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Original Article

High temperatures promote the uptake of hydrophobic pollutants by *Cucurbita pepo* via altered gene expression levels of major latex-like proteins

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Cucurbitaceae family members are accumulators of hydrophobic pollutants. Such pollutants have been detected in cucurbits at levels above the maximum residue limit. Since major latex-like proteins (MLPs) are involved in hydrophobic pollutant uptake, changes in MLP expression can increase or decrease contamination. MLP expression levels were altered in the roots of *Cucurbita pepo* 'Magda', and MLP-PG1 was detected in the xylem sap of Magda when cultivated at a high temperature (35°C). Day length also influenced MLP expression levels but only induced minor changes in the amount of MLPs. The concentration of pyrene, a hydrophobic pollutant, significantly increased with increasing MLP levels in the xylem sap of Magda when cultivated at 35°C. Thus, high temperatures promote the pollution of cucurbits by hydrophobic pollutants. These results can be used to develop novel techniques to reduce crop contamination and establish efficient phytoremediation.

Keywords: cucurbitaceae family, high temperature, hydrophobic pollutant, major latex-like protein, xylem sap.

Electronic supplementary materials: The online version of this article contains supplementary materials (Supplemental Table S1), which are available at <http://www.jstage.jst.go.jp/browse/jpestics/>.

Introduction

Cucurbits, including cucumbers, pumpkins, and zucchini, are known to be high accumulators of hydrophobic pollutants, such as the insecticides dieldrin,¹⁾ dichlorodiphenyltrichloroethane (DDT) and its metabolite dichlorodiphenyldichloroethylene (DDE),²⁾ chlordane,³⁾ and heptachlor and its metabolite heptachlor epoxide.⁴⁾ Dieldrin, DDT, chlordane, and heptachlor are regarded as persistent organic pollutants (POPs) and are immunotoxic and carcinogenic to wildlife and human beings. Although these insecticides have not been used in the cultivation of cucurbit crops since the 1970s, they are sometimes detected in their fruits. For example, pollutants, such as dieldrin and heptachlor, were found to have remained in field conditions without chemical and biological degradation for more than 40 years.¹⁾ Once crops have been contaminated with insecticide

pollutants at concentrations over their maximum residue limits, all harvests from the same fields of the contaminated crops are collected from the market and discarded. This disposal has severe monetary and reputational consequences for the farmers. Therefore, the cultivation of crops with pollutant levels below the maximum residue limit is important for farmers as well as for consumers. Because POPs have relatively high hydrophobicity ($\log K_{ow} > 4$), plants generally cannot take them up into their shoots (and fruits), although they can accumulate them in the roots.^{5,6)} The molecular mechanisms underlying the remarkable uptake of hydrophobic pollutants by the Cucurbitaceae family have not been clarified, although it has been suggested that the xylem vessel serves as the route *via* which hydrophobic pollutants are transported from the roots to the shoots⁴⁾ and that protein-like materials in the xylem sap are responsible for this transport.⁷⁾ In 2013, it was revealed that major latex-like proteins (MLPs) are involved in the accumulation of hydrophobic pollutants, known as polychlorinated biphenyls (PCBs), in zucchini (*Cucurbita pepo*) by aiding their transport from the roots to the shoots *via* xylem sap.⁸⁾ Three MLP genes (*MLP-PG1*, *MLP-GR1*, and *MLP-GR3*) were mainly expressed in the roots, and the MLPs in the roots and xylem sap bound to the PCBs.^{8,9)} MLP-PG1 and MLP-GR3 apparently serve different functions, probably including defense responses to pathogens, as indicated by their different levels of gene expression and protein accumu-

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lation.⁹⁾ Recombinant MLPs also bound to 17 β -estradiol, 4-*t*-octylphenol, and dieldrin.⁹⁾ In fact, zucchini plants have been reported to accumulate PCBs^{10,11)} and dieldrin^{1,12)} in their shoots when cultivated in contaminated soil. Many plant species have MLP genes in their genomes: for example *Cucumis sativus* and *Arabidopsis thaliana* genomes contain 38 and 25 genes, respectively.¹³⁾

MLPs and pathogenesis-related proteins of class 10 (PR-10) are members of the Bet v1 family, which is a pollen allergen. These proteins have similar 3D structures that contain an internal hydrophobic cavity. This structure is responsible for ligand binding: birch Bet v1 has been shown to bind to the brassinosteroid-like compound deoxycholate,¹⁴⁾ the secondary plant metabolite naringenin, and the plant hormone kinetin¹⁵⁾; and yellow lupine PR-10 has been shown to bind to the synthetic cytokinin *N,N'*-diphenylurea¹⁶⁾ and the plant hormone trans-zeatin.¹⁷⁾ Thus, the binding of hydrophobic pollutants to *C. pepo* MLPs seems to be caused by its cavity, and the possession of MLPs is responsible for the contamination of zucchini plants. Numerous reports have demonstrated the changes in the expression level of MLP genes in response to biotic and abiotic stresses. In *A. thaliana*, the MLP type 3 gene was induced by the inoculation of *Alternaria brassicicola*.¹⁸⁾ The GhMLP and GhMLP28 genes were expressed in response to the toxins of *Verticillium dahliae* and the inoculation of *V. dahliae*.^{19,20)} The HMLX56 gene from the mulberry was expressed as a result of the treatment of *Pseudomonas syringae*.²¹⁾ Plant hormones, such as brassinosteroids, abscisic acid (ABA), methyl jasmonate, and salicylic acid, were shown to alter the MLP gene expression in cucumber²²⁾ and grape (*Vitis vinifera*) plants.¹³⁾ Abiotic stresses, such as cold, heat, drought, salt, and darkness, also influence the expression of MLP43 in *A. thaliana*,²³⁾ MLP151 in Korean ginseng (*Panax ginseng*),²⁴⁾ and VvMLPs in *V. vinifera*.¹³⁾ These reports suggest that environmental stresses can alter the expression levels of MLP genes and thus influence the contaminant levels in plants.

In this study, we (1) determined the expression levels of two MLP genes, MLP-PG1 and MLP-GR3, and (2) identified the MLPs in the roots and xylem sap of two zucchini cultivars, 'Patty Green' (PG) and 'Magda' (MG), under different cultivation conditions (temperature and day length). Since MLP-PG1 and MLP-GR1 showed high amino acid identity (> 99%) in 156 amino acids, MLP-PG1 and MLP-GR3 were selected to determine the gene expression characteristics. PG and MG cultivars have different accumulation abilities for DDE,²⁵⁾ dioxins, and PCBs.^{9,11)} Furthermore, we investigated the changes in the pyrene contamination level in zucchini plants incubated in experimentally polluted soil. Pyrene, which is a hydrophobic pollutant, was used as a model POP compound because it is highly accumulated in the shoots of zucchini plants with high POP accumulation ability²⁶⁾ and is simply quantified using its fluorescence.

Materials and Methods

1. Effect of cultivation temperature and day length on MLPs and associated genes in *C. pepo*

1.1. Plant materials and incubation conditions

Cucurbita pepo L. ssp. *ovifera* PG and ssp. *pepo* MG were purchased from Johnny's Selected Seeds (Albion, ME, USA). After the seed coat was peeled off, the seeds were immersed in tap water overnight at 4°C. Plastic pots (top diameter: 13.5 cm; height: 11.5 cm; bottom diameter: 9.5 cm) were filled with ~400 g of commercial soil (Hyponex Japan Corp., Ltd., Osaka, Japan). Three seeds were sown per pot, and one healthy seedling was selected (the others removed) after incubation for 1 week at 25°C in a plant incubator under a photoperiod of 16 hr light/8 hr dark. The selected seedlings were incubated for another 2 weeks. Seedlings were then subjected to different cultivation temperatures and day lengths, 15°C and 35°C and 8 hr light/16 hr dark and 12 hr light/12 hr dark, respectively, for 1 week. All plants were incubated for 4 weeks. Stem solution (sap) samples from each plant were tested using pH test papers. When the samples were shown to be acidic (pH ~5.6), xylem sap (~500 μ L) was collected in a 1.5 mL tube from a cut made just below the cotyledon. Roots were washed with tap water after the xylem sap collection and stored at -80°C.

1.2. Expression analyses of MLP genes in the roots

Root samples were ground in liquid nitrogen using a mortar and pestle. Total RNA was extracted using TRIzol Reagent (Thermo Fisher Scientific Inc., Waltham, MA, USA), and a ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd., Osaka, Japan) was used to synthesize the cDNA according to the manufacturer's instructions. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was conducted using a Thunderbird SYBR qPCR Mix (Toyobo) with the primers for *actin*, MLP-PG1, and MLP-GR3 genes (Table S1) under the following conditions: 1 min at 95°C; 40 cycles of 15 sec at 95°C and 30 sec at 60°C; 5 sec at 95°C; and 1 min at 65°C (Light Cycler 480 II, Roche Applied Science, Indianapolis, IN, USA). The relative expression levels of the MLP-PG1 and MLP-GR3 genes were calculated using the $\Delta\Delta C_T$ method and revised using the *actin* gene expression level.

1.3. Western blotting of MLPs in the roots and xylem sap

Root proteins were extracted using the buffer [50 mM potassium phosphate buffer (pH 7.0), 200 mM sodium chloride, 10 mM EDTA, 0.1% (v/v) Triton-X100, 0.1% (v/v) *N*-lauroylsarcosine sodium salt] including 10 mM 2-mercaptoethanol. After vigorous mixing, the mixture was centrifuged at 20,700 $\times g$ for 15 min at 4°C, and the supernatant was collected. Proteins in the root extracts were quantified using the Bradford method.²⁷⁾ Samples for SDS-PAGE were prepared by adding a sample buffer solution containing a reducing reagent (Nacalai Tesque, Inc., Kyoto, Japan) to 10 μ g of root proteins and 10 μ L of xylem sap and then heating them in a water bath at 98°C for 5 min. Proteins were separated using a 15% polyacrylamide gel and transferred to a PVDF-Plus membrane (Maine Manufacturing, LLC,

Sanford, ME, USA) to detect the MLPs using anti-MLP-PG1 and anti-MLP-GR3 antibodies.⁹⁾

2. Uptake of pyrene by *C. pepo* incubated at different cultivation temperatures

2.1. Plant materials and incubation conditions

Purchased soil (Hyponex) was autoclaved for 15 min at 120°C, dried, and then mixed (1 kg) with acetone (500 mL) containing dissolved pyrene (2.5 mM). After the acetone had completely evaporated, 180 g of the pyrene-contaminated soil was transferred to a glass jar (top and bottom diameter: 6 cm; central diameter: 8 cm; height: 13 cm). Seeds of the MG cultivar were peeled and soaked in tap water overnight at 4°C. Two seeds were sown per jar, and one healthy seedling was selected (the other removed) after incubation for 1 week. Plants were incubated for another 3 weeks at 25°C under a photoperiod of 16 hr light/8 hr dark. Plants were then divided into three groups, each of which was incubated at either 15°C, 25°C, and 35°C, for another week.

2.2. Quantification of pyrene levels in the xylem sap

Xylem sap was collected as previously described. The fluorescence from pyrene was measured in the xylem sap containing 50% dimethyl sulfoxide (DMSO) at 330 nm excitation and 390 nm emission (Microplate Reader SH-9000, Corona Electric Co., Ltd., Hitachinaka, Ibaraki, Japan).

2.3. Western blotting of MLPs in the xylem sap

MLPs in the xylem sap were detected using western blotting as previously described. The intensities of bands corresponding to MLP-PG1 and MLP-GR3 were measured using ImageJ.²⁸⁾

3. Statistical analysis

Student's *t*-test was used to demonstrate significant differences in the gene expression of MLPs in roots, protein levels in xylem sap, and pyrene concentrations. Differences with *p* values less than 0.05 were considered significant.

Results

1. Responses of the PG and MG cultivars to different cultivation temperatures

In the PG cultivar, compared to incubation at 25°C, the incubation at 15°C resulted in significant but slightly lower and higher changes in the expression levels of the *MLP-PG1* and *MLP-GR3* genes, respectively (Fig. 1A). In contrast, both genes were significantly induced at 35°C, at expression levels of more than twice those induced at 25°C. Two bands were observed around 17 kDa after the reaction with the anti-MLP-PG1 antibody (Fig. 1B). The accumulation of MLP-PG1 in the roots showed the same pattern as the *MLP-PG1* gene expression, i.e., a lower accumulation at 15°C and a higher accumulation at 35°C compared to that at 25°C. Additionally, two bands were detected when the anti-MLP-GR3 antibody was used. However, the band intensity of MLP-GR3 did not increase at 15°C but showed a slight increase at 35°C. No MLPs were detected in the xylem sap of the PG cultivar at any incubation temperature.

The expression of the *MLP-PG1* gene in the MG cultivar at

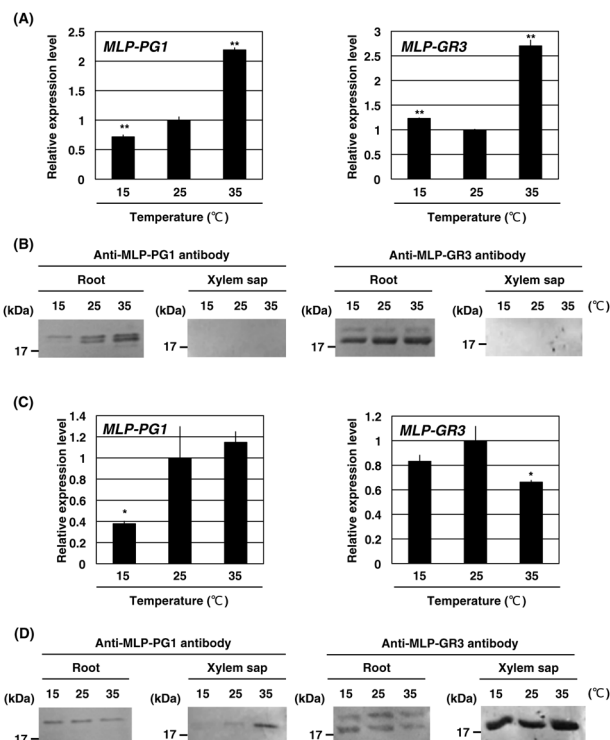


Fig. 1. Expression analysis of major latex-like protein (MLP) genes in the roots (A and C) and accumulation of MLPs in the roots and xylem sap (B and D) of *Cucurbita pepo* cultivars Patty Green (A and B) and Magda (C and D) under different cultivation temperatures. After cultivation for 3 weeks in a plastic pot supplemented with soil, plants were incubated for 1 week at 15°C, 25°C, and 35°C. Total RNA was extracted from the roots and subjected to qRT-PCR with primers to detect the *MLP-PG1* and *MLP-GR3* genes. Root proteins were extracted, and xylem sap was collected from cut stems. These samples were subjected to SDS-PAGE, and the MLPs were detected using the corresponding antibodies. Error bars indicate standard deviations ($n=3$). Asterisks indicate significant differences compared to the treatment at 25°C (* $p<0.05$; ** $p<0.01$; Student's *t*-test).

15°C was suppressed to half that at 25°C, whereas no significant changes in expression level were observed at 35°C (Fig. 1C). The expression of the *MLP-GR3* gene was significantly suppressed at 35°C. Both MLPs in the roots were accumulated less at 35°C than at other temperatures (Fig. 1D). Two bands were detected by the anti-MLP-GR3 antibody, but one band was detected in the xylem sap. The xylem sap of the MG cultivar incubated at 35°C contained a higher amount of MLP-PG1 than that at 15°C or 25°C. In contrast, all temperature conditions showed an accumulation of MLP-GR3, and a slight increase was observed at 35°C.

2. Responses of the PG and MG cultivars to different day lengths

In the PG cultivar, both short and long days significantly suppressed the expression of the *MLP-PG1* gene (Fig. 2A). In contrast, suppression of the *MLP-GR3* gene was dependent on the day length. The *MLP-PG1* in the roots showed higher accumulations and the *MLP-GR3* showed lower accumulations on longer days (Fig. 2B). Two bands were detected in the roots when the

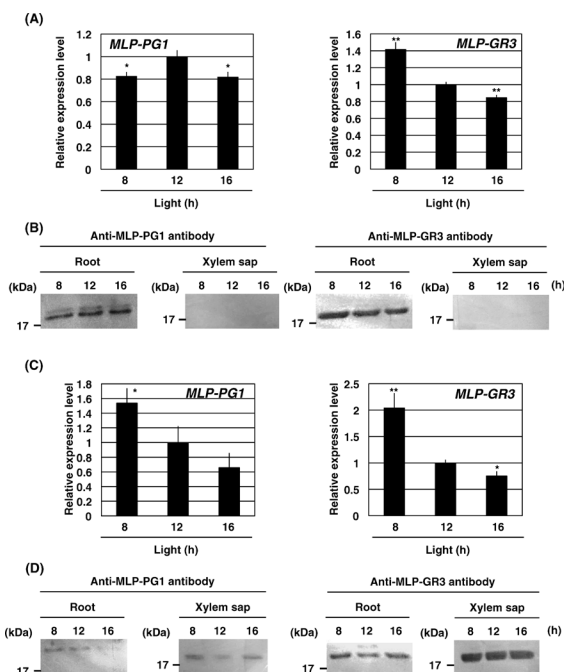


Fig. 2. Expression analysis of major latex-like protein (MLP) genes in the roots (A and C) and accumulation of MLPs in the roots and xylem sap (B and D) of the *Cucurbita pepo* cultivars Patty Green (A and B) and Magda (C and D) under different day length treatments. After cultivation for 3 weeks in a plastic pot supplemented with soil, plants were incubated for 1 week under light/dark cycles of 8 hr/16 hr, 12 hr/12 hr, and 16 hr/8 hr. Total RNA was extracted from the roots and subjected to qRT-PCR with primers to detect the *MLP-PG1* and *MLP-GR3* genes. Root proteins were extracted, and xylem sap was collected from cut stems. These samples were subjected to SDS-PAGE, and the MLPs were detected using the corresponding antibodies. Error bars indicate standard deviations ($n=3$). Asterisks indicate significant differences compared to the treatment under a light/dark cycle of 12 hr/12 hr (* $p<0.05$; ** $p<0.01$; Student's t -test).

anti-MLP-PG1 antibody was used. As mentioned in the experiments at different temperatures, the PG cultivar did not accumulate either of the MLPs in the xylem sap.

In the MG cultivar, suppression of the expression of both *MLP* genes was dependent on the day length (Fig. 2C). The amount of *MLP-PG1* in the roots of the MG cultivar was dependent on the expression levels of the *MLP* genes, whereby the 8 hr day length showed a higher accumulation than did the 16 hr day length (Fig. 2D). In contrast, *MLP-GR3* was accumulated more in the roots under the 8 hr and 16 hr day length treatments than under the 12 hr day length treatment. The MLPs in the xylem sap were constantly accumulated in all day length treatments.

3. Pyrene accumulation in the xylem sap of the MG cultivar

To confirm whether changes in the cultivation temperature can induce the accumulation of hydrophobic pollutants in the shoots, MG was cultivated in soil containing the hydrophobic pollutant pyrene under different temperature conditions. The *MLP-PG1* levels in the xylem sap significantly increased with the increase in temperature (Fig. 3A and B). In contrast, *MLP-GR3* was constantly accumulated under all temperature condi-

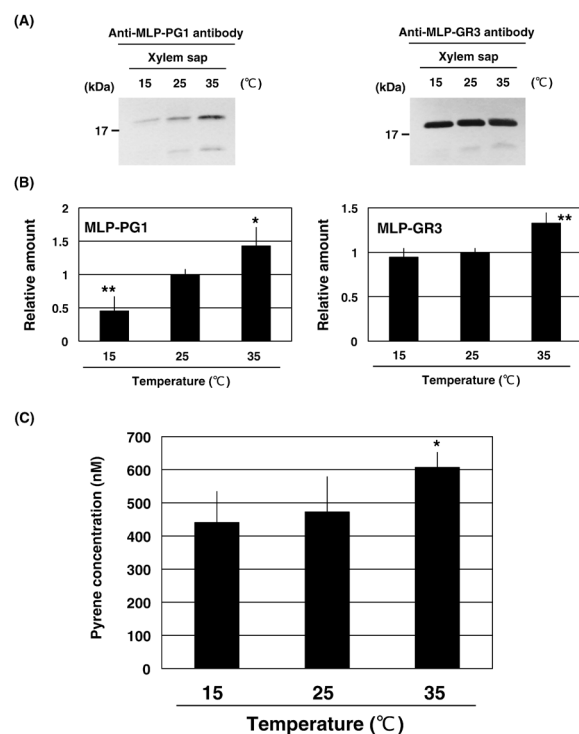


Fig. 3. The amount of major latex-like proteins (MLPs) and the concentration of pyrene in the xylem sap of *Cucurbita pepo* 'Magda' under different cultivation temperatures. After cultivation for 3 weeks at 25°C in a glass jar supplemented with pyrene-contaminated soil (1.25 mmol/kg), plants were incubated at experimental temperatures of 15°C, 25°C, and 35°C. Xylem sap was collected from cut stems after 1 week of incubation and subjected to SDS-PAGE and western blot analysis. MLPs were detected using the corresponding antibodies (A). Band intensity corresponding to the MLPs at around 17 kDa was measured using ImageJ (B). Error bars indicate standard deviations ($n=5$). Asterisks indicate significant differences compared to the treatment at 25°C (* $p<0.05$; ** $p<0.01$; Student's t -test). The fluorescence from pyrene in the xylem sap was measured at 330 nm excitation and 390 nm emission (C). Error bars indicate standard deviations ($n=5-6$). Asterisks indicate significant differences compared to the treatment at 25°C (* $p<0.05$; Student's t -test).

tions but was significantly accumulated when the plants were incubated at 35°C. Incubation at 35°C resulted in a significant increase in the concentration of pyrene in the xylem sap (Fig. 3C). In contrast, the pyrene concentration in the xylem sap of plants was not significantly different between the incubation treatments at 15°C and 25°C.

Discussion

In this study, we examined the influence of environmental conditions, such as cultivation temperature and day length, on the contamination of two zucchini cultivars by hydrophobic pollutants. MLPs are reportedly responsible for the contamination of PCBs in zucchini plants⁸⁾ because MLPs are detected in the roots and xylem sap of zucchini plants and bind to PCB.⁹⁾ Xylem vessels have been shown to be pathways for pollutants as well as nutrients that are taken up and transported from the roots to the upper parts of plants *via* the xylem sap.^{4,9,29)} Hydrophobic

compounds, such as PCBs, are only slightly soluble in water, and MLPs have been shown to play a role in dissolving them in the xylem sap.⁸⁾ There is, therefore, a significant positive correlation between the amount of MLPs in xylem sap and the bioaccumulation factors of PCB in the aerial parts of plants.⁹⁾ Interestingly, the amounts of each PCB congener in the aerial parts and xylem sap are very different despite their structures being similar.^{9,11)} These results suggest that the amount of MLPs in xylem sap determines the level of contamination, and the variety of MLPs determines the kinds of pollutants in the plants. Thus, a change in the amount of MLPs synthesized in the roots can alter the contamination levels and the variety of pollutants. If environmental conditions influence the gene expression of MLPs, the contamination level and the variety of pollutants in zucchini plants will be affected.

In the MG cultivar, incubation temperatures of 25°C and 35°C induced higher expression levels of the *MLP-PG1* gene than did those at 15°C (Fig. 1C). However, the high accumulation of the corresponding protein was not observed in the roots at 25°C and 35°C (Fig. 1D). In contrast, a high amount of MLP-PG1 at 35°C was detected in xylem sap. These results suggest that a portion of the MLP-PG1 in the roots is translocated to the xylem sap. Although the expression level of the *MLP-GR3* gene was suppressed at 35°C, the protein level in xylem sap did not show lower accumulation levels (Fig. 1C and D). These results indicate that the expression levels of *MLP* genes and the accumulation levels of MLPs are not always correlated. This tendency was also observed when day length was altered (Fig. 2C and D). These findings suggest that the stability of MLPs or the excretion of MLPs to the outside of cells (or both) is also influenced by environmental conditions.

Since high temperatures increased the amount of MLPs in xylem sap, MG was cultivated in the contaminated soil with the polyaromatic hydrocarbon pyrene. Pyrene is a hydrophobic pollutant from the exhausted gas from cars and incinerators. The pyrene concentration in xylem sap at 35°C was higher than that at 15°C and 25°C (Fig. 3C). This may be due to the higher MLP amounts in the xylem sap at 35°C than at 25°C (Fig. 3B). The PG cultivar did not show an accumulation of MLPs in xylem sap under different temperatures and day lengths, although the expression of *MLP* genes did vary (Figs. 1B and 2B). These results imply that the contamination of the PG cultivar by hydrophobic pollutants does not occur under the observed changes in temperature and day length. Differences in day length did, however, alter the expression of *MLP* genes in the roots (Fig. 2A and C). This result suggests that information on day length recognized by the leaves is transmitted to the roots. Light signals have been reported to influence the expression of the *xylem sap protein 30* gene in the roots of cucumber plants.³⁰⁾ When *P. ginseng* was treated with high light intensity, the *MLP151* gene was expressed.²⁴⁾ In contrast, a dark condition decreased the expression. Light and dark treatments can, therefore, influence the contamination levels of hydrophobic pollutants in zucchini plants through the change in the expression levels of *MLP*

genes. The volume of xylem sap (transpiration volume) also influences the accumulation of POPs in the shoots. Namiki *et al.* reported that the culture conditions, such as the addition of ABA in hydroponic medium and dark treatment, decreased the volume of xylem sap, followed by the decreased accumulation of β -1,2,3,4,5,6-hexachlorocyclohexane and dieldrin.³¹⁾ The changes in cultivation temperature and day length may contribute to the contamination levels in zucchini plants *via* the change in xylem sap volume.

In conclusion, high temperatures contribute to the high pollution of cucurbit plants by hydrophobic pollutants through the expression of *MLP* genes in the roots, followed by an accumulation of MLPs in the xylem sap. MLP-PG1 is thought to be responsible for inducing pollutant accumulation, and MLP-GR3 is related to the maintenance of basal levels of pollutants. Global warming is predicted to increase temperatures, both locally and globally. This phenomenon will influence the contamination levels of pollutants in crops. Therefore, to reduce the contamination level of pollutants, it is important to cultivate crops belonging to the Cucurbitaceae family under moderate temperatures, especially in the early stages of growth, since the expression levels of *MLP* genes at an early stage were higher than those at a later stage.⁹⁾ This study furthers our understanding of the effects of environmental conditions on crop contamination and provides valuable reference data for the control of contamination levels for efficient phytoremediation and the cultivation of safe crops.

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