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Pesticide treatment reduces hydrophobic pollutant contamination in *Cucurbita pepo* through competitive binding to major latex-like proteins

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Abstract

Hydrophobic pollutants are still present in agricultural soil. The Cucurbitaceae family accumulates hydrophobic pollutants through roots, resulting in the contamination of aerial parts. Major latex-like proteins (MLPs), found in the Cucurbitaceae family, play an important role in the contamination by binding to these hydrophobic pollutants. Thus far, efficient cultivation methods for the production of safe crops with lower concentrations of hydrophobic pollutants have not been developed. Herein, we competitively inhibited the binding of MLPs to hydrophobic pollutants, pyrene and dieldrin, in roots by using MLP binding pesticides. By conducting a chemical array screening, we found that MLPs bound compounds with indole- and quinazoline-like structures. Commercially available pesticides

amisulbrom and pyriproxyfen, which possess such structures, successfully inhibited the binding of MLPs to pyrene and dieldrin *in vitro*. When zucchini plants were cultivated in the contaminated soil with 1.25 mmol/kg pyrene and 12.5 μ mol/kg dieldrin, the concentration of pyrene and dieldrin in xylem sap was significantly decreased by 30% and 15%, respectively. Our results demonstrate that the pesticides binding to MLPs competitively inhibited the binding of MLPs to pyrene and dieldrin in roots, resulting in the reduction of overall contamination. This study proposes a novel approach to cultivate safer crops and advances the utilization of unknown functions of pesticides.

(184 words)

Key words

competitive binding inhibition; *Cucurbita pepo*; hydrophobic pollutant; major latex-like protein; pesticides

Capsule

Pesticides binding major latex-like proteins competitively inhibited the binding of MLPs to hydrophobic pollutants and decreased the concentrations of them in xylem sap of zucchini plants.

49

50 **1. Introduction**

51 The contamination of crops by hydrophobic pollutants such as persistent organic pollutants (POPs)
52 and polycyclic aromatic hydrocarbons (PAHs) is currently a severe problem in terms of food safety
53 and quality. For example, the insecticides dieldrin and heptachlor, categorized as POPs, were detected
54 in cucumber and pumpkin, respectively, over the residue limits, although they have been prohibited
55 from use more than 40 years ago (Hashimoto, 2005). Hydrophobic pollutants registered as POPs were
56 discontinued in 181 countries in 2020 because of their persistence, long-distance mobility,
57 bioaccumulation, and toxicity. However, they were detected in crops, such as the Cucurbitaceae family,
58 because of the contamination of agricultural soil by the use of insecticides determined as POPs and
59 the incomplete combustion of straw after field burning (Shen et al., 2013). Hydrophobic pollutants are
60 reported to show carcinogenicity (Bortey-Sam et al., 2017) and neurotoxicity (Shi et al., 2019).
61 Therefore, the intake of such contaminated crops can lead to these diseases in humans.

62 It is well known that the Cucurbitaceae family such as cucumber, melon, squash, watermelon, pumpkin,
63 and zucchini, can accumulate POPs in their stems and leaves (Otani et al., 2007). In addition, when
64 dieldrin is detected in cucumber over the Japanese residue limit (0.02 ppm), farmers have to discard
65 all crops cultivated in the same farmland, leading to enormous economic loss (Hashimoto, 2005). Thus,
66 many approaches, such as degradation of POPs by microorganisms (Xiao et al., 2011) and adsorption
67 of POPs by active carbon (Murano et al., 2009), have been attempted for the suppression of crop
68 contamination. However, these methods show low efficiency and take a long time and high cost.
69 Therefore, it is crucially important to develop novel technologies to produce safe crops efficiently.

70 The accumulation of hydrophobic pollutants in the Cucurbitaceae family is mainly due to the transport
71 of them to aerial plant tissue. In certain Cucurbitaceae species, POPs such as chlordane were
72 transported from roots to aerial tissues via the xylem sap (Mattina et al., 2004); in another study on

the uptake of the POP dieldrin by cucurbits, it was found that dieldrin-binding proteins were responsible for the solubilization of dieldrin in the xylem sap and possibly the subsequent transport of dieldrin to aerial tissues (Murano et al., 2010). Major-latex like proteins (MLPs) were identified as one of the POP transporting factors in zucchini plants (Inui et al., 2013).

MLPs are a member of the birch pollen allergen (Bet v 1) family (Radauer and Breiteneder, 2007), which includes pathogenesis-related proteins of class 10 (PR-10s) (Fernandes et al., 2013), cytokinin specific-binding proteins (Pasternak et al., 2006), and fruit allergens such as kiwi allergen Act d 11 (D'Avino et al., 2011), and strawberry allergen (Ishibashi et al., 2017). Proteins in this family have a similar 3D structure; they all contain three α helices and seven β sheets, but have a variable amino acid sequence (Choi et al., 2015). Remarkably, these structural elements are assembled in such a way that a large internal hydrophobic cavity, which enables the proteins to bind hydrophobic compounds such as plant hormones and secondary metabolites, is created (Fernandes et al., 2013). For example, Bet v 11 (a naturally occurring isoform of Bev v 1) can bind brassinosteroids (Markovic'-Housley et al., 2003), and Fra a proteins in strawberries can bind flavonoids such as quercetin, myricetin, and catechin (Casañal et al., 2013). MLP-PG1 and MLP-GR3 proteins, identified in zucchini plants, exhibit binding activity towards PCBs, dieldrin, and other hydrophobic pollutants (Goto et al., 2019). MLPs from the Cucurbitaceae family are key factors in the hydrophobic pollutant contamination of their aerial tissue: they bind hydrophobic pollutants in roots, which are then transported to fruits via xylem vessels (Goto et al., 2019).

In this study, reduction of hydrophobic pollutant contamination was attempted in a Cucurbita species via the use of targeted pesticides: by treatment with MLP binding pesticides, Raimei and Colt, competitive inhibition could decrease the binding of MLPs to hydrophobic pollutants, pyrene and dieldrin, resulting in their decreases in the xylem sap, and consequently a reduction in the transportation of them to the aerial parts of plants. This approach proposes a practical reduction of

contamination reduction, as treatments consist of commercially available pesticides. To the best of our knowledge, this is the first study to focus on the reduction of such contamination through competitive inhibition of MLP and contaminant binding.

2. Materials & Methods

2.1 Chemical array screening

Chemical array screening was performed primarily to identify compounds with a positive response to MLPs. The arrays were prepared using previously described method (Kondoh et al., 2015). We used 22,097 compounds from the RIKEN NPDepo for the screening. Recombinant MLP-PG1 and MLP-GR3 proteins fused with His-tags at the C-terminus were produced using *Escherichia coli*, as described previously (Inui et al., 2013). To prevent non-specific binding, the chemical arrays were incubated in a blocking solution of TBS-T (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% [v/v] Tween 20) and 1% skimmed milk for 1 h at room temperature. After washing with TBS-T and potassium phosphate buffer (20 mM potassium phosphate buffer [pH 5.6], 100 mM KCl, 1 mM MgCl₂, 0.2 mM CaCl₂, and 0.2 mM EDTA), 4 µM of recombinant MLP-PG1 or MLP-GR3 in potassium phosphate buffer was applied onto the chemical arrays and incubated at 30°C for 1 h. After another washing with potassium phosphate buffer and TBS-T, the chemical arrays were incubated with anti-His antibody (GE Healthcare, Chicago, IL), followed by a second antibody (goat anti-mouse IgG) with Cy5 fluorescence dye (Thermo Fisher Scientific, Waltham, MA). Finally, the chemical arrays were scanned with a microarray scanner GenePix 4300A (Molecular Devices, Sunnyvale, CA), using the Cy5 channel, with the fluorescence excitation wavelength set at 635 nm and emission wavelength set at 655–695 nm. A structure search of MLP-binding compounds was performed in SciFinder (<https://www.cas.org/products/scifinder>).

2.2 Plant materials, growing conditions, and collection of xylem sap

The seeds of *Cucurbita pepo* subspecies *pepo*, cultivars 'Magda (MG)' and 'Raven (RA)', were purchased from Johnny's Selected Seeds (Albion, ME). Soil was obtained from Hyponex Japan Corp., Ltd. (Osaka, Japan), autoclaved at 120°C for 15 min and completely dried. Two types of soil were prepared: non-contaminated soil (1 kg of the dry soil mixed with 500 mL of acetone), and contaminated soil (1 kg of the dry soil mixed with 500 mL of acetone with 2.5 mM pyrene or 25 µM dieldrin). Acetone was then evaporated from the soil. The spiked concentration was six times higher than that in the agricultural field (Hashimoto, 2005). After peeling the seed coat off to promote germination, seeds of the two cultivars of *C. pepo* subsp. *pepo* were incubated in tap water at 4°C for 1 d and then sown in a glass jar supplemented with 200 g of non-contaminated or contaminated soil. Zucchini plants cultivated in non-contaminated soil and contaminated soil without the treatment of pesticides were regarded as the negative control and positive control, respectively.

After 27-day of cultivation at 26°C under a 16/8 h light/dark cycle, xylem sap was collected following procedures described previously (Inui et al., 2013). Briefly, the stem was cut at 1 cm below the cotyledon, and the weight of stem and leaf tissues was measured. A glass tube washed with acetone or hexane (for the quantification of pyrene or dieldrin, respectively) was put on the stem until the amount of xylem sap reached approximately 500 µL. Collected xylem sap was stored at 4°C until time of use.

The fungicide Raimei (Nissan Chemical Co., Ltd., Tokyo, Japan) and the insecticide Colt (Kumiai Chemical Industry Co., Ltd., Tokyo, Japan), chosen based on the results of the chemical assay, were applied to the plants by spray according to the manufacturer's instruction at the recommended timing at two doses (the standard dose, or thrice the standard dose). The number of collected xylem sap ranged from 5 to 12 and from 8 to 19 under the cultivation of contaminated soil with pyrene and dieldrin, respectively.

2.3 Quantification of pyrene and dieldrin

The obtained xylem sap (50 μ L) was mixed with 50 μ L of dimethyl sulfoxide (DMSO) in a 96-well black microtiter plate. Fluorescence of pyrene in the samples was measured using Microplate Reader SH-9000 (Corona Electric Co., Ltd., Hitachinaka, Ibaraki, Japan) with the excitation wavelength set at 330 nm and the emission wavelength set at 390 nm. Zucchini plants cultivated in non-contaminated soil and contaminated soil without the treatment of pesticides were regarded as the negative control and positive control.

The xylem sap (250 μ L) was mixed with 20 μ L of 200 ng/mL 13 C-labeled dieldrin as an internal standard ([Cambridge Isotope Laboratories, Inc., Tewksbury, MA], offered by Tohoku Ryokka Kankyohozen Co., Ltd. [Sendai, Japan]). Dieldrin was extracted using two washes of 1 mL hexane. After dehydration by anhydrous sodium sulfate, 50 μ L of nonane were added, and samples were concentrated by nitrogen gases at 35°C until the total amount reached approximately 100 μ L. We added 10 μ L of 50 ng/mL MBP-15, 70, 101, 153 (Wellington Laboratories Inc., Guelph, Canada) as a syringe spike, and dieldrin concentration was quantified by high-resolution gas chromatography and high-resolution mass spectrometry (HRGC/HRMS: GC, 6890N [Agilent Technologies, Tokyo, Japan]; MS, JMS-800D [JEOL Ltd., Tokyo, Japan]) equipped with a DB-5MS column (Agilent Technologies) using DioK Ver. 4.02 (JEOL, Ltd., Tokyo, Japan). The values of the method detection limits and the method quantification limits were 0.19 nM and 0.63 nM, respectively.

2.4 Competitive binding assay

Recombinant MLPs were diluted with a sodium phosphate buffer (50 mM sodium phosphate buffer [pH 7.0], 200 mM NaCl, and 150 mM imidazole) at a final concentration of 0.2 mg/mL. Two different quantities (5 mM or 10 mM) of amisulbrom (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and pyrifluquinazon (Wako Pure Chemical Industries), dissolved in DMSO, were added into MLP

solutions at a final concentration of 25 μ M or 50 μ M, respectively. Finally, pyrene (1 mM), dissolved in DMSO, was added at a final concentration of 10 μ M to the solutions. These solutions were incubated with rotation at 20°C for 1 h. Fluorescence of pyrene was measured in 100 μ L of each solution using Microplate Reader SH-9000, as described above.

Dieldrin (200 mM), dissolved in dimethylformamide (DMF), was bound to 1 mg of epoxy beads (Tamagawa Seiki, Co., Ltd, Nagano, Japan) according to the manufacturer's instruction. After dispersion and spindown of dieldrin-binding beads, magnetic separation was performed to remove the supernatant, and the binding buffer (50 mM potassium phosphate buffer [pH 5.6], 100 mM KCl, 1 mM MgCl₂, 0.2 mM CaCl₂, 0.2 mM EDTA, 10% [v/v] glycerol, 0.1% [v/v] Nonidet P-40, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, and 0.1% *N*-lauroylsarcosine sodium salt) was added. Washing was performed three times. Recombinant MLP-PG1 or MLP-GR3 was diluted with the binding buffer, until the final concentration of each MLP was 0.1 mg/mL. The solutions were centrifuged at 4°C for 30 min at 20,700 \times g, and the supernatants were collected. Then, 20 mM amisulbrom and pyrifluquinazon, dissolved in DMSO, were added into the solutions at a final concentration of 0.2 mM. The solutions were incubated with rotation at 4°C for 2 h. After incubation, 200 μ L of the solutions were added to 0.25 mg of beads and incubated at 4°C for 4 h. Magnetic separation was performed, the supernatant removed, and the binding buffer added. Washing with binding buffer was repeated eight times. The binding buffer (35 μ L) and 7 μ L of Sample Buffer Solution with Reducing Reagent for SDS-PAGE (Nacalai Tesque, Inc., Kyoto, Japan) were added to the beads, and the beads with the solution were heated at 98°C for 5 min. After magnetic separation, the samples were subjected to SDS-PAGE on a 15% acrylamide gel, and gels were stained using a Silver Staining kit (Wako Pure Chemical Industries). Band intensities were measured using the ImageJ software (Schneider et al., 2012).

2.5 Western blot analysis

Roots from RA were ground, and root proteins were extracted as reported previously (Goto et al., 2019). Extracted root proteins were quantified by the Bradford method (Bradford, 1976)(Bradford, 1976). Sample Buffer Solution with Reducing Reagent for SDS-PAGE (Nacalai Tesque) was added to the samples, and they were subjected to SDS-PAGE on 15% acrylamide gel. Western blot analysis was performed with anti-MLP-PG1 and anti-MLP-GR3 antibodies, as reported previously (Goto et al., 2019). Band intensities were quantified using the ImageJ software (Schneider et al., 2012).

2.6 Statistical analysis

Each experiment for quantification of pyrene and dieldrin was performed independently with at least three biological replicates. One-way analysis of variance was performed, and Dunnett's multiple comparison test was applied to judge significant differences among treatments using R software ver. 3.6.2 (<https://www.R-project.org/>).

3. Results

3.1 Selection of compounds binding MLPs and selection of pesticides

Chemical array screening was performed for the identification of positive signals after incubation with MLPs. Of the 22,097 compounds from the RIKEN NPDepo, the compounds 166 and 176 had positive signals with different fluorescence intensities for recombinant MLP-PG1 and MLP-GR3, respectively (Table 1). The number of total compounds with positive signals was 242, and 100 compounds had positive signals for both MLPs. Thirty plant-related compounds, such as plant hormones and secondary metabolites, were chosen from the 242 compounds by SciFinder, and two compounds with indole-like structures were proposed

(Table 2). Amisulbrom was finally selected as a pesticide containing an indole-like structure (Figure 1A).

Compounds whose positive signal showed ++ or +++ to both MLPs were selected. Amongst them, ten compounds that had quinazoline-, quinoxaline-, and quinoline-like structures were identified (Table 3). Pyrifluquinazon was finally selected as a pesticide with a quinazoline-like structure (Figure 1B).

3.2 Competitive binding activity of pesticides

When pyrene was incubated with MLPs, the fluorescence of pyrene dramatically increased (Figure 2). With MLP-PG1, the addition of 50 μ M of amisulbrom led to a decrease of 24% in the fluorescence of pyrene, as compared to without pesticides (Figure 2A). With the addition of 25 μ M and 50 μ M of pyrifluquinazon, the fluorescence of pyrene was decreased by 26% and 61%, respectively (Figure 2A). With MLP-GR3, the addition of 25 μ M and 50 μ M of amisulbrom led to a decrease of 50% and 40% in the fluorescence of pyrene, as compared to without pesticides (Figure 2B). Similarly, with the addition of 25 μ M and 50 μ M of pyrifluquinazon, the fluorescence of pyrene was decreased by 32% and 28%, respectively (Figure 2B). Without pesticides, the fluorescence when incubated with MLP-GR3 roughly doubled as compared to that with MLP-PG1 (Figure 2). When pyrene was incubated with pesticides without MLPs, the fluorescence of pyrene was not increased (Figure S1A). When MLPs were incubated with pesticides without pyrene, the fluorescence was also not increased (Figure S1B).

In MLP-PG1 and MLP-GR3, a band was detected when dieldrin-binding beads were added (Figure 3). With the addition of amisulbrom, the band intensity was not clearly decreased in MLP-PG1, but was decreased by 49% in MLP-GR3 (Figure 3A). In contrast, with the addition of pyrifluquinazon, the band intensity was decreased by 19% in MLP-PG1 and by 78% in MLP-GR3, respectively (Figure

3B). No bands were detected in the beads without dieldrin (Figure 3). There were no bands in the supernatant from the last washing step (Figure S2).

3.3 Reduced contamination in zucchini plants cultivated in the contaminated soil

The fungicide Raimei containing amisulbrom and the insecticide Colt containing pyrifluquinazon were selected as pesticides to accomplish the reduction of pyrene and dieldrin contamination in zucchini plants. In the Raimei treatment, pyrene concentration was significantly decreased by 40% with the triple dose, but not with the usual dose (Figure 4A). In the Colt treatment, the pyrene concentration was not significantly decreased with the triple doses but was significantly decreased by 30% with the usual dose (Figure 4B). The fresh weights of aerial parts in each treatment were not significantly changed (Figure S3).

In the Raimei treatment during the cultivation of zucchini plants in dieldrin-contaminated soil, dieldrin concentration was not significantly decreased, even upon the use of the triple dose (Figure 5). In the Colt treatment, dieldrin concentration was significantly decreased by 15% upon administration of the triple dose; the usual dose tended to also decrease the concentration, but not significantly so (Figure 5). The fresh weights of aerial parts in each treatment were not significantly changed (Figure S4). Furthermore, with the treatment of the triple dose of Colt, the amount of MLP-PG1 and MLP-GR3 in roots was not significantly decreased (Figure S5).

4. Discussion

Chemical array screening is a suitable method to identify compounds with a high ability to bind MLPs as shown by Maeda *et al.* (2017) that the inhibitor for the synthase responsible for production of a fungal toxin was successfully obtained (Maeda *et al.*, 2017). The MLPs used in this study bound onto 242 compounds in the chemical arrays (Table 1). Almost all of the compounds had aromatic rings,

which suggested that MLPs were likely to bind to relatively hydrophobic compounds. It had been reported that Act d 11, which has a similar 3D structure to MLPs, had a significant ability to bind compounds with indole-like structures (Chruszcz et al., 2013). Thus, it was thought that MLPs could bind indole compounds. Amongst 30 compounds, there were two compounds with indole-like structures (Table 2), and amisulbrom (contained in the fungicide Raimei) was selected for the competitive inhibition of MLP binding to hydrophobic pollutants (Figure 1). Amongst compounds with a high affinity to bind MLPs, there were 10 compounds with quinazoline-like, quinoxaline-like, and quinoline-like structures (Table 3), and the compound pyrifluquinazon (with a quinazoline-like structure), contained in the insecticide Colt, was selected (Figure 1). Notably, this is the first report to find MLPs that could bind compounds with quinazoline-like, quinoxaline-like, and quinoline-like structures. Raimei and Colt are registered for the application on the Cucurbitaceae family, so treatment with these pesticides is a practical method for reduction of hydrophobic pollutant contamination. Amisulbrom competitively inhibited the binding of MLPs to pyrene and dieldrin. Inhibition activity was higher in MLP-GR3 than in MLP-PG1 (Figure 2 and 3A). This result was supported by the result of the chemical array screening. MLP-GR3 had a higher ability to bind compounds with indole-like structures than MLP-PG1 (Table 2). Pyrifluquinazon also competitively inhibited the binding of MLPs to pyrene and dieldrin (Figure 3B). Pyrifluquinazon was better at inhibiting the binding of MLP-PG1 to pyrene than that of MLP-GR3 to pyrene (Figure 2). It is thought that MLPs have a binding preference toward certain compounds, due to their structural difference. Based on these results, amisulbrom and pyrifluquinazon can both be applied to reduce the contamination by hydrophobic pollutants in the Cucurbitaceae family.

The hydrophobic pollutant perylene was found to mainly localize in the plasma membrane of the endodermis and pericycle of root tissue (Yamazaki et al., 2015). Therefore, MLPs produced in root cells will bind hydrophobic pollutants on the plasma membrane of these tissues. Then, MLP-

hydrophobic pollutant complexes translocate to xylem vessel (Goto et al., 2019). The estimated log K_{ow} of amisulbrom and pyrifluquinazon is 4.4 and 3.12, respectively, which indicates their relative hydrophobic properties (<https://pubchem.ncbi.nlm.nih.gov/>). Based on these results, these pesticides must also localize in the plasma membrane of the endodermis and pericycle and competitively inhibit the binding of MLPs to hydrophobic pollutants.

Pyrene concentration in the xylem sap was significantly decreased by the treatment of Raimei and Colt, which suggests that amisulbrom and pyrifluquinazon competitively inhibited the binding of MLPs to pyrene in roots (Figure 4). Since POP concentration in xylem sap had a positive correlation with that in aerial parts, the amount of pyrene in aerial parts would also be decreased (Goto et al., 2019) (Figure 3). Dieldrin concentration was significantly decreased by the treatment of Colt, but not Raimei, although the mole of pyrifluquinazon applied to plants was 425 times lower than that of amisulbrom (Figure 5). One of the reasons for this is the difference in the competitive inhibition activity between amisulbrom and pyrifluquinazon. The inhibition activity of MLP binding to dieldrin by pyrifluquinazon was higher than that of MLPs to dieldrin by amisulbrom (Figure 3). Another reason for the high potency of Colt is the difference in the solubility of the two pesticides in water. The solubility of pyrifluquinazon is more than ten times higher than that of amisulbrom. Hence, pyrifluquinazon can easily spread to the rhizosphere and is taken up into roots. Furthermore, another possible cause is the different binding ability of MLPs toward these hydrophobic pollutants. Hydrophobicity of the compounds is a critical factor in explaining differences in binding affinity. However, the estimated log K_{ow} of pyrene and dieldrin is 5.18 (Miller et al., 1985) and 5.20 (Namiki et al., 2018), respectively, indicating that water solubility is almost the same. This suggests that these compounds in zucchini plants accumulate nearly at the same concentrations. In contrast, the difference in the bulkiness between hydrophobic pollutants such as PCBs made a big difference in their accumulation in zucchini plants. The amount of bulky PCBs containing chlorines at *ortho*-positions

such as 2,3',4,4',5-pentachlorobiphenyl (CB118) in zucchini plants was several times higher than that of planner PCBs not containing such chlorines such as 3,3',4,4',5-pentachlorobiphenyl (CB126) (Matsuo et al., 2011), although their estimated log K_{ow} is almost the same (Hawker and Connell, 1988). Pyrene has a planner structure, but dieldrin has a bulky structure. Thus, in the study, MLPs had a higher ability to bind dieldrin than pyrene. Therefore, with the Raimei treatment, pyrene concentration was significantly decreased, while dieldrin concentration was not significantly decreased. Hence, only a triple dose of Colt was effective in significantly decreasing dieldrin concentration in xylem sap.

5. Conclusion

In this study, the contamination of zucchini plants by the hydrophobic pollutants, pyrene and dieldrin, in the agricultural field was reduced by the treatment of pesticides with the ability to bind MLPs. Our findings show that selected pesticides competitively inhibited the binding of MLPs to pyrene and dieldrin, and the treatment of Colt decreased the concentration of pyrene and dieldrin by 30% and 15%, respectively, in xylem sap, potentially leading to a decrease of contamination in the fruits, although there are no reports on the positive correlation between the concentrations of pollutants in the xylem sap and fruits. However, this study did not show the contamination levels in the fruits, and analysis of the fruits should be undertaken. Selected pesticides showed highly selective toxicity, and so they are safe for human beings. This is the first report to reduce crop contamination based on molecular mechanisms in the uptake of pyrene and dieldrin. Previous studies attempted the remediation of agricultural soil by physical and chemical methods for the production of safe crops. This study proposes the novel method to reduce contamination based on the molecular mechanisms of the uptake of hydrophobic pollutants by the Cucurbitaceae family. Since zucchini, pumpkin, and cucumber have MLPs binding hydrophobic pollutants (Iwabuchi et al., 2020), this approach can apply to many Cucurbitaceae family plants. With the treatment of pesticides that bind MLPs, safe crops can

be produced, even in contaminated soil. Furthermore, this study advances the utilization of unknown functions of pesticides.

CRedit authorship contribution statement

Kentaro Fujita: Data curation, Formal analysis, Writing - original draft, Writing - review & editing.
Kondoh Yasumitsu: Data curation, Formal analysis. **Kaori Honda:** Data curation, Formal analysis.
Yuki Haga: Data curation, Formal analysis. **Hiroyuki Osada:** Data curation, Formal analysis.
Chisato Matsumura: Data curation, Formal analysis. **Hideyuki Inui:** Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure legends

Figure 1. Structures of amisulbrom (the fungicide Raimei) (A) and pyrifluquinazon (the insecticide Colt) (B).

Figure 2. Competitive binding of major latex-like proteins (MLPs) to pyrene by amisulbrom and pyrifluquinazon.

The 0.2 mg/mL recombinant MLP-PG1 (A) and MLP-GR3 (B) were incubated with 10 μ M pyrene and 25 μ M or 50 μ M amisulbrom and pyrifluquinazon for 1 h at 20°C. The fluorescence of pyrene was measured. –, the incubation without MLPs or pesticides; +, the incubation with 0.2 mg/mL MLPs or 25 μ M pesticides; ++, the incubation with 50 μ M pesticides. The white, gray, and black bars represent control (no pesticides), + (25 μ M pesticides), and ++ (50 μ M pesticides), respectively. Error bars indicate standard deviations ($n = 3-8$). Asterisks indicate significant differences compared to the fluorescence incubated with MLPs without pesticides (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Figure 3. Competitive binding of major latex-like proteins (MLPs) to dieldrin by amisulbrom (A) and pyrifluquinazon (B).

The 0.1 mg/mL recombinant MLPs were incubated with 0.2 mM amisulbrom (A) and pyrifluquinazon (B) for 2 h at 4°C. The magnetic beads binding 100 mM dieldrin were added and incubated for 4 h at 4°C. After the heat elution, samples were subjected to SDS-PAGE, and MLPs were detected by silver staining. C; beads without dieldrin, D; dieldrin-binding beads; –, the incubation with magnetic beads without binding of dieldrin or without pesticides; +, the incubation with magnetic beads binding dieldrin or with 0.2 mM pesticides. Band intensities were quantified by ImageJ.

Figure 4. Pyrene concentration in xylem sap from zucchini plants under the treatment of Raimei (A) and Colt (B).

The *Cucurbita pepo* cultivar ‘Magda’ was cultivated in the contaminated soil with pyrene (1.25 mmol/kg) for 27 days at 26°C under a 16/8 h light/dark cycle. –, the cultivation in the non-contaminated soil with pyrene or the non-treatment of pesticides; +, the cultivation in the contaminated soil with pyrene or the treatment of pesticides at usual dose; +++, the treatment of pesticides at the triple dose. The white, gray, and black bars represent control (no pesticides), + (the treatment of pesticides at usual dose), and +++ (the treatment of pesticides at the triple dose), respectively. Error bars indicate standard deviations (A, $n = 5-12$, B, $n = 6-7$). Asterisks indicate significant differences compared to pyrene concentration cultivated in the contaminated soil with pyrene without the treatment of pesticides (*, $p < 0.05$; ***, $p < 0.001$).

Figure 5. Dieldrin concentration in xylem sap from zucchini plants under the treatment of Raimei and Colt.

The *Cucurbita pepo* cultivar ‘Raven’ was cultivated in the contaminated soil with dieldrin (12.5 $\mu\text{mol/kg}$) for 27 days at 26°C under a 16/8 h light/dark cycle. –, the cultivation in the non-contaminated soil with dieldrin or the non-treatment of pesticides; +, the cultivation in the contaminated soil with dieldrin or the treatment of pesticides at usual dose; +++, the treatment of pesticides at the triple dose. The white, gray, and black bars represent control (no pesticides), + (the treatment of pesticides at usual dose), and +++ (the treatment of pesticides at the triple dose), respectively. Error bars indicate standard deviations ($n = 8-19$). Asterisks indicate significant differences compared to dieldrin concentration cultivated in the contaminated soil with dieldrin without the treatment of pesticides (*, $p < 0.05$).

Supplementary Figure 1. Competitive binding of major latex-like proteins (MLPs) to pyrene by amisulbrom and pyrifluquinazon.

The 10 μ M pyrene and 5 μ M or 50 μ M amisulbrom and pyrifluquinazon were incubated without recombinant MLPs for 1 h at 20°C (A). The 0.2 mg/mL recombinant MLPs were incubated with 5 μ M or 50 μ M amisulbrom and pyrifluquinazon without pyrene (B). The fluorescence of pyrene was measured. –, the incubation without pesticides; +, the incubation with 5 μ M pesticides; ++, the incubation with 50 μ M pesticides. The white, gray, and black bars represent control (no pesticides), + (25 μ M pesticides), and ++ (50 μ M pesticides), respectively. Error bars indicate standard deviations ($n = 3-4$).

Supplementary Figure 2. Competitive binding of major latex-like proteins (MLPs) to dieldrin by amisulbrom (A) and pyrifluquinazon (B).

The 0.1 mg/mL recombinant MLPs were incubated with 0.2 mM amisulbrom (A) and pyrifluquinazon (B) for 2 h at 4°C. The magnetic beads binding 100 mM dieldrin were added and incubated for 4 h at 4°C. Eluates at the eighth washing step were subjected to SDS-PAGE, and MLPs were detected by silver staining. C; beads without dieldrin, D; dieldrin-binding beads; –, the incubation with magnetic beads without binding of dieldrin or without pesticides; +, the incubation with magnetic beads binding dieldrin or with 0.2 mM pesticides.

Supplementary Figure 3. Fresh weight of aerial parts of zucchini plants cultivated in the contaminated soil with pyrene under the treatment of Raimei (A) and Colt (B).

The *Cucurbita pepo* cultivar ‘Magda’ was cultivated in the contaminated soil with pyrene (1.25 mmol/kg) for 27 days at 26°C under a 16/8 h light/dark cycle. Aerial parts were collected from each plant, and the fresh weight of them was measured. –, the cultivation in the non-contaminated soil with

pyrene or the non-treatment of pesticides; +, the cultivation in the contaminated soil with pyrene or the treatment of pesticides at usual dose; +++, the treatment of pesticides at the triple dose. The white, gray, and black bars represent control (no pesticides), + (the treatment of pesticides at usual dose), and +++ (the treatment of pesticides at the triple dose), respectively. Error bars indicate standard deviations (A, $n = 5-12$, B, $n = 6-7$).

Supplementary Figure 4. Fresh weight of aerial parts of zucchini plants cultivated in the contaminated soil with dieldrin under the treatment of Raimei and Colt.

The *Cucurbita pepo* cultivar 'Raven' was cultivated in the contaminated soil with dieldrin (12.5 $\mu\text{mol/kg}$) for 27 days at 26°C under a 16/8 h light/dark cycle. Aerial parts were collected from each plant, and the fresh weight of them was measured. –, the cultivation in the non-contaminated soil with dieldrin or the non-treatment of pesticides; +, the cultivation in the contaminated soil with dieldrin or the treatment of pesticides at usual dose; +++, the treatment of pesticides at the triple dose. The white, gray, and black bars represent control (no pesticides), + (the treatment of pesticides at usual dose), and +++ (the treatment of pesticides at the triple dose), respectively. Error bars indicate standard deviations ($n = 8-19$).

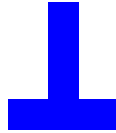
Supplementary Figure 5. The change in the amount of major latex-like proteins by the treatment of Colt.

The *Cucurbita pepo* cultivar 'Raven' was cultivated in the contaminated soil with dieldrin (12.5 $\mu\text{mol/kg}$) for 27 days at 26°C under a 16/8 h light/dark cycle. Roots were collected, and root proteins were extracted. They were subjected to SDS-PAGE, and reacted with anti-MLP-PG1 antibody and anti-MLP-GR3 antibody, respectively. –, the non-treatment of Colt; +++, the treatment of Colt at the triple dose. The white and black bars represent control (no pesticides) and +++ (the treatment of

546 pesticides at the triple dose), respectively. Error bars indicate standard deviations ($n = 6$). The band
547 intensities were quantified by ImageJ.

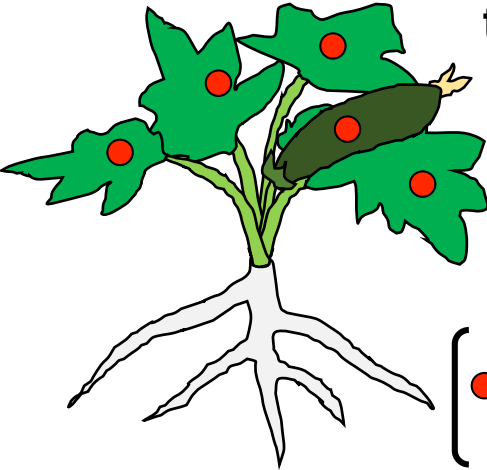
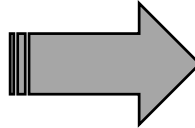
Pesticides
(Raimei and Colt)

High
toxicity



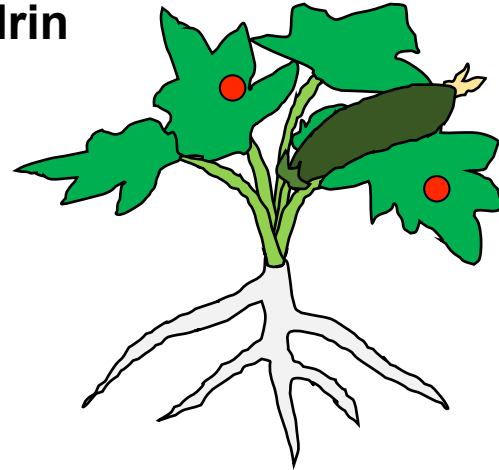
Less
toxicity

Binding of MLPs
to pyrene and dieldrin

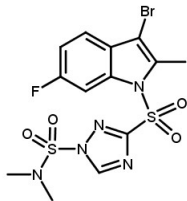


● Pyrene
Dieldrin

Zucchini
(*Cucurbita pepo*)



(A)



(B)

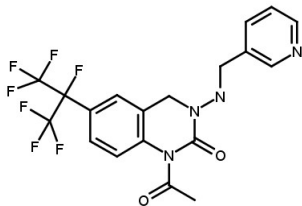


Figure 1

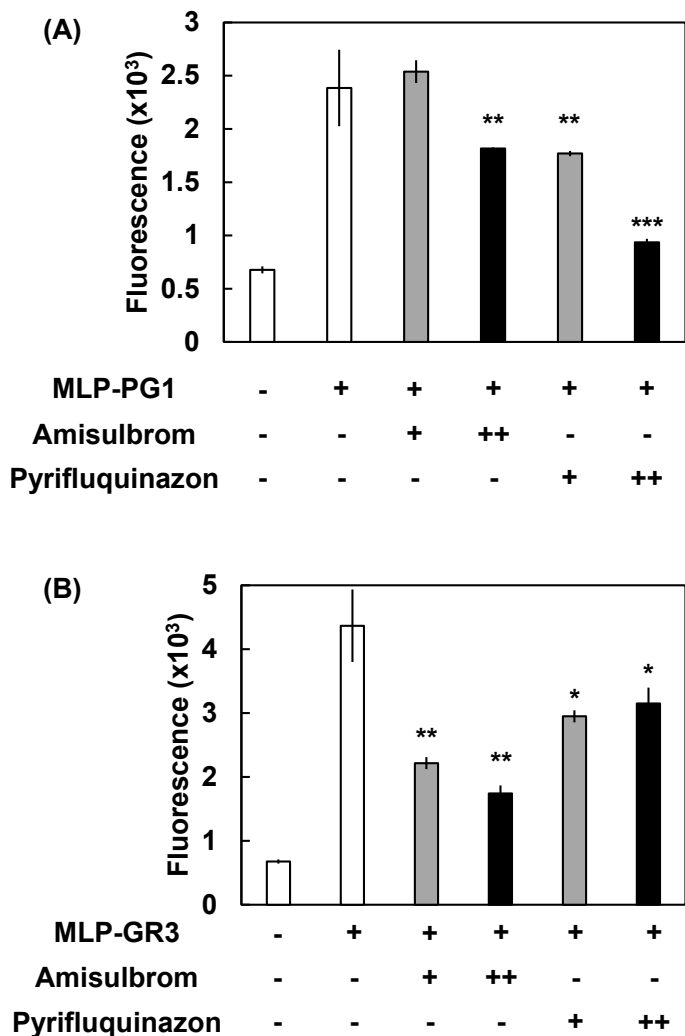


Figure 2

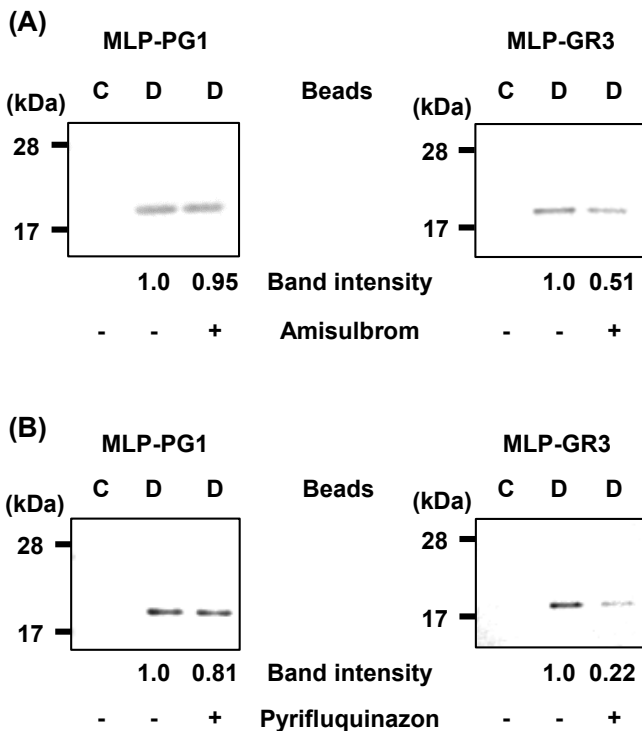


Figure 3

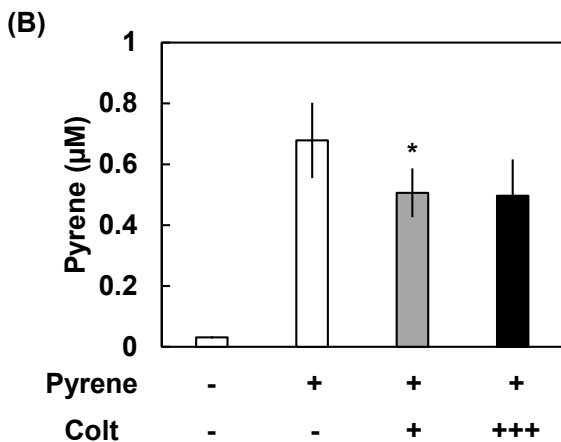
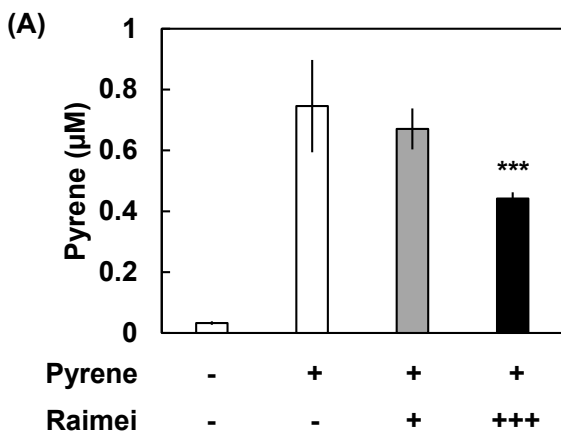


Figure 4

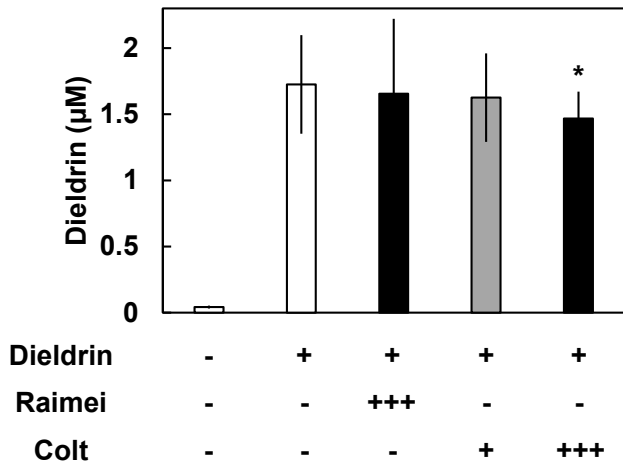


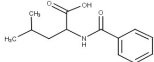
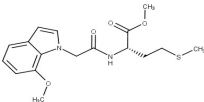
Figure 5

Table 1. MLP-binding compounds in chemical array screening.

MLP	Number of compounds			Total
	Binding affinity			
	+	++	+++	
MLP-PG1	98	48	20	166
MLP-GR3	99	55	22	176


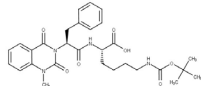
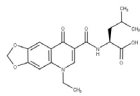

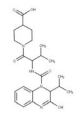
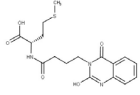
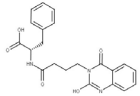
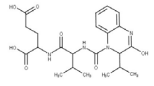
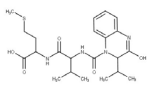
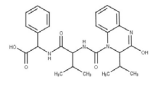
The chemical arrays were incubated with 4 μ M of recombinant MLP-PG1 and MLP-GR3, sequentially anti-His antibody, and secondary antibody with Cy5. The fluorescence of Cy5 was detected. For the reference arrays, the chemical arrays were incubated only with anti-His antibody and Cy5-labeled secondary antibody. The Z-score of the difference in fluorescence intensities of each spot between the MLPs-treated arrays and the reference arrays was calculated (Inui et al. 2013). The marks +, ++, and +++ were defined as Z-score >1, >2, and >3, respectively.

Table 2. Compounds with an indole-like structure binding MLPs.

Name	Structure	Binding affinity	
		MLP-PG1	MLP-GR3
NPD9013		+	+
NPD14496		+	+++

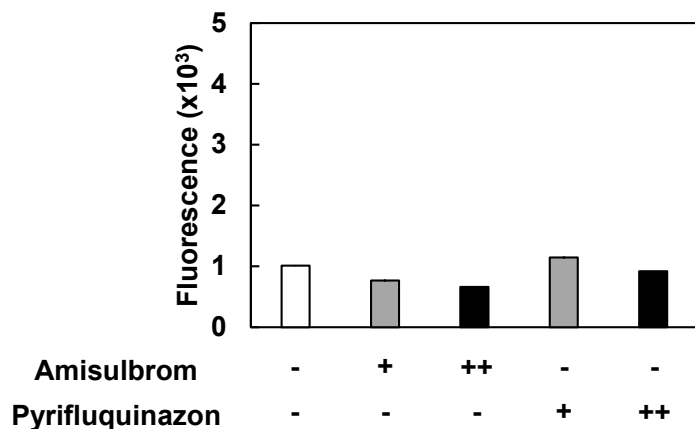
By chemical array screening, 242 MLP-binding compound candidates were identified. Publications about selected compounds were searched by SciFinder, and 30 compounds related to plants, such as plant hormones and secondary metabolites, were identified. The marks + and +++ were described in the legend of Table 1.

Table 3. Compounds containing quinazoline-, quinoxaline-, and quinoline-like structures with a high affinity to bind MLPs.

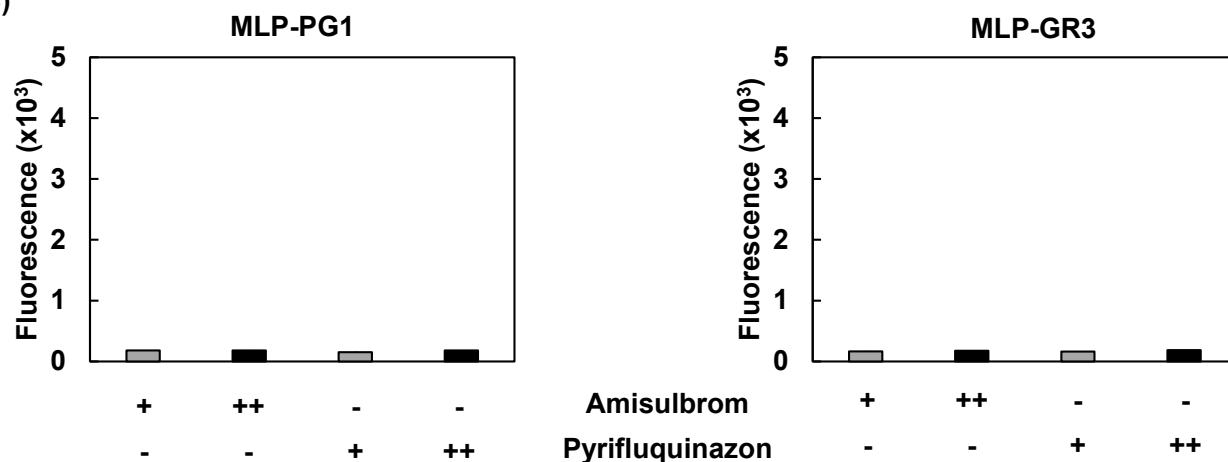
Name	Structure	Binding affinity	
		MLP-PG1	MLP-GR3
NPD8605		++	++
NPD12285		++	++
NPD13695		++	++
NPD13698		+++	++
NPD13699		++	++
NPD13700		+++	+++
NPD13711		+++	++
NPD13717		++	+++
NPD13718		++	+++
NPD13719		+++	+++

By chemical array screening, 242 compounds binding MLPs were identified. The 37 compounds with the high affinity (++ and +++) to bind both MLPs were selected. There were 10 compounds with quinazoline-like, quinoxaline-like, and quinoline-like structures in 37 compounds. The marks ++ and +++ were described in the legend of Table 1.

(A)



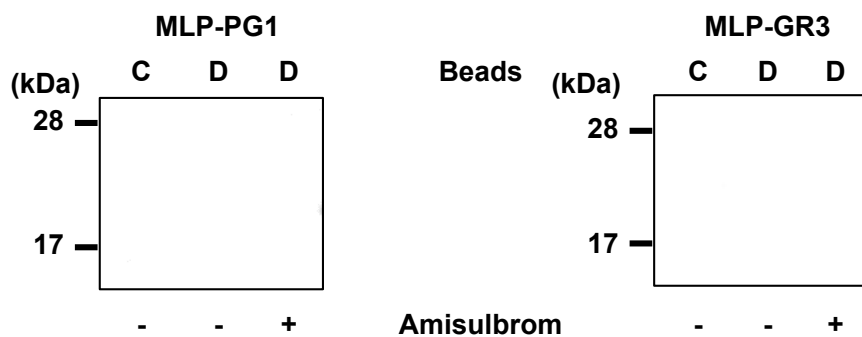
(B)



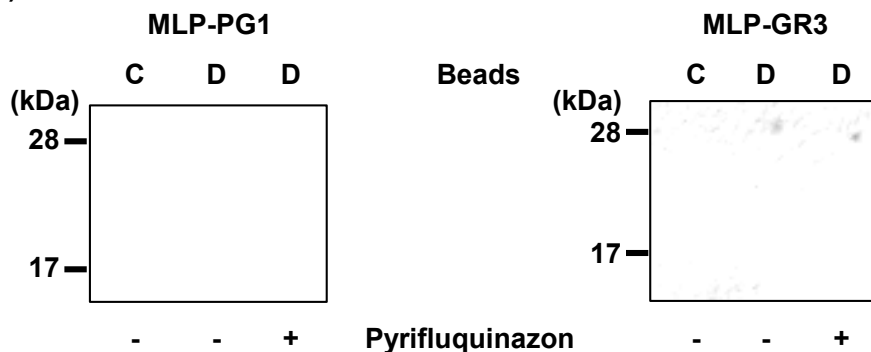
Supplementary Figure 1. Competitive binding of major latex-like proteins (MLPs) to pyrene by amisulbrom and pyrifluquinazon.

The 10 μ M pyrene and 5 μ M or 50 μ M amisulbrom and pyrifluquinazon were incubated without recombinant MLPs for 1 h at 20° C (A). The 0.2 mg/mL recombinant MLPs were incubated with 5 μ M or 50 μ M amisulbrom and pyrifluquinazon without pyrene (B). The fluorescence of pyrene was measured. –, the incubation without pesticides; +, the incubation with 5 μ M pesticides; ++, the incubation with 50 μ M pesticides. The white, grey, and black bars represent control (no pesticides), + (25 μ M pesticides), and ++ (50 μ M pesticides), respectively. Error bars indicate standard deviations ($n = 3-4$).

(A)

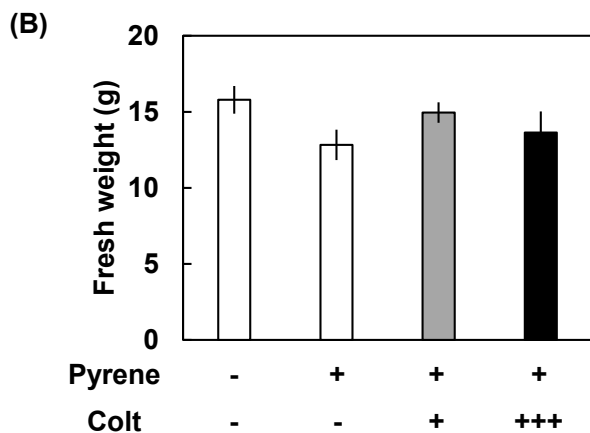
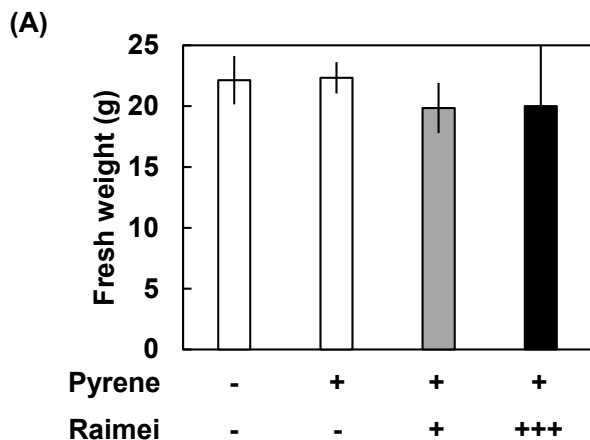


(B)



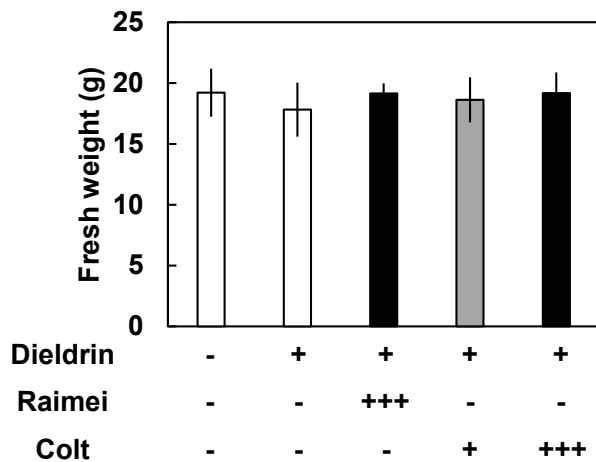
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The 0.1 mg/mL recombinant MLPs were incubated with 0.2 mM amisulbrom (A) and pyrifluquinazon (B) for 2 h at 4° C. The magnetic beads binding 100 mM dieldrin were added and incubated for 4 h at 4° C. Eluates at the eighth washing step were subjected to SDS-PAGE, and MLPs were detected by silver staining. C; beads without dieldrin, D; dieldrin-binding beads; –, the incubation with magnetic beads without binding of dieldrin or without pesticides; +, the incubation with magnetic beads binding dieldrin or with 0.2 mM pesticides.



Supplementary Figure 3. Fresh weight of aerial parts of zucchini plants cultivated in the contaminated soil with pyrene under the treatment of Raimei (A) and Colt (B).

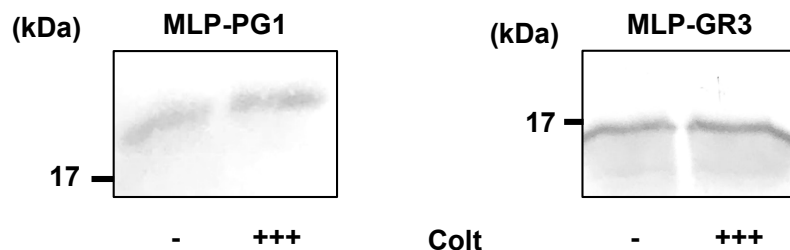
The *Cucurbita pepo* cultivar ‘Magda’ was cultivated in the contaminated soil with pyrene (1.25 mmol/kg) for 27 days at 26° C under a 16/8 h light/dark cycle. Aerial parts were collected from each plant, and the fresh weight of them was measured. –, the cultivation in the non-contaminated soil with pyrene or the non-treatment of pesticides; +, the cultivation in the contaminated soil with pyrene or the treatment of pesticides at usual dose; +++, the treatment of pesticides at the triple dose. The white, grey, and black bars represent control (no pesticides), + (the treatment of pesticides at usual dose), and +++ (the treatment of pesticides at the triple dose), respectively. Error bars indicate standard deviations (A, $n = 5-12$, B, $n = 6-7$).



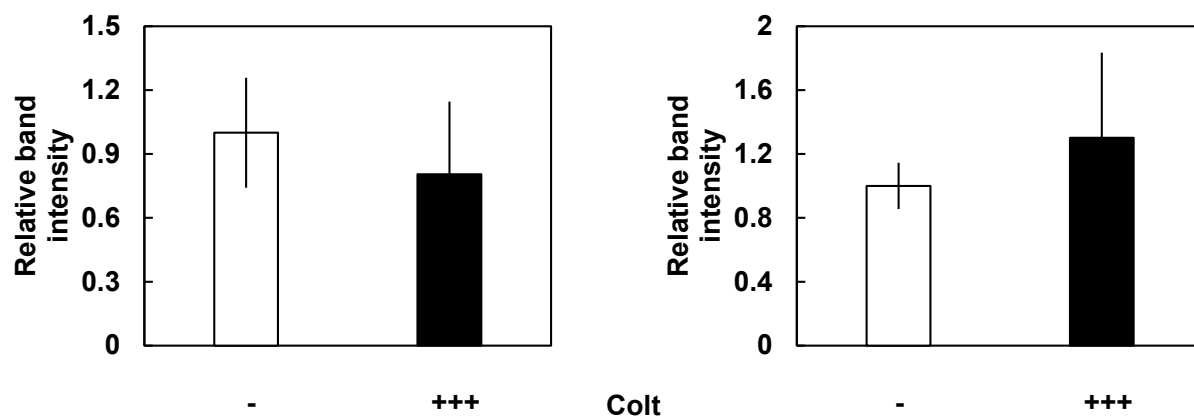
Supplementary Figure 4. Fresh weight of aerial parts of zucchini plants cultivated in the contaminated soil with dieldrin under the treatment of Raimei and Colt.

The *Cucurbita pepo* cultivar ‘Raven’ was cultivated in the contaminated soil with dieldrin (12.5 $\mu\text{mol/kg}$) for 27 days at 26° C under a 16/8 h light/dark cycle. Aerial parts were collected from each plant, and the fresh weight of them was measured. –, the cultivation in the non-contaminated soil with dieldrin or the non-treatment of pesticides; +, the cultivation in the contaminated soil with dieldrin or the treatment of pesticides at usual dose; +++, the treatment of pesticides at the triple dose. The white, grey, and black bars represent control (no pesticides), + (the treatment of pesticides at usual dose), and +++ (the treatment of pesticides at the triple dose), respectively. Error bars indicate standard deviations ($n = 8-19$).

(A)



(B)



Supplementary Figure 5. The change in the amount of major latex-like proteins by the treatment of Colt. The *Cucurbita pepo* cultivar 'Raven' was cultivated in the contaminated soil with dieldrin (12.5 $\mu\text{mol/kg}$) for 27 days at 26° C under a 16/8 h light/dark cycle. Roots were collected, and root proteins were extracted. They were subjected to SDS-PAGE, and reacted with anti-MLP-PG1 antibody and anti-MLP-GR3 antibody, respectively. -, the non-treatment of Colt; +++, the treatment of Colt at the triple dose. The white and black bars represent control (no pesticides) and +++ (the treatment of pesticides at the triple dose), respectively. Error bars indicate standard deviations ($n = 6$). The band intensities were quantified by ImageJ.