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Regular Article

Major latex-like proteins show pH dependency in their binding to hydrophobic organic pollutants

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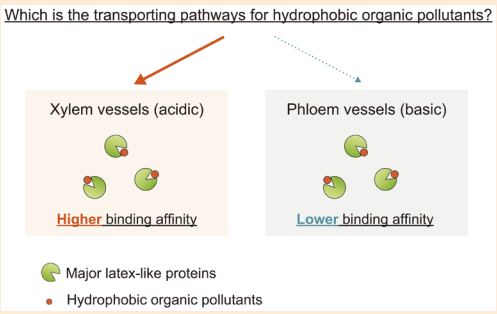
Supplementary material

The Cucurbitaceae family accumulates hydrophobic organic pollutants in its aerial parts at high concentrations. Major latex-like proteins (MLPs) were identified in zucchini (*Cucurbita pepo*) as a transporting factor for hydrophobic organic pollutants. MLPs bind to hydrophobic organic pollutants in the roots, are secreted to xylem vessels as complexes, and are transported to the aerial parts. However, the suitable conditions for binding MLPs to hydrophobic organic pollutants remain elusive. In the present study, we show that MLPs bind to the hydrophobic organic pollutant pyrene with higher affinity under acidic conditions. Our results demonstrated that pH regulates the binding of MLPs to hydrophobic organic pollutants.



Keywords: acidity, crop contamination, *Cucurbita pepo*, hydrophobic organic pollutants, major latex-like protein.

Introduction

Hydrophobic organic pollutants contaminate the environment, and agricultural fields are not exceptional.[1)] The organochlorine insecticide dieldrin is still detected in agricultural fields in France,[2)] Japan,[3)] and the United States,[4)] although its use as an insecticide is prohibited. Pyrene, a polycyclic aromatic hydrocarbon, is produced by the incomplete burning of straw, and the amount of pyrene in agricultural fields is increased by slash-and-burn agriculture.[5)] Hydrophobic organic pollutants show a wide variety of toxicities toward human beings, such as carcinogenicity[6)] and neurotoxicity,[7)] thereby leading to severe syndromes due to the intake of contaminated crops. Commercial crops would be basically safe under the current food safety regulation.

Unlike other plant families, members of the Cucurbitaceae family, such as cucumber (*Cucumis sativus*), squash (*Cucurbita maxima*), melon (*Cucumis melo*), and zucchini (*Cucurbita pepo*), accumulate hydrophobic organic pollutants in the aerial parts at high concentrations.[8)] *C. sativus* and *C. pepo* accumulate hydrophobic organic pollutants from the roots to the aerial parts *via* the xylem vessels.[9)] This indicates that the xylem sap contains transporting factors for hydrophobic organic pollutants.[10)] Major latex-like proteins (MLPs) identified in the xylem sap of *C. pepo* play a crucial role in contamination.[11)] MLPs bind to hydrophobic organic pollutants in root cells, and it is thought that MLP–hydrophobic organic pollutant complexes are secreted into xylem vessels in the only Cucurbitaceae family. Hydrophobic organic pollutants are transported to the aerial parts.[12,13)] Contamination with hydrophobic organic pollutants occurs in the Cucurbitaceae family.

MLPs elicit abiotic and biotic stress responses in several monocots and dicots.[14)] MLPs confer drought tolerance through interactions with drought-related proteins[15,16)] and promote resistance through the induction of *pathogenesis-related protein* genes.[17–19)] MLPs are members of Bet v 1 family, such as *patho-*

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genesis-related protein class 10 (PR-10).[14] The most remarkable feature of the members is their internal hydrophobic cavity, which enables them to bind to hydrophobic compounds.[20] Proteins of Bet v 1 family consist of three α-helices and seven β-sheets as their secondary structures. The cavity is formed by $\beta1$–$\beta7$ wrapped around a long C-terminus $\alpha3$, and there are several entrances to the cavity. MLPs bind to a wide variety of hydrophobic organic pollutants including steroids,[12,21] biphenyl,[13] quinazoline,[22] and lysophosphatidic acid.[23] These results indicate that the ligand binding of MLPs plays a crucial role in plant physiology. MLPs have been detected in the xylem (acidity) and phloem (basicity) sap of several plants.[11,13,18,24] MLPs from the Cucurbitaceae family transport hydrophobic organic compounds *via* xylem vessels[11,13]; however, the decisive factor for ligand binding of MLPs has not been extensively studied.

In the present study, the optimal pH conditions for the binding of MLP–PG1 and MLP–GR3 (identified from *C. pepo*) to pyrene, a hydrophobic fluorescent organic pollutant, were identified. First, the existence of MLPs in vascular bundles of the members of the Cucurbitaceae family was clarified. Furthermore, the pH conditions for the binding of MLPs to pyrene were optimized. The binding affinity of MLPs with pyrene was investigated by detecting the amount of MLPs binding to pyrene and the increase in pyrene fluorescence. Our study revealed the crucial pH conditions required for contamination of the members of the Cucurbitaceae family with hydrophobic organic pollutants by focusing on binding MLPs to hydrophobic pollutants.

Materials and methods

1. Collection of xylem and phloem sap

The seeds of *C. pepo* subspecies *ovifera* cultivar 'Starship' (ST) and ssp. *pepo* cv. 'Black Tosca' (BT) were purchased from Johnny's Selected Seeds (Albion, ME) and Sakata Seed Co. (Kanagawa, Japan), respectively. After peeling off the seed coat, the seeds were incubated overnight in tap water at 4°C and sown into sterile soil in a glass jar. They were cultivated under a 16/8 hr light/dark cycle at 26°C for three weeks, and the stem below the cotyledons was cut. Because the pH of xylem and phloem sap is acidic and basic, respectively, the basic droplet was collected as the phloem sap (20–40 μL). After confirming that the pH of the stem surface was acidic, a tube was inserted into the stem and incubated for 8 hr, and the solution was collected as xylem sap (1.1–2.8 mL). The xylem and phloem sap were stored at 4°C until further use.

2. SDS-PAGE and western blot analysis

A sample buffer solution with a reducing reagent for SDS-PAGE (Nacalai Tesque, Inc., Kyoto, Japan) was added to the xylem and phloem sap of *C. pepo* ssp. *ovifera* cv. ST and ssp. *pepo* cv. BT. The mixture was boiled at 98°C for 5 min and subjected to SDS-PAGE on 15% acrylamide gel. The gels were stained with Coomassie Brilliant Blue Stain One (Nacalai Tesque), and the bands were detected.

Western blotting was performed as described in our previous report (Goto *et al.*, 2019). First, the gels were transferred to PVDF membranes. After blocking with TTBS buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, and 0.05% [v/v] Tween 20) supplemented with 1% BSA, PVDF membranes were incubated with anti-MLP–PG1 or anti-MLP–GR3 antibodies (Medical & Biological Laboratories Co., Ltd., Aichi, Japan) diluted at 1 : 500 and 1 : 1000, respectively, for 45 min at room temperature (approximately 20°C) and washed with TTBS buffer. PVDF membranes were reacted with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO) for 30 min at room temperature and washed with TTBS buffer. PVDF membranes were incubated in AP9.5 buffer (10 mM Tris-HCl [pH 9.5], 100 mM NaCl, and 5 mM $MgCl_2$) for 5 min and transferred to AP9.5 buffer containing 75 μg/mL nitroblue tetrazolium and 50 μg/mL 5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt, and bands were detected.

3. Magnetic bead binding assay

Codon-optimized *MLP–PG1* and *MLP–GR3* genes were inserted into a pET-28a (+) vector (Novagen, Madison, WI), and a 6×His tag followed by a tobacco etch virus (TEV) protease site was attached to the N-terminus of MLP–PG1 and MLP–GR3. *Escherichia coli* BL21 Star (DE3) (Invitrogen, Waltham, MA) expressing *MLP–PG1* and *MLP–GR3* was cultured in lysogeny broth medium containing 100 μg/mL kanamycin. After the optical density reached approximately 0.7, 0.5 mM isopropyl β-D-thiogalactopyranoside was added, and *E. coli* was cultured for 20 hr at 18°C. *E. coli* precipitate was harvested by centrifugation and sonicated in Buffer A (20 mM Tris-HCl [pH 8.0], 10% glycerol, 200 mM NaCl, and 5 mM imidazole) containing a complete Protease Inhibitor Cocktail tablet (Roche, Basel, Switzerland). The sonicated solution was centrifuged at 20,000×*g* for 1 hr at 4°C, and the supernatant was collected. The HiTrap TALON column (Cytiva, Marlborough, MA) was equilibrated with Buffer A, and the supernatant was loaded. After washing with Buffer A, the elution was collected with Buffer B (20 mM Tris-HCl [pH 8.0], 10% glycerol, 200 mM NaCl, and 200 mM imidazole). After the addition of TEV protease, the sample was dialyzed overnight in 20 mM Tris-HCl (pH 8.0, 10% glycerol, and 200 mM NaCl) at 4°C. The dialyzed sample was purified using a HisTrap HP (Cytiva). The column was equilibrated with Buffer A, and the dialyzed sample was loaded. The elution was collected with Buffer A, concentrated by ultrafiltration, and further purified by gel filtration using HiLoad 16/60 Superdex 75 prep grade (Cytiva). The column was equilibrated with 20 mM Tris-HCl (pH 8.0), and the sample was loaded. The existence of MLPs in the elution was confirmed with CBB staining, and the elution was frozen in liquid nitrogen and stored at −80°C until use.

Two hundred microliters of 50 mM 1-pyrenecarboxylic acid dissolved in dimethylformamide was immobilized on 1 mg of NH_2 magnetic beads (Tamagawa Seiki Co., Ltd., Nagano, Japan), according to the manufacturer's instructions. The 1-pyrenecarboxylic acid-binding beads (0.25 mg) were washed three times

with Buffer C (50 mM potassium phosphate [pH 5.6], 100 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM EDTA, 10% [v/v] glycerol, 0.1% [v/v] Nonidet P-40, 1 mM dithiothreitol [DTT], 0.2 mM phenylmethylsulfonyl fluoride [PMSF], 0.1% Triton X-100, and 0.1% *N*-lauroylsarcosine sodium salt), Buffer D (100 mM HEPES-NaOH [pH 7.2], 100 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM EDTA, 10% [v/v] glycerol, 0.1% [v/v] Nonidet P-40, 1 mM DTT, 0.2 mM PMSF, 0.1% Triton X-100, and 0.1% *N*-lauroylsarcosine sodium salt), or Buffer E (100 mM Tris-HCl [pH 8.8], 100 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 0.2 mM EDTA, 10% [v/v] glycerol, 0.1% [v/v] Nonidet P-40, 1 mM DTT, 0.2 mM PMSF 0.1% Triton X-100, and 0.1% *N*-lauroylsarcosine sodium salt). Recombinant MLP–PG1 and MLP–GR3 were diluted to 0.2 mg/mL in Buffer C, D, or E and centrifuged at 4°C for 30 min at 20,700×*g*. The supernatant (200 μL) was incubated with 0.25 mg of 1-pyrenecarboxylic acid-binding beads at 4°C for 4 hr. The solution was washed eight times with Buffer C, D, or E *via* magnetic separation, and the supernatant was collected after eight washes with salt elution. Buffer C, D, or E (30 μL) and 10 μL of 4× sample buffer for SDS-PAGE were added to the beads, and the solution was boiled at 98°C for 5 min. After magnetic separation, the supernatant was collected and subjected to heat elution. The samples were subjected to SDS-PAGE on a 12.5% acrylamide gel, and the bands were detected using a Silver Staining kit (Fujifilm Wako Pure Chemical Industries, Ltd., Osaka, Japan).

4. Pyrene fluorescence assay

Recombinant MLP–PG1 and MLP–GR3 were purified as described in our previous report,[22] and diluted in Buffer C, D, or E at final concentrations of 0.2 mg/mL. Pyrene dissolved in dimethyl sulfoxide (DMSO) was added at final concentrations of 0, 10, and 20 μM (the estimated water solubility of pyrene; 644 nM).[25] These solutions were mixed well at room temperature, and pyrene fluorescence was measured in a 96-well black microplate using a Microplate Reader SH-9000 (Corona Electric Co., Ltd., Hitachinaka, Ibaraki, Japan) with the excitation wavelength set at 330 nm and emission wavelength set at 390 nm.

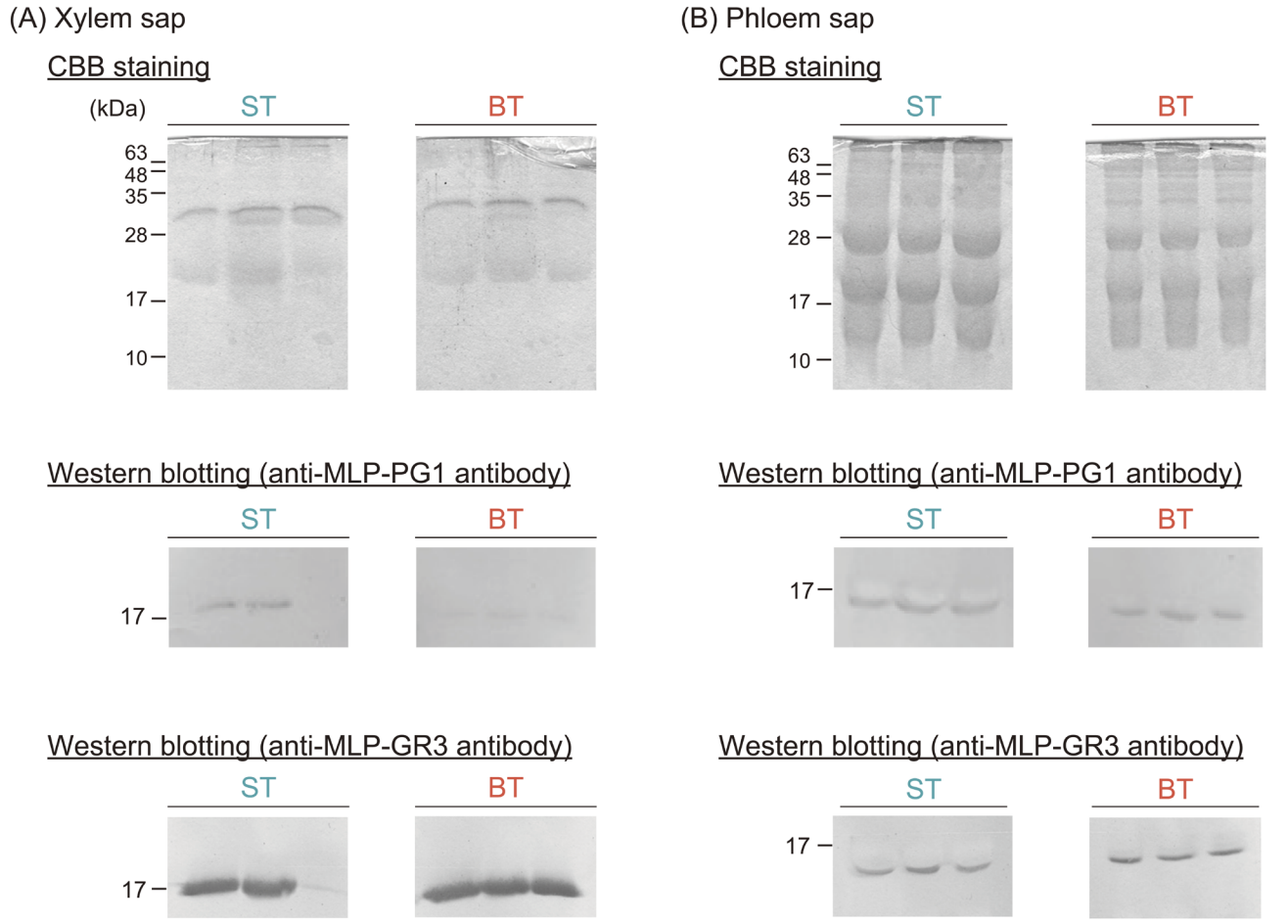

Fig. 1. Major latex-like proteins (MLPs) are detected in the xylem and phloem sap of *Cucurbita pepo*. *C. pepo* subspecies *ovifera* cultivar 'Starship' (ST) and ssp. *pepo* cv. 'Black Tosca' (BT) were cultivated under a 16/8 hr light/dark cycle at 26°C for three weeks. (A) Xylem and (B) phloem sap were collected and boiled in sample buffer solution, and the mixture was subjected to SDS-PAGE. Bands were detected using Coomassie Brilliant Blue (CBB) staining. Western blot analysis was performed by the reaction with the anti-MLP–PG1 or anti-MLP–GR3 antibodies, and bands were detected. The different lanes showed biological replicates.

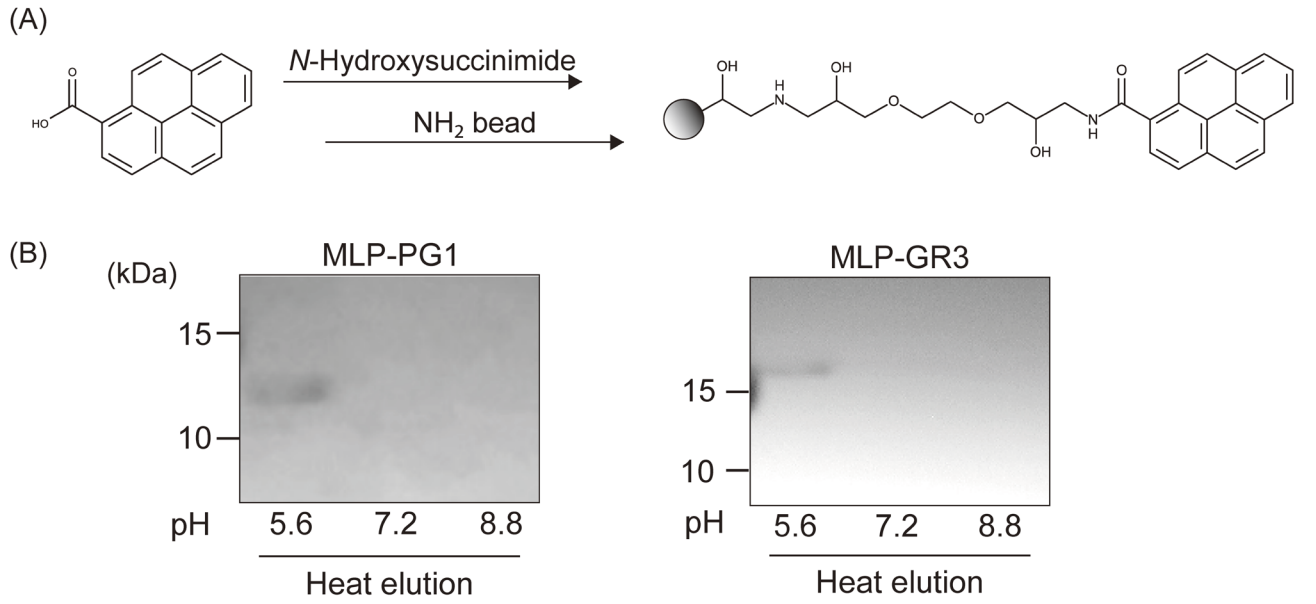

Fig. 2. Major latex-like proteins (MLPs) bind to 1-pyrenecarboxylic acid under acidic conditions. (A) Immobilization reaction of 1-pyrenecarboxylic acid to NH_2 magnetic beads. (B) Binding assay of recombinant MLPs with 1-pyrenecarboxylic acid. 1-Pyrenecarboxylic acid immobilized with NH_2 magnetic beads reacted with recombinant MLP–PG1 and MLP–GR3 in the buffer with different pH (5.6, 7.2, and 8.8) at 4°C for 4 hr. After washing eight times by magnetic separation, the supernatant from the eighth wash was collected. The buffer and sample buffer for SDS-PAGE were added to the beads, and the solution was boiled at 98°C for 5 min. After magnetic separation, the supernatant was collected as heat elution. The samples were subjected to SDS-PAGE, and bands were detected by silver staining.

5. Statistical analysis

One-way analysis of variance with Tukey's *post-hoc* test was applied to determine significant differences among treatments using RStudio software version 2023.03.0+386 (http://www.rstudio.com). Asterisks in figures indicate significant differences (*, $p<0.05$; **, $p<0.01$: ****, $p<0.0001$).

Results

1. Detection of MLPs in xylem and phloem sap

Xylem and phloem sap were collected from *C. pepo* ssp. *ovifera* cv. ST and *pepo* cv. BT plants to detect MLPs in vascular bundles. The samples were subjected to SDS-PAGE, and bands were detected by CBB staining and western blot analysis using anti-MLP antibodies. CBB staining detected three bands (approximately >17, >28, and >75 kDa) in the xylem sap of *C. pepo* ssp. *ovifera* cv. ST and *pepo* cv. BT plants (Fig. 1A). After the reaction with the anti-MLP–PG1 antibody, the bands at approximately 17 kDa in the xylem sap from *C. pepo* ssp. *ovifera* cv. ST plants showed a higher intensity than *C. pepo* ssp. *pepo* cv. BT plants (Fig. 1A). In contrast, after the reaction with the anti-MLP–GR3 antibody, the bands at approximately 17 kDa in the xylem sap from *C. pepo* ssp. *ovifera* cv. ST plants showed an intensity equivalent to that of *C. pepo* ssp. *pepo* cv. BT plants (Fig. 1A).

After reaction with anti-MLP–PG1 and anti-MLP–GR3 antibodies, the bands at approximately 17 kDa in the phloem sap from *C. pepo* ssp. *ovifera* cv. ST plants showed an intensity equivalent to that of *C. pepo* ssp. *pepo* cv. BT plants (Fig. 1B).

2. Binding of MLPs with pyrene

1-Pyrenecarboxylic acid was activated by *N*-hydroxysuccinimide and immobilized using NH_2 magnetic beads (Fig. 2A). After the reaction with recombinant MLP–PG1 and MLP–GR3 at pH 5.6, 7.2, and 8.8, magnetic separation was performed, and the supernatant was collected as wash fraction samples. The precipitation was boiled, and magnetic separation was performed. The supernatant was collected as heat elution samples. Xylem and phloem sap show approximately pH 6.0 and 8.0, respectively. In this study, we set slightly higher acidity and basicity to emphasize the pH effects. A band at approximately 15 kDa was detected in the heat elution samples of MLP–PG1 and MLP–GR3 at pH 5.6 (Fig. 2B). The bands detected in the heat elution samples of MLP–PG1 and MLP–GR3 were similar to those in the input samples (Fig. S1). In contrast, the bands were hardly detected in the heat elution samples of MLP–PG1 and MLP–GR3 at pH 7.2 and 8.8 (Fig. 2B). The band was not detected in wash samples of MLP–PG1 and MLP–GR3 under the investigated pH conditions (Fig. S1).

When MLPs are incubated with pyrene, pyrene solubility is promoted, and fluorescence increases.[22] In this study, we applied this principle to investigate suitable pH conditions for the binding of MLPs to pyrene (Fig. 3A). Recombinant MLP–PG1 and MLP–GR3 were incubated without pyrene, and almost no fluorescence was observed under the investigated pH conditions (Fig. 3B). Pyrene (10 and 20 μM) incubated with MLP–PG1 at pH 5.6 showed significantly higher fluorescence than at pH 7.2 and 8.8 (Fig. 3B). Ten micromolar of pyrene incubated with MLP–GR3 showed similar fluorescence at the investigated pH

conditions, and 20 μM pyrene incubated with MLP–GR3 at pH 5.6 and 8.8 showed significantly higher fluorescence than at pH 7.2 (Fig. 3B). The pyrene fluorescence incubated without MLPs did not show the pH effects (Fig. S2).

Discussion

In the present study, MLPs were found to tend to bind to hydrophobic organic pollutants with a high affinity under acidic conditions. Although MLP–PG1 and MLP–GR3 were detected in the xylem (acidity) and phloem (basicity) sap (Fig. 1), respectively, both MLPs showed remarkably higher affinity for pyrene under acidic conditions (Fig. 2B). This highlights the importance of xylem vessels as the main transport pathway for hydrophobic organic pollutants through the binding of MLPs.

The effect of temperature, as an environmental factor, on MLP homologs has been extensively studied.[25–27] AsPR-1 and AsPR-2 from the Chinese medicinal plant *Angelica sinensis* exhibit the highest RNase activity at 50°C.[29] Our previous study showed that high temperatures induced the expression of *MLP* genes and promoted the uptake of a hydrophobic organic pollutant in *C. pepo*.[28] This suggests that environmental factors control the uptake of hydrophobic organic pollutants through the regulation of MLPs.

Previous studies have shown that pH regulates the activity of MLP homologs. MtPhBP shows a higher binding affinity toward gibberellic acid under acidity (pH 5.5) than under neutrality (pH 7.4).[30] CaPR-10 and JcPR-10 exhibit the highest RNase and DNase activities, respectively, under acidic conditions.[30,31] In contrast, Gly m 4l exhibited the highest RNase activity in acidic and neutral regions.[33] These results indicate that MLP homologs have an optimum pH for their high activity, which is related to their biological functions. Xylem sap is acidic, and MLP–PG1 and MLP–GR3 are clearly shown to bind to hydrophobic organic pollutants in xylem vessels, but not in phloem vessels.

In contrast, MLPs were detected in phloem sap, which is basic (Fig. 1B), although MLPs may not show binding activity toward hydrophobic organic pollutants. These results raise two possibilities for the biological function of MLPs in phloem vessels: the transport of other compounds and signal transduction by MLPs. MLPs bind to various hydrophobic compounds such as long-chain fatty acids and steroids.[21,23] This suggests that MLPs bind to and transport hydrophobic compounds in the phloem sap other than hydrophobic organic pollutants. Since phloem vessels are a pathway for systemic acquired resistance (SAR) signals,

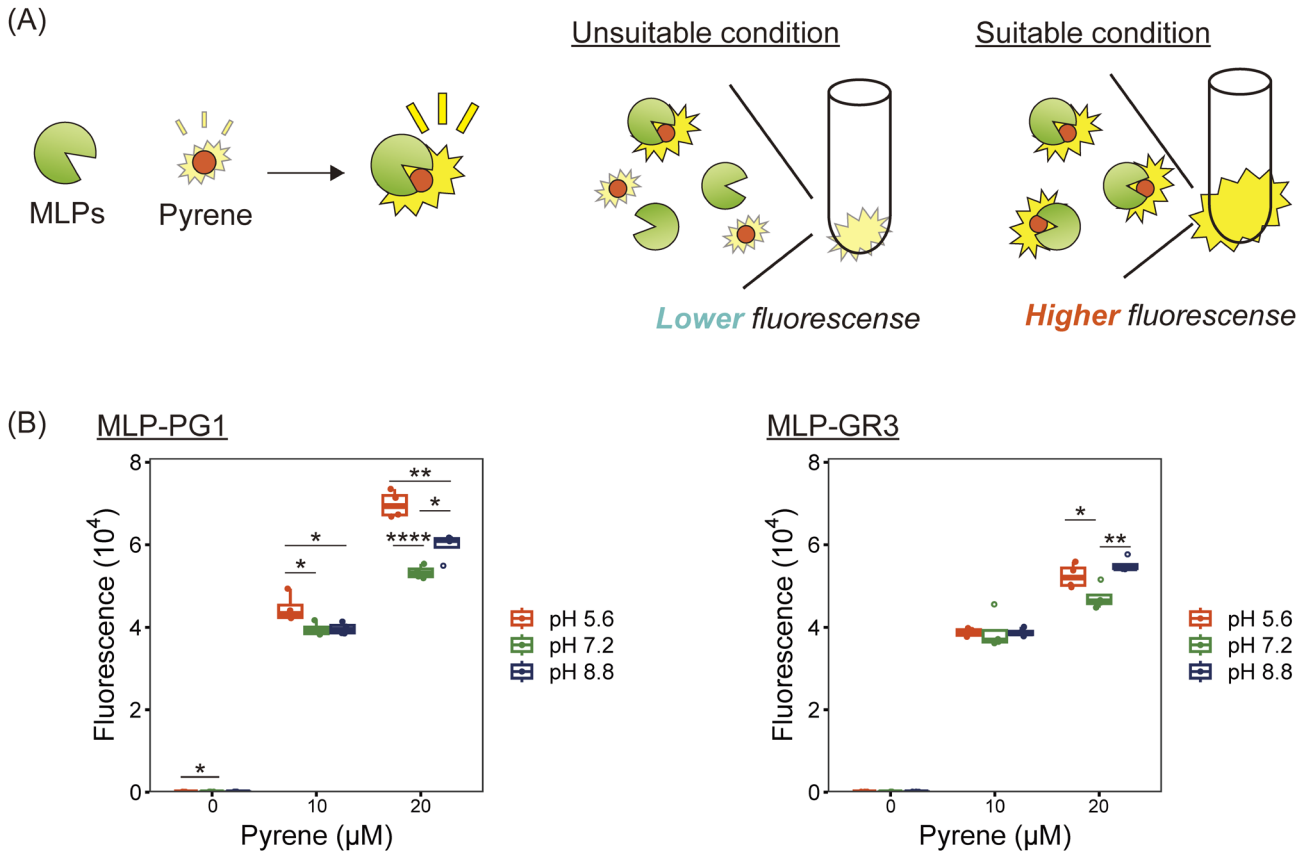

Fig. 3. Fluorescence of pyrene mixed with major latex-like proteins (MLPs) increased under acidic condition. (A) Increase in the fluorescence of pyrene through the binding of MLPs to pyrene. (B) Pyrene fluorescence assay mixed with recombinant MLPs. Recombinant MLP–PG1 and MLP–GR3 (0.2 mg/mL) diluted in the buffer with different pH (5.6, 7.2, and 8.8) was mixed with 0, 10, or 20 μM pyrene dissolved in dimethyl sulfoxide at room temperature, and the pyrene fluorescence was measured.

MLPs are thought to be involved in SAR and transduce pathogen attack signals to other organs.[24,33]

8-Aniline-1-naphthalene sulfonic acid (ANS) displacement assay was employed to identify the hydrophobic ligands of the protein.[35] ANS binds to the hydrophobic cavity of proteins and increases the fluorescence. Upon the addition of ligand candidates, the fluorescence decreases, and the compound is identified as the ligand. This principle has been applied to the identification of ligands of MLP homologs, and the binding affinity of MLP homologs to ligands has been quantified. Previous studies have identified fatty acids, flavonoids, and steroids as ligands of MLP homologs.[35,36] The binding affinity of VrCSBP, an MLP homolog, with natural and synthesized cytokinins was calculated using the ANS displacement assay.[38] These results indicated that the fluorescent compound displacement assay can be applied to MLP homologs. Because MLP–PG1 and MLP–GR3 bind to pyrene, a fluorescent hydrophobic organic pollutant,[22] we investigated the binding affinity of MLPs with pyrene through the detection of pyrene fluorescence. The pyrene incubated with MLP–PG1 under acidic conditions showed the highest fluorescence at 10 and 20 μM pyrene (Fig. 3B). In MLP-GR3, pH conditions did not influence the pyrene fluorescence at 10 μM pyrene, and acidic conditions showed higher fluorescence than neutral conditions and similar fluorescence with basic conditions at 20 μM pyrene. These results suggest that acidic conditions have less effect on the binding affinity of MLP-GR3 with pyrene. One of the reasons for this is the high binding affinity of MLP–GR3 for pyrene. MLP–GR3 possesses more hydrophobic amino acids than MLP–PG1,[11] suggesting that the effect of pH on the binding of MLP–GR3 to pyrene is limited by the detection of pyrene fluorescence.

In conclusion, we have advanced our understanding of the mechanisms underlying MLPs binding to hydrophobic organic pollutants. We detected MLPs in the xylem and phloem sap (Fig. 1); however, MLPs showed binding activity toward pyrene under acidic conditions (Fig. 2B). Because xylem and phloem sap show acidity and basicity, respectively, xylem vessels are the main pathways for the transport of hydrophobic organic pollutants by MLPs in the Cucurbitaceae family. Depending on the pH of the rhizosphere, the uptake of hydrophobic organic pollutants through MLPs can be changed.

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Conflicts of interest

The authors declare no competing interests.

Author contribution

KF designed the research; KF, CS, MC, and HI performed the experiments; KF and HI provided the materials; KF analyzed the data; KF and HI wrote the manuscript, which was revised and approved by all authors.

Electronic supplementary materials

The online version of this article contains supplementary materials (Fig. S1, S2), which are available at https://www.jstage.jst.go.jp/browse/jpestics/.

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