REDUCTION OF POP CONTAMINATION IN CUCURBITACEAE FAMILY FOCUSING ON THE TRANSPORTING FACTORS FOR POPS BY THE TREATMENT OF PESTICIDES

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Introduction:

In recent years, one of the most concerned things in the security of foods is residual pesticides in crops. In Japan, for example, pesticides, such as dieldrin and heptachlor, were detected in crops, especially Cucurbitaceae family, although they were prohibited for using and registered as persistent organic pollutants (POPs). POPs are so toxic for mammals including human beings and wildlife that intake of crops polluted by POPs is harmful to their health in carcinogenicity, neurotoxicity, and immunotoxicity. Once crops above a residue limit are found, farmers have to discard all crops in their farmland, and economic loss is huge. Since even today there is still so much amount of POPs in the soil of farmland in Japan, many attempts to remediate the soil were carried out, but it was difficult to remediate POP-contaminated soil because of the cost and effectiveness. However, we hope to harvest non-contaminated crops even if we cultivate them in POP-contaminated soil.

Many plants do not accumulate POPs in their aerial parts like leaves, stems, and fruits, but it is well-known that Cucurbitaceae family, including cucumber, melon, watermelon, squash, and zucchini can accumulate POPs such as dieldrin[1], dichlorodiphenyltrichloroethane (DDT)[2], polychlorinated dibenzo-*p*-dioxins (PCDDs)[3], polychlorinated dibenzofurans (PCDFs)[3], and polychlorinated biphenyls (PCBs)[4]. Our research group has identified a major latex-like protein (MLP) in zucchini (*Cucurbita pepo*) as a transporting factor for POPs[5]. MLPs have a big internal hydrophobic cavity formed by long third α-helix and seven β-sheet and binding affinity to POPs, such as PCB and dieldrin[6]. We proposed the pathways for POP contamination in Cucurbitaceae family[6]: MLPs bind to POPs in root cells, MLP-POP complexes translocate into xylem vessel, and finally, they are transported to aerial parts (Figure 1).

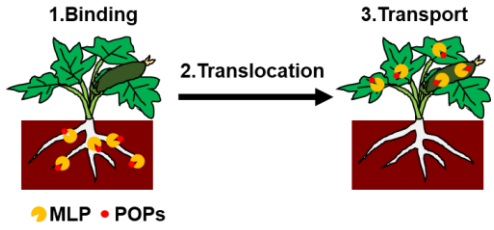

Figure 1 POP contamination in Cucurbitaceae family by MLPs

Therefore, it is possible that POP contamination in Cucurbitaceae family is reduced by the regulation of MLPs. In this study, the transportation of POPs is regulated by the treatment of commercially available pesticides. First, the amount of MLPs in roots is decreased by the suppression of the expression of *MLP* genes by the treatment of pesticides (Figure 2A). The amount of MLPs translocating to the xylem vessel is decreased, resulting that the amount of POPs transported to aerial parts is decreased.

Second, binding of MLPs to POPs is competitively inhibited by the treatment of pesticides which contain MLP-binding active compounds (Figure 2B). In roots, the amount of MLPs binding to POPs is decreased. As a result, the amount of MLPs

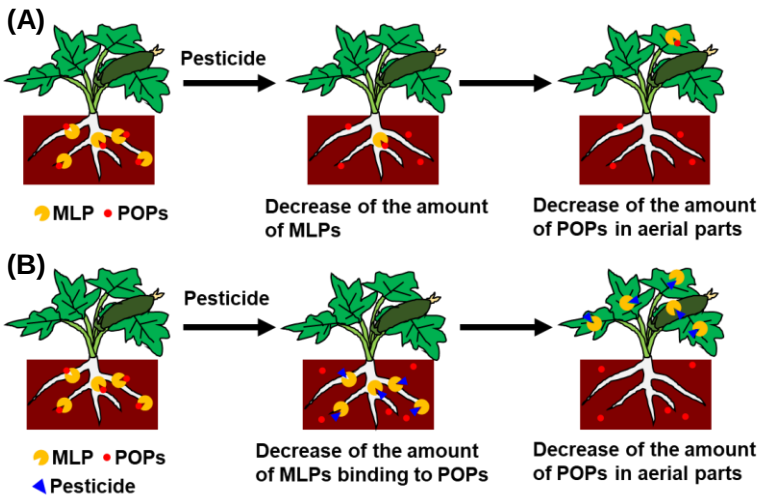

Figure 2 Two proposed methods to reduce POP contamination in Cucurbitaceae family
(A)Suppression of the expression of *MLP* genes,
(B)Competitive inhibition of binding of MLPs to POPs

binding to POPs translocating to xylem vessel is decreased, and the amount of POPs transported to aerial parts is decreased.

Materials and methods:

Screening of pesticides suppressing the expression of MLP genes (Figure 3)

Seed coats of *C. pepo* subspecies *pepo* cultivar 'Magda' were removed, and seeds were sown in a pot containing the contaminated soil with the hydrophobic fluorescent contaminant, perylene. Magda cultivars were cultivated for 27 days and treated with pesticides registered for the cultivation of Cucurbitaceae family. After cultivation, xylem sap was collected in the glass tube until the volume reached 1 mL, and the concentration of perylene was quantified. *β-Glucuronidase* (*GUS*) gene was combined at the downstream of the cloned promoter region of *MLP* gene. Transgenic tobacco plants which have this construct were produced and incubated with Murashige-Skoog (MS) medium for 2 weeks. The seedlings were transferred to water containing 1% dimethyl sulfoxide (DMSO) or active compounds of pesticides. After 2 days, roots were collected and used for fluorometric measurement of GUS activity with the substrate 4-methyl-umbelliferyl-β-D-glucuronide.

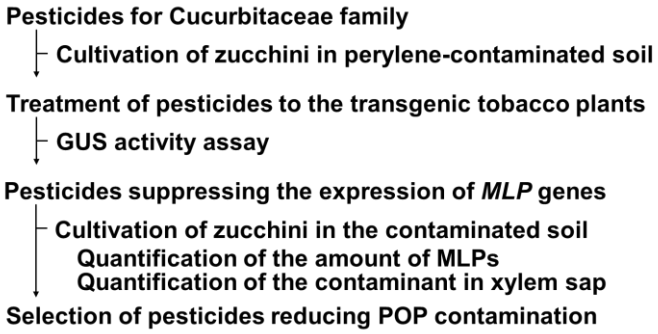

Figure 3 Scheme of the reduction of POP contamination by the suppression of *MLP* genes' expression

Magda cultivars were cultivated in the contaminated soil with the hydrophobic fluorescent contaminant, pyrene, under the treatment of selected pesticides. After 27 days, xylem sap and roots were collected. Proteins in xylem sap and roots were subjected to western blot analysis using anti-MLP antibodies in order to quantify the amount of MLPs. Furthermore, the concentration of pyrene in the xylem sap was quantified.

Screening of compounds binding to MLPs (Figure 4)

Chemical arrays with 22097 compounds from RIKEN compound library (NPDepo) were used for screening of compounds binding to MLPs[7]. Recombinant MLPs with His-tag were added and incubated at 30°C for 1 h. After addition of the anti-His antibody, the secondary antibody with Cy5 fluorescence dye was added, and chemical arrays were scanned by Cy5 channel for the quantification of fluorescence. The pesticides which have the structure similar to MLP-binding compounds were selected (Figure 5).

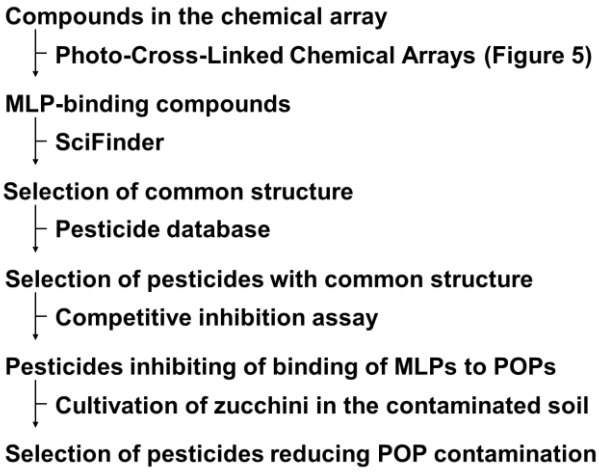

Figure 4 Scheme of the reduction of POP contamination by the competitive ihibition of MLPs to POPs

In order to confirm that the selected pesticides competitively inhibit binding of MLPs to pyrene or dieldrin *in vitro*, the competitive binding assay was performed. Pyrene and the selected pesticide were incubated with MLPs, and the fluorescence of pyrene was measured. Furthermore, dieldrin-binding magnetic beads were prepared and incubated with recombinant MLPs. MLPs were eluted after magnetic separation. On the other hand, recombinant MLPs were incubated with the selected pesticide before incubation with dieldrin-binding magnetic beads, and these eluates were subjected to SDS-PAGE, and the band intensity was quantified at the molecular weight of MLPs.

Magda cultivars were cultivated in the contaminated soil with pyrene and treated with selected pesticides. After 27 days, xylem sap was collected, and the concentration of pyrene in xylem sap was quantified.

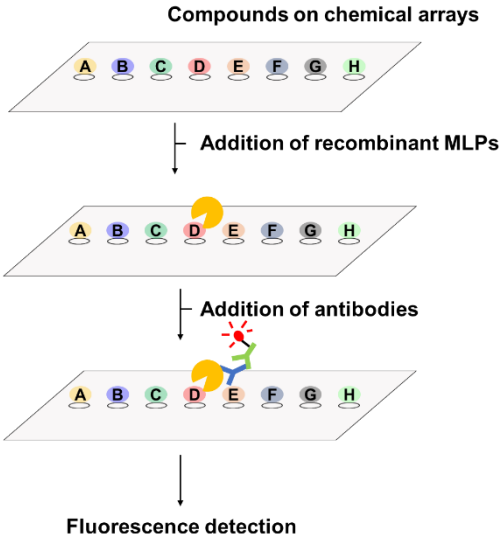

Figure 5 Screening of MLP-binding compounds by Photo-Cross-Linked Chemica Arrays

Results and discussion:

Suppression of the expression of MLP genes

When Magda cultivars were cultivated in perylene-contaminated soil, they were treated with each of five pesticides applicable for Cucurbitaceae family. By the treatment of two pesticides, the concentration of perylene in xylem sap was decreased. So, they were selected as the pesticides which possibly suppress the expression of *MLP* genes. In general, pesticides contain active compounds for insects, fungi or bacteria, detergents, and so on. In this study, in order to confirm that an active compound in the pesticides suppresses the expression of *MLP* genes, GUS activity assay was performed. Transgenic tobacco plants which had the *GUS* gene as a reporter gene at the downstream of the promoter of the *MLP* gene were treated with active compounds of selected pesticides. One of them significantly decreased GUS activity, and it showed that an active compound of the pesticide suppressed the transcription of *MLP* genes. So, the pesticide will suppress the expression of *MLP* genes.

When Magda cultivars were cultivated in pyrene-contaminated soil and treated with the selected pesticide, the band intensity of MLPs in roots and xylem sap was clearly decreased. It showed that the treatment of the pesticide decreased the amount of MLPs in roots followed by xylem sap. In fact, the concentrations of pyrene in xylem sap were decreased by the treatment of the pesticide.

To sum up the results, this strategy confirms that the active compound suppressed the expression of *MLP* genes in roots of zucchini plants and decreased the amount of MLPs in roots, so the amount of MLPs which translocated to xylem vessel was decreased. As a result, the concentration of pyrene in xylem sap was significantly decreased, probably leading to low contamination of POPs in Cucurbitaceae family.

Competitive inhibition of binding of MLPs to POPs

Chemical array screening was performed for the selection of MLP-binding compounds. After the purification of recombinant MLPs, recombinant MLPs were added into chemical arrays with 22097 compounds in NPDepo. Positive signals were seen in more than 200 compounds by the addition of recombinant MLPs. If MLP-binding compounds would be treated to the contaminated soil for the cultivation of zucchini plants, they do not accumulate POPs. The published papers related to MLP-binding compounds were searched by SciFinder, and compounds which had the relationship to plants were selected. Some of MLP-binding compounds had indole-like structure. So, by the treatment of pesticides which contain indole-like structures as an active compound, it binds to MLPs, and binding of MLPs to POPs will be competitively inhibited. And then, the amount of POPs transported to aerial parts is decreased. The active compound which has indole-like structure was selected, and the competitive binding activities of MLPs to pyrene were investigated. When pyrene was incubated with MLPs, fluorescence was increased because pyrene was more solubilized by binding of MLPs to pyrene. However, fluorescence was significantly decreased by the addition of the active compound, and it showed that the active compound competitively inhibited the binding of MLPs to pyrene by binding to MLPs. The competitive binding activities of MLPs to dieldrin were also investigated. A band to show binding of MLPs to dieldrin was detected when dieldrin-binding beads were used. By the addition of the active compound, the band intensity of MLPs was clearly decreased. Any bands were not detected when dieldrin-binding beads were not used. So, it was confirmed that the active compound could competitively inhibit the binding of MLPs to pyrene and dieldrin.

The pesticide for Cucurbitaceae family containing the active compound was selected for the reduction of POP contamination by the competitive inhibition of binding of MLPs to POPs. Magda cultivars were cultivated in pyrene-contaminated soil and treated with the pesticide. Since the pesticide did not contain so much amount of the active compound compared with other pesticides, the pesticide was treated by not only the usual dosage but 3 times dosage. By the treatment of the pesticide, the concentration of pyrene was decreased concentration-dependently. Although the usual dosage of the pesticide was not significantly decreased, higher dosage showed a significant decrease of pyrene in xylem sap. This strategy confirms that the pesticide, including the active compound which has an indole-like structure, competitively inhibits the binding of MLPs to POPs in roots and decreased the amount of MLPs binding to POPs in roots. Therefore, the amount of MLPs binding to POPs which translocated into xylem vessel was decreased, resulting that the concentration of POPs in xylem sap was significantly decreased.

In conclusion, by the treatment of the pesticides, which could suppress the expression of *MLP* genes and competitively inhibit the binding of MLPs to contaminants, the concentration of contaminants was decreased in xylem sap. It suggests that transport of POPs to fruits is inhibited. This study proposes a new method that we can harvest non-contaminated crops in POP-contaminated soil.

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