) was added to 0.5 mg of each bead, and the beads were dispersed for washing. The supernatants were discarded after magnetic separation. A 10 × 100 mM KCl buffer supplemented with 10% (v/v) Triton X-100, 10% (v/v) *N*-lauroyl sarcosine sodium salt, 10 mM DTT, and 2 mM PMSF was added to xylem sap to decrease its final

concentration to one-tenth, and 100 mM KCl buffer supplemented with 1% (v/v) Triton X-100, 1% (v/v) *N*-lauroyl sarcosine sodium salt, 1 mM DTT, and 0.2 mM PMSF was added to recombinant MLPs. Samples were centrifuged at 22,000×*g* for 15 min at 4°C. Supernatants containing 40 μg of protein were added to 0.5 mg of PCB-bound beads, dieldrin-bound beads, or control beads without any bound compounds. After dispersion of the beads, solutions were incubated for 4 h at 4°C with continuous rotation, and after magnetic separation, supernatants were removed. A 200-μL volume of 100 mM KCl buffer was added to the remaining beads, and the beads were dispersed for washing; this washing step was repeated eight times. After washing, 35 μL of 100 mM KCl buffer and 7 μL of the sample buffer solution were added to these beads, and the beads were heated for 5 min at 98°C. After magnetic separation, the supernatants were subjected to SDS-PAGE on 15% acrylamide gels, and gels were stained using a Silver Stain Kit. Band intensity was quantified by ImageJ (Schneider et al., 2012). Cross-reactivities of the antibodies were confirmed using recombinant MLPs, and homologies of epitopes toward *C. pepo* MLPs were determined by CLUSTALW (http://www.genome.jp/tools/clustalw/) (Fig. S2).

3. Results

3.1. Detection of 17-kDa proteins in xylem sap and roots and their PCB and dieldrin binding activities

One or more proteins with molecular weight of approximately 17 kDa were found in xylem sap and root extracts of all tested cucurbits (Fig. 1A). The number of proteins

detected in the xylem sap was lower than that in roots and the amount of 17-kDa proteins in xylem sap varied among the tested members of the Cucurbitaceae family. In the xylem sap of pumpkin, fig leaf squash, white-flowered gourd, and watermelon, proteins were detected in the PCB-immobilized bead treatment but not in the control bead treatment. In contrast, there were no protein bands in cucumber and loofah in both PCB-immobilized bead and control bead treatments. In soybean and tomato, the 17-kDa proteins were not detected in the xylem sap, but they were present in roots (Fig. 1A).

Proteins with molecular weight of approximately 17 kDa were detected in the xylem sap of all tested cucurbits in the dieldrin-immobilized bead treatment but not in the control bead treatment (Fig. 1B). Interestingly, in cucumber and loofah, only the dieldrin-binding proteins were detected in the xylem sap (Fig. 1).

3.2. Cloning of MLP cDNAs in members of the Cucurbitaceae family

Ten *MLP* cDNAs were amplified from root extracts of pumpkin, fig leaf squash, cucumber, white-flowered gourd, watermelon, and loofah. Three *MLP* genes from pumpkin were designated as *CmMLP1*, *CmMLP2*, and *CmMLP3*. Similarly, two from fig leaf squash, one from cucumber, one from watermelon, two from loofah, and one from white-flowered gourd were designated as *CfMLP1* and *CfMLP2*; *CsMLP1*; *ClMLP1*; *LcMLP1* and *LcMLP2*; and *LsMLP1*, respectively. The DNA sequences of *CsMLP1*, *ClMLP1*, and *LcMLP1* were identical to those of *CmMLP1*. Deduced amino acid sequences consisted of 156 to 158 amino acids with a calculated molecular weight of 17.8 to 18.1 kDa (Fig. S1). The similarity of amino acid sequences inferred from the cloned *MLP* cDNAs ranged from 52.9 to 100% (Table S2). The search of all MLPs

using Pfam (http://pfam.xfam.org) confirmed the presence of Bet v 1 motif in all obtained MLPs. Phylogenetic analysis based on the MLPs from the members of the Cucurbitaceae family under study and other non-cucurbitaceous plant species resolved two clades, one with cucurbits and the other with non-cucurbits (Fig. 2).

3.3. Detection of PCB- and dieldrin-binding activity of recombinant MLPs

The recombinant MLPs CmMLP1, CmMLP2, CfMLP1, CfMLP2, and LcMLP2 were successfully produced in *E. coli*. All recombinant MLPs, except for LcMLP2, were detected in the PCB-immobilized bead treatments, whereas they were not present, or were present at lower levels in the control bead treatment (Fig. 3A). Band intensity of CmMLP1 and CmMLP3 was higher than that of CfMLP1 or CfMLP2. In contrast, all tested recombinant MLPs showed dieldrin binding activity (Fig. 3B). MLPs ranked as follows, based on band intensity: CmMLP3 and LcMLP2 > CmMLP1 and CfMLP2 > CfMLP1.

3.4. Detection of MLPs in cucurbits by anti-CpMLP antibodies

The anti-CpMLP-PG1 and the anti-CpMLP-GR3 antibodies detected CmMLP1 and CmMLP3, which contain identical epitopes to those in CpMLP-PG1 epitope 2 and CpMLP-GR3 epitope 2, respectively (Fig. S2). MLPs were detected in root extracts of all tested cucurbits using the anti-CpMLP-PG1 antibody (Fig. 4A, dotted arrow, and Fig. S3A, solid arrows), but not in the xylem sap of cucurbits, except for pumpkin. MLPs with a different molecular weight from that in root extracts were detected in the

xylem sap of pumpkin; these MLPs showed PCB-binding affinity (Fig. 4A, solid arrow). In contrast, the anti-CpMLP-GR3 antibody detected MLPs in the xylem sap and root extracts of pumpkin, but not in other species (Fig. 4B and Fig. S3B). These MLPs in pumpkin showed the same mobility in SDS-PAGE, and the MLPs in xylem sap effectively bound PCB.

4. Discussion

In this study, we aimed to explain why the uptake of POPs might be limited to cucurbits (Otani et al., 2007). Because the molecular weight of MLPs responsible for uptake of PCBs in *C. pepo* is approximately 17 kDa (Inui et al., 2013), we focused on 17-kDa proteins present in xylem sap and roots. Three *MLP* genes were expressed in roots of *C. pepo*, and the corresponding MLPs were translocated to shoots via xylem sap (Inui et al., 2013). PCBs were detected in xylem sap, and the level to which they accumulated in shoots and the amount of both MLP-PG1 and MLP-GR3 correlated positively (Goto et al., 2019). Furthermore, these MLPs were observed to bind PCBs. Therefore, we conclude that MLPs are directly involved in the mechanism underlying POP accumulation in cucurbits.

In general, xylem vessels play a major role in transporting not only water but various other substances as well, such as *t*-zeatin riboside, glutamine, methylglycine, *myo*-inositol, fructose, glucan, and pectins contained in the xylem sap of squash (Satoh, 2006). Some proteins, such as chitinase, glycine-rich protein, peroxidase, subtilase, and xylem-sap protein 30 are also present in the xylem sap of pumpkin and cucumber, although the combination of these proteins differs among plant species (Buhtz et al.,

2004). Supposedly, these proteins are produced in root cells and translocated to xylem vessels, where they participate in repair and defense reactions. The cucurbits used in this study contained xylem sap proteins with molecular weight near 17 kDa as well as the root extracts (Fig. 1A). Western blotting showed that some of these proteins in xylem sap and roots of cucurbits were MLPs (Fig. 4 and Fig. S3). In contrast, proteins with molecular weight near 17 kDa were present in lower quantities or were altogether absent from xylem sap of non-cucurbits, such as soybean and tomato (Fig. 1A). It was reported that all cucurbits used in this study showed a high accumulation of dieldrin in their shoots, but not in soybean and tomato (Otani et al., 2007). These results suggest that the movement of MLPs produced in root cells into the xylem vessels of the stele is a crucial step in the uptake of POPs by members of the Cucurbitaceae.

Although the chemical structures of PCB and dieldrin are not similar, some xylem sap proteins, including MLPs and recombinant MLPs, bound PCB and/or dieldrin (Figs. 1 and 3). Cucurbits are known for their capacity to take up an array of structurally diverse POPs (Hülster et al., 1994; Incorvia Mattina et al., 2000; White et al., 2003; Otani et al., 2007; Inui et al., 2008). These results suggest that the binding affinity of MLPs is likely determined by the characteristics they have in common, such as hydrophobicity, rather than the specificity of each structure. The detected 17-kDa xylem sap proteins in cucumber and loofah can bind dieldrin but not PCB (Fig. 1), suggesting that extremely high hydrophobicity interfered with binding: the reported values of log K_{ow} for dieldrin and 2′,3,3′,4′,5′-pentachlorobiphenyl (CB106) are 5.40 (De Bruijn et al., 1989) and 6.64 (Hawker and Connell, 1988), respectively. Thus, it is likely that cucumber and loofah accumulate more dieldrin than PCB. Interestingly, our previous study revealed differential accumulation of certain PCB congeners, such as 2-

chlorinated PCBs, in high and low accumulators among *C. pepo* cultivars (Inui et al., 2011; Matsuo et al., 2011; Goto et al., 2019). The 2-chlorinated PCBs show structural bulkiness owing to inhibition of rotation of a biphenyl ring. Such structural bulkiness observed in 2-chlorinated PCBs and dieldrin may also promote the binding of the MLPs. MLPs are members of the PR-10 or Bet v 1-like super family (Radauer et al., 2008; Fernandes et al., 2013). The important structural feature of these proteins is a large Y-shaped hydrophobic cavity that is responsible for binding diverse relatively high-hydrophobic ligands, such as flavonoids, cytokinins, and brassinosteroids (Fernandes et al., 2013). This structural similarity explains why all tested recombinant MLPs bound either PCB or dieldrin (Fig. 3). These results strongly suggest that the binding affinity of MLPs for hydrophobic compounds resides in their internal cavity. In other words, the efficiency and specificity of POP accumulation in the shoots of cucurbits are determined by the specific MLPs produced by the root.

MLP cDNAs were cloned from root extracts of cucurbits, as xylem sap proteins synthesized in root cells and diffuse into the xylem vessels via apoplast (Buhtz et al., 2004). The amino acid sequences deduced from the 10 *MLP* genes shared 52.9−100% similarity (Table S2). These MLPs are relatively conserved within Cucurbitaceae, whereas *Arabidopsis* MLP28 (Lytle et al., 2009), MLP43 (Wang et al., 2016), and MLP423 (Litholdo et al., 2016) showed only 20.3–27.4% homology to those of Cucurbitaceae. The MLPs from the cucurbits tested here were not classified in the clade containing MLPs of non-cucurbits (Fig. 2), suggesting that the movement of MLPs into the xylem vessels and POPs-binding capacity of MLPs are distinctive features of the Cucurbitaceae family. Interestingly, the sequence of LcMLP2 was weakly homologous to the sequences of other MLPs (Table S2), which may be the basis of a specific binding

activity—LcMLP2 binds dieldrin, but not PCB (Fig. 3).

It has been reported that cucumber Csf2 (Suyama et al., 1999) and bell pepper Sn-1 (Pozueta-Romero and Klein, 1995) are involved in fruit development, but the detailed physiological mechanisms controlled by these two proteins have not been identified. Recent studies revealed several functions of MLPs from non-cucurbits. Cotton GhMLP28 and *Arabidopsis* MLP43 are involved in defense responses against fungal pathogens and drought tolerance as positive regulators of ethylene and abscisic acid responses, respectively (Yang et al., 2015; Wang et al., 2016). Overexpression of cotton *Gh-MLP* in *Arabidopsis* induced an increase in flavonoid content (Chen and Dai, 2010). Similarly, strawberry PR-10 Fra a, with similar 3D structure to MLPs, reportedly controlled flavonoid biosynthesis by binding its metabolic intermediates (Casañal et al., 2013). Furthermore, *Arabidopsis* MLP423 is related to normal development of leaf pattern and morphology (Litholdo et al., 2016). The accumulation pattern of CpMLP-PG1 and CpMLP-GR3 in xylem sap was different, suggesting that each MLP has different functions (Goto et al., 2019).

Although fig leaf squash, cucumber, white-flowered gourd, watermelon, and loofah, all contained xylem sap proteins, MLPs were not detected by the anti-CpMLP antibodies in this case (Fig. S3). Western blot analysis showed that MLPs, probably CmMLP1, in root extracts of pumpkin are identical to those in cucumber, watermelon, and loofah, because these cucurbits expressed the same *MLP* genes (Table S2). As these MLPs were not detected in xylem sap of cucumber, watermelon, or loofah, they presumably represent proteins of a kind that does not move into the xylem vessels (Fig. 4A, dotted arrow, and Fig. S3A, solid arrows). This is supported by the results of the analysis of root extracts of *C. pepo*, in which MLP-PG1 (upper band near 17 kDa) was

not detected in xylem sap (Goto et al., 2019). In contrast, MLPs that do move into the xylem vessels were detected by the anti-CpMLP-PG1 and the anti-CpMLP-GR3 antibodies only in pumpkin (Fig. 4). Moreover, these MLPs showed binding affinity for PCB. Thus, it is likely that MLPs detected in pumpkin by anti-MLP-PG1 (Fig. 4A) and anti-CpMLP-GR3 (Fig. 4B) are CmMLP1, and CmMLP2 and CmMLP3, respectively (Fig. S2). These results suggest that some of MLPs produced in root cells function in facilitating the movement of POPs into xylem vessels and their transport to the aerial parts of the plant. Furthermore, the localization of MLPs in root tissues is important because MLPs must interact with POPs to bind them. It was reported that the hydrophobic compound was accumulated in endodermis and pericycle cells (Yamazaki et al., 2015). It is necessary for MLPs to be detected in these tissues.

5. Conclusions

The accumulation of POPs in members of the Cucurbitaceae family seems to be determined mainly by two factors, namely, 1) apoplastic movement of MLPs produced in root cells into the xylem vessels within the stele, and 2) the structural capacity of MLPs for binding a range of compounds on the basis of shared properties, such as the degree of hydrophobicity of POPs. The effects of both factors are complex, resulting in substantial variability among cucurbits for the level of accumulation of POPs. Thus, MLPs were responsible for the accumulation of POPs in the members of the Cucurbitaceae family under scrutiny here. Our study lays a solid theoretical foundation for the development of phytoremediation strategies that may use the overexpression of *MLP* genes in plants and for breeding varieties of cucurbit crop species with low

accumulation of contaminants through the downregulation of *MLP* genes. However, the reasons for Cucurbitaceae family members to have evolved MLPs that bind POPs, and the physiological function of MLPs in these species, remain unknown and deserve investigation.

Accession numbers: Sequence data in this article can be found in the GenBank/EMBL data libraries under accession numbers LC177368 (*CmMLP1*), LC177369 (*CmMLP2*), LC177370 (*CmMLP3*), LC177371 (*CfMLP1*), LC177372 (*CfMLP2*), LC177373 (*LcMLP2*), and LC177374 (*LsMLP1*).

Declaration of interest

The authors declare no conflicts of interest.

Acknowledgements

We express our gratitude to Prof. Shigeo Takumi, Prof. Ryo Fujimoto, Prof. Takeshi Takasaki-Yasuda, and Dr. Daisuke Matsuoka of the Graduate School of Agricultural Science, Kobe University, for their helpful discussion of the manuscript contents.

References

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Buhtz, A., Kolasa, A., Arlt, K., Walz, C., Kehr, J., 2004. Xylem sap protein

composition is conserved among different plant species. Planta 219, 610–618.

Casañal, A., Zander, U., Muñoz, C., Dupeux, F., Luque, I., Botella, M.A., Schwab, W., Valpuesta, V., Marquez, J.A., 2013. The strawberry pathogenesis-related 10 (PR-10) Fra a proteins control flavonoid biosynthesis by binding to metabolic intermediates. J. Biol. Chem. 288, 35322–35332.

Chen, J.-Y., Dai, X.-F., 2010. Cloning and characterization of the *Gossypium hirsutum* major latex protein gene and functional analysis in *Arabidopsis thaliana*. Planta 231, 861–873.

De Bruijn, J., Busser, F., Seinen, W., Hermens, J., 1989. Determination of octanol/water partition coefficients for hydrophobic organic chemicals with the "slow-stirring" method. Environ. Toxicol. Chem. 8, 499–512.

Fernandes, H., Michalska, K., Sikorski, M., Jaskolski, M., 2013. Structural and functional aspects of PR-10 proteins. FEBS J. 280, 1169–1199.

Gent, M.P.N., White, J.C., Parrish, Z.D., Isleyen, M., Eitzer, B. rianD, Mattina, M.J.I., 2007. Uptake and translocation of *p*,*p*′-dichlorodiphenyldichloroethylene supplied in hydroponics solution to Cucurbita. Environ. Toxicol. Chem. 26, 2467–2475.

Goto, J., Iwabuchi, A., Yoshihara, R., Kodama, N., Matsui, T., 2019. Uptake mechanisms of polychlorinated biphenyls in *Cucurbita pepo* via xylem sap containing major latex-like proteins. Environ. Exp. Bot. 162, 399–405.

Hawker, D.W., Connell, D.W., 1988. Octanol-water partition coefficients of polychlorinated biphenyl congeners. Environ. Sci. Technol. 22, 382–387.

Hülster, A., Müller, J.F., Marschner, H., 1994. Soil–plant transfer of polychlorinated dibenzo-*p*-dioxins and dibenzofurans to vegetables of the cucumber family (Cucurbitaceae). Environ. Sci. Technol. 28, 1110–1115.

Incorvia Mattina, M.J., Iannucci-Berger, W., Dykas, L., 2000. Chlordane uptake and its translocation in food crops. J. Agric. Food Chem. 48, 1909–1915.

Inui, H., Sawada, M., Goto, J., Yamazaki, K., Kodama, N., Tsuruta, H., Eun, H., 2013. A major latex-like protein is a key factor in crop contamination by persistent organic pollutants. Plant Physiol. 161, 2128–2135.

Inui, H., Wakai, T., Gion, K., Kim, Y.S., Eun, H., 2008. Differential uptake for dioxin-like compounds by zucchini subspecies. Chemosphere 73, 1602–1607.

Inui, H., Wakai, T., Gion, K., Yamazaki, K., Kim, Y.-S., Eun, H., 2011. Congener specificity in the accumulation of dioxins and dioxin-like compounds in zucchini plants grown hydroponically. Biosci. Biotechnol. Biochem. 75. 705–710.

Litholdo, C.G., Parker, B.L., Eamens, A.L., Larsen, M.R., Cordwell, S.J., Waterhouse, P.M., 2016. Proteomic identification of putative microRNA394 target genes in *Arabidopsis thaliana* identifies major latex protein family members critical for normal development. Mol. Cell. Proteomics. 15, 2033–2047.

Lytle, B.L., Song, J., De La Cruz, N.B., Peterson, F.C., Johnson, K.A., Bingman, C.A., Phillips, G.N., Volkman, B.F., 2009. Structures of two *Arabidopsis thaliana* major latex proteins represent novel helix-grip folds. Proteins Struct. Funct. Bioinforma. 76, 237–243.

Matsuo, S., Yamazaki, K., Gion, K., Eun, H., Inui, H., 2011. Structure-selective accumulation of polychlorinated biphenyls in *Cucurbita pepo*. J. Pestic. Sci. 36. 363–369.

Mattina, M.I., Eitzer, B.D., Iannucci-Berger, W., Lee, W.Y., White, J.C., 2004. Plant uptake and translocation of highly weathered, soil-bound technical chlordane residues: Data from field and rhizotron studies. Environ. Toxicol. Chem. 23, 2756–

2762.

Murano, H., Otani, T., Seike, N., 2010. Dieldrin-dissolving abilities of the xylem saps of several plant families, particularly *Cucurbita pepo* L. Environ. Toxicol. Chem. 29, 2269–2277.

Neumann, G., Martinoia, E., 2002. Cluster roots - An underground adaptation for survival in extreme environments. Trends Plant Sci. 7, 162–167.

Otani, T., Seike, N., Sakata, Y., 2007. Differential uptake of dieldrin and endrin from soil by several plant families and Cucurbita genera. Soil Sci. Plant Nutr. 53, 86–94.

Pozueta-Romero, J., Klein, M., 1995. Characterization of a family of genes encoding a fruit-specific wound-stimulated protein of bell pepper (*Capsicum annuum*): identification of a new family of transposable elements. Plant Mol. Biol. 79230, 1011–1025.

Radauer, C., Lackner, P., Breiteneder, H., 2008. The Bet v 1 fold: An ancient, versatile scaffold for binding of large, hydrophobic ligands. BMC Evol. Biol. 8, 1–19.

Satoh, S., 2006. Organic substances in xylem sap delivered to above-ground organs by the roots. J. Plant Res. 119, 179–187.

Schneider, C.A., Rasband, W.S., Eliceiri, K.W., 2012. NIH Image to ImageJ: 25 years of image analysis. Nat. Methods 9, 671.

Suyama, T., Yamada, K., Mori, H., Takeno, K., Yamaki, S., 1999. Cloning cDNAs for genes preferentially expressed during fruit growth in cucumber. J. Am. Soc. Hortic. Sci. 124, 136–139.

Wang, Y., Yang, L., Chen, X., Ye, T., Zhong, B., Liu, R., Wu, Y., Chan, Z., 2016. Major latex protein-like protein 43 (MLP43) functions as a positive regulator during abscisic acid responses and confers drought tolerance in *Arabidopsis*

thaliana. J. Exp. Bot. 67, 421–434.

White, Jason C., Mattina, M.I., Lee, W.Y., Eitzer, B.D., Iannucci-Berger, W., 2003a. Role of organic acids in enhancing the desorption and uptake of weathered *p*,*p*′-DDE by *Cucurbita pepo*. Environ. Pollut. 124, 71–80.

White, Jason C, Wang, X., Gent, M.P.N., Iannucci-berger, W., Eitzer, B.D., Schultes, N.P., Arienzo, M., Mattina, M.I., 2003b. Subspecies-level variation in the phytoextraction of weathered *p*,*p*′-DDE by *Cucurbita pepo*. Environ. Sci. Technol. 37, 4368–4373.

Yamazaki, K., Tsuruta, H., Inui, H., 2015. Different uptake pathways between hydrophilic and hydrophobic compounds in lateral roots of *Cucurbita pepo*. J. Pestic. Sci. 40, 99–105.

Yang, C.L., Liang, S., Wang, H.Y., Han, L.B., Wang, F.X., Cheng, H.Q., Wu, X.M., Qu, Z.L., Wu, J.H., Xia, G.X., 2015. Cotton major latex protein 28 functions as a positive regulator of the ethylene responsive factor 6 in defense against *Verticillium dahliae*. Mol. Plant 8, 399–411.

Yoshihara, R., Matsuo, S., Iwata, T., Inui, H., 2014. Effect of amending soil with organic acids on perylene uptake into *Cucurbita pepo*. J. Pestic. Sci. 39, 162–164.

Figure legends

Fig. 1. Polychlorinated biphenyl (PCB)-binding proteins in xylem sap of Cucurbitaceae and non-cucurbits (A) and dieldrin-binding proteins in xylem sap (B). The 12.5 mM of 4-hydroxy-2′,3,3′,4′,5′-pentachlorobiphenyl (A) and 100 mM dieldrin (B) were bound to the magnetic beads. Non-binding beads (0 mM) were used as a control. Proteins were detected by silver staining. Xs, xylem sap; Re, root extract; –, xylem sap or root extracts were loaded without binding experiments. Arrows show PCB- and dieldrin-binding proteins.

Fig. 2. Phylogenetic tree of the Cucurbitaceae family and other plant species based on the amino acid sequences deduced from *MLP* cDNAs. DNA sequences of *Arabidopsis MLP28*, *Arabidopsis MLP43*, cotton *GhMLP28*, peach *Pp-MLP1*, cucumber *Csf2*, ginseng *MLP151*, bell pepper *Sn-1*, tobacco *FB7-4*, *Arabidopsis MLP423*, and opium poppy *gMLP15* were from NM_001124107, NM_105756, DQ123838, AF239177, AB008847, EU939308, X79230, S44872, NM_001198146, and X54306, respectively. The genes for CpMLP-PG1, CpMLP-GR1, and CpMLP-GR3 of *Cucurbita pepo* were from AB753855, AB753856, and AB753857, respectively. The phylogenetic tree was prepared in CLUSTALW.

Fig. 3. Binding activities of recombinant major latex-like proteins (MLPs) to polychlorinated biphenyl (PCB) (A) and dieldrin (B). The 25 mM of 4-hydroxy-2′,3,3′,4′,5′-pentachlorobiphenyl (A) and 100 mM dieldrin (B) were bound to the magnetic beads. Non-binding beads (0 mM) were used as a control. –, recombinant MLPs were loaded without binding experiments. Proteins were detected by silver

staining. Arrows show recombinant MLPs that bind PCB or dieldrin.

Fig. 4. Major latex-like proteins (MLPs) in xylem sap and roots of pumpkin detected by the anti-CpMLP-PG1 (A) and anti-CpMLP-GR3 (B) antibodies. The 12.5 mM of 4-hydroxy-2′,3,3′,4′,5′-pentachlorobiphenyl were bound to the magnetic beads. Non-binding beads (0 mM) were used as a control. Xs, xylem sap; Re, root extract; –, xylem sap or root extracts were loaded without binding experiments. Solid arrows show polychlorinated biphenyl (PCB)-binding MLPs in xylem sap. A dotted arrow shows MLPs in root extracts that are not detected in xylem sap.

Fig. S1. Alignment of deduced amino acid sequences of the major latex-like proteins (MLPs) from the members of the Cucurbitaceae family. Alignment was carried out in CLUSTALW. Asterisks show identical amino acids among MLPs.

Fig. S2. Cross-reactivity of anti-CpMLP antibodies raised to CpMLP-PG1/GR1 and CpMLP-GR3 derived from *Cucurbita pepo.*
(A) Homology between epitopes in major latex-like proteins (MLPs) obtained in this study and *C. pepo* MLPs. (Inui et al., 2013)
(B) Detection of recombinant MLPs by anti-CpMLP-PG1/GR1 (left) and anti-CpMLP-GR3 antibodies (right). Solid arrows show detectable MLPs.

Fig. S3. Detection of polychlorinated biphenyl (PCB)-binding major latex-like proteins (MLPs) in xylem sap and roots of Cucurbitaceae by anti-CpMLP-PG1 (A) and anti-CpMLP-GR3 antibodies (B).

Proteins were detected by anti-CpMLP antibodies raised to CpMLP-PG1 and CpMLP-GR3 from *Cucurbita pepo*. Xs, xylem sap; Re, root extract; –, xylem sap or root extracts were loaded without binding experiments. Solid arrows show MLPs in root extracts that are not detected in xylem sap.

Table S1. The sequences of primers used in this study

Primer name	Sequence	Amplified gene/Accession number
5'-UTR	5'-CGGTACCCGGGGATCCATCACATCAAAAGA-3'	-
3'-UTR	5'-CGACTCTAGAGGATCCAAACTTAAGATCAC-3'	
H1-sIF	5'-AGGAGATATACCATGGTCCAAACTGATAGCATTTG-3'	*CmMLP1*
H1-asIF	5'-TCAGTGGTGGTGGTGGTGGTGGTTCCTGGAGAAATAAGCAT-3'	LC177368
H2-sIF	5'-AGGAGATATACCATGGGCAAAAGTGATAGCATTTG-3'	*CmMLP2*
H2-asIF	5'-TCAGTGGTGGTGGTGGTGGTGGTTGTTGGAGAAATAAGCATC-3'	LC177369
H2-sIF	5'-AGGAGATATACCATGGGCAAAAGTGATAGCATTTG-3'	*CmMLP3*
H1-asIF	5'-TCAGTGGTGGTGGTGGTGGTGGTTCCTGGAGAAATAAGCAT-3'	LC177370
K1-sIF	5'-AGGAGATATACCATGAGCAAAAGTGATAGCATTTG-3'	*CfMLP1*
K1-asIF	5'-TCAGTGGTGGTGGTGGTGGTGGTTCTTGGAGAAATAAGCAT-3'	LC177371
K1-sIF	5'-AGGAGATATACCATGAGCAAAAGTGATAGCATTTG-3'	*CfMLP2*
H2-asIF	5'-TCAGTGGTGGTGGTGGTGGTGGTTGTTGGAGAAATAAGCATC-3'	LC177372
F2-sIF	5'-AGGAGATATACCATGAGCCAAACTGAAAGCATTTG-3'	*LcMLP2*
F2-asIF	5'-TCAGTGGTGGTGGTGGTGGTGGTTCTTGGAAAAATAAGCATC-3'	LC177373
H1-sIF	5'-AGGAGATATACCATGGTCCAAACTGATAGCATTTG-3'	*LsMLP1*
H1-asIF	5'-TCAGTGGTGGTGGTGGTGGTGGTTCCTGGAGAAATAAGCAT-3'	LC177374

Table S2. Homology of deduced amino acid sequences of *MLP* cDNAs cloned in this study

	CmMLP1	CmMLP2	CmMLP3	CfMLP1	CfMLP2	LcMLP1	LcMLP2	CsMLP1	ClMLP1	LsMLP1
CmMLP1		78.2	79.5	79.5	79.5	100	55.1	100	100	99.4
CmMLP2			98.1	88.6	89.2	78.2	52.9	78.2	78.2	77.6
CmMLP3				88.6	88.6	79.5	54.1	79.5	79.5	78.8
CfMLP1					99.4	79.5	53.5	79.5	79.5	78.8
CfMLP2						79.5	52.9	79.5	79.5	78.8
LcMLP1							55.1	100	100	99.4
LcMLP2								55.1	55.1	55.1
CsMLP1									100	99.4
ClMLP1										99.4
LsMLP1										

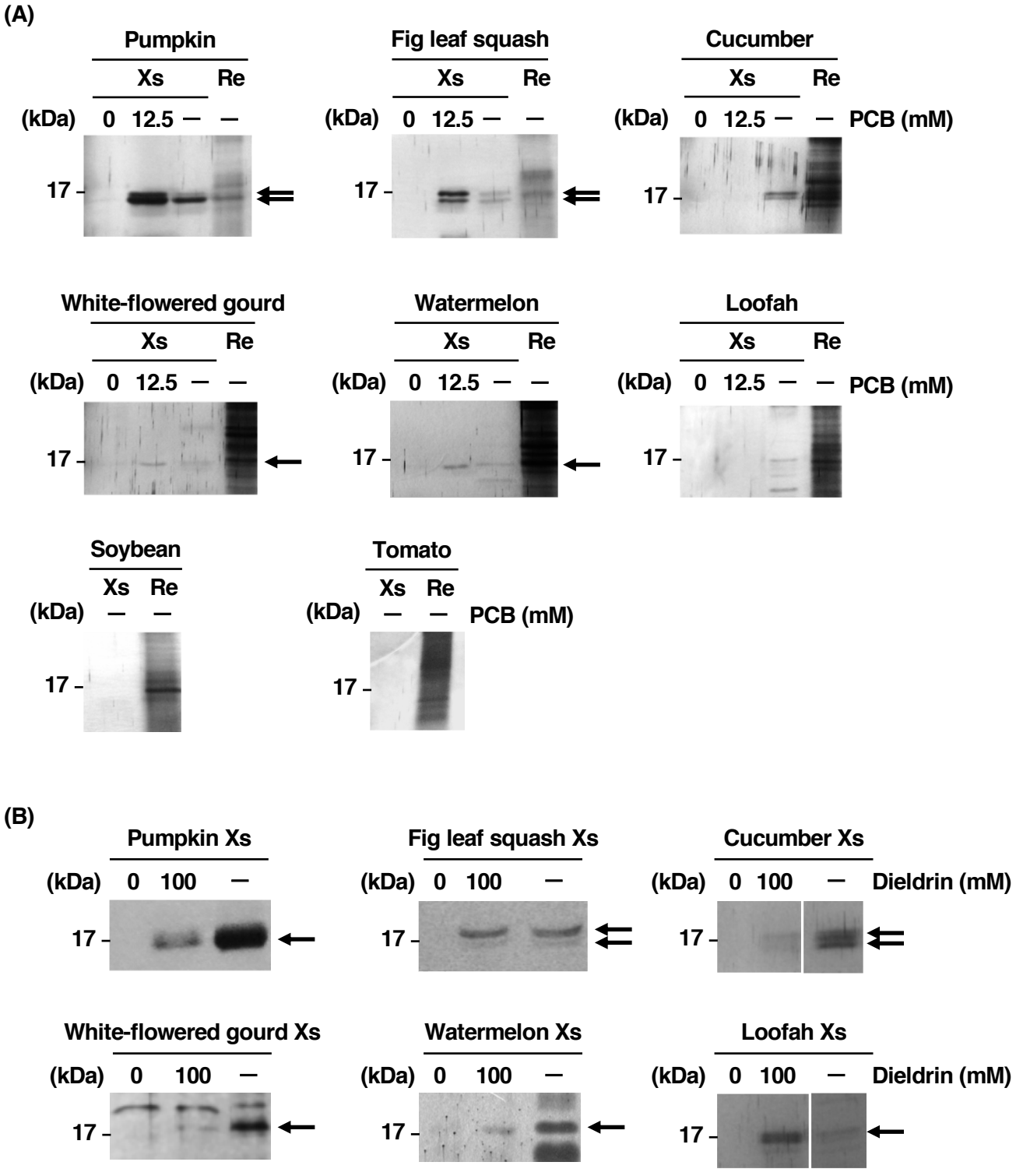
(A)
Pumpkin
Xs
Re
(kDa)
0
12.5
—
—
17
Fig leaf squash
Cucumber
PCB (mM)
White-flowered gourd
Watermelon
Loofah
Soybean
Tomato
(B)
Pumpkin Xs
100
Dieldrin (mM)
Fig leaf squash Xs
Cucumber Xs
White-flowered gourd Xs
Watermelon Xs
Loofah Xs

Fig. 1

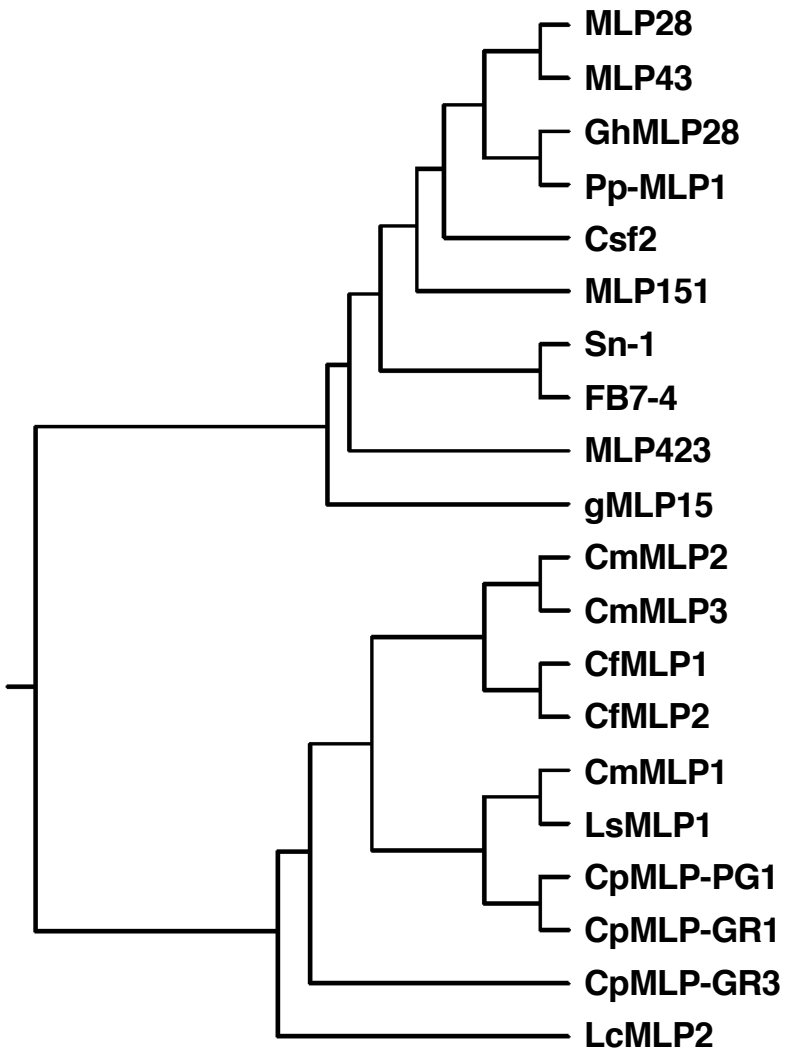
MLP28
MLP43
GhMLP28
Pp-MLP1
Csf2
MLP151
Sn-1
FB7-4
MLP423
gMLP15
CmMLP2
CmMLP3
CfMLP1
CfMLP2
CmMLP1
LsMLP1
CpMLP-PG1
CpMLP-GR1
CpMLP-GR3
LcMLP2

Fig. 2

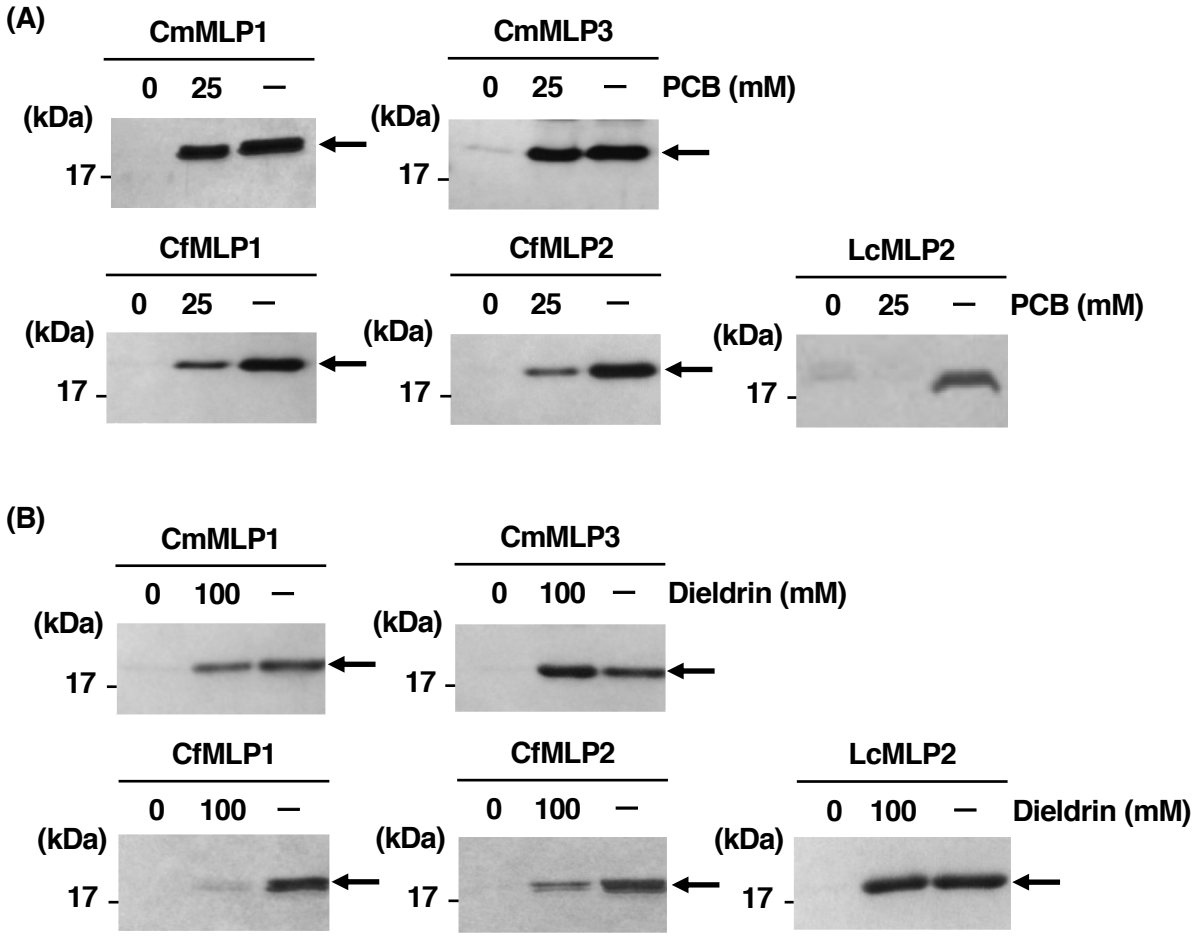
(A)
CmMLP1
0 25 —
(kDa)
17
CmMLP3
0 25 — PCB (mM)
(kDa)
17
CfMLP1
0 25 —
(kDa)
17
CfMLP2
0 25 —
(kDa)
17
LcMLP2
0 25 — PCB (mM)
(kDa)
17
(B)
CmMLP1
0 100 —
(kDa)
17
CmMLP3
0 100 — Dieldrin (mM)
(kDa)
17
CfMLP1
0 100 —
(kDa)
17
CfMLP2
0 100 —
(kDa)
17
LcMLP2
0 100 — Dieldrin (mM)
(kDa)
17

Fig. 3

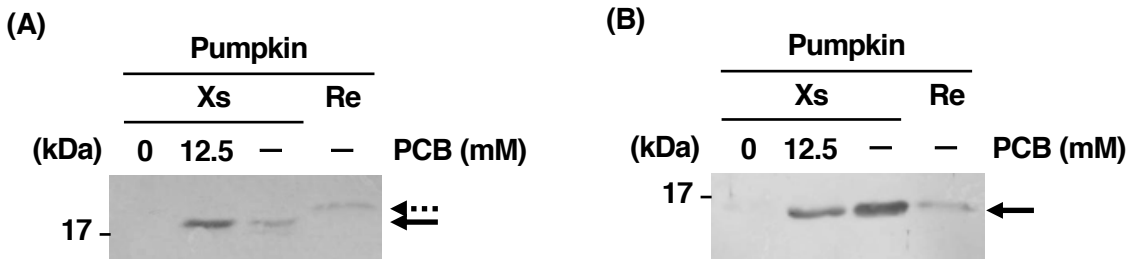
(A)
Pumpkin
Xs
Re
(kDa)
0
12.5
—
—
PCB (mM)
17
(B)
Pumpkin
Xs
Re
(kDa)
0
12.5
—
—
PCB (mM)
17

Fig. 4