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Title: Transport enhancement of hydrophobic pollutants by the expression of zucchini major latex-like protein genes in tobacco plants

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Abstract

The environmental spread of hydrophobic pollutants has been receiving attention because of specific characteristics of these compounds that make them resistant to degradation, thus causing various toxic effects on humans as a result of their bioaccumulation. Here, we report the role of zucchini major-latex like proteins (MLPs) on the accumulation of hydrophobic pollutants, as consumption of contaminated crops is one of the main routes for accumulation. Transgenic tobacco plants expressing an aryl hydrocarbon receptor (*AhR*) gene with a β-glucuronidase (GUS) inducible expression system were transformed with one of the three zucchini *MLP* genes (*PG1*, *GR1,* and *GR3*). MLP transgenic plants showed a significant increase in the fold induction of GUS activity compared to the parental AhR tobacco plants when one of the most toxic polychlorinated biphenyl (PCB) congeners, 3,3',4,4',5-pentachlorobiphenyl (CB126), was applied. GUS activity was detected in both aerial parts and roots after treatment with the strong carcinogen 3-methylcholanthrene. Phenotypic changes in the MLP tobacco during incubation with CB126 were also observed. The MLP transgenic plant PG1 responded to treatment with 0.32 nM CB126, whereas vector control plants significantly induced GUS activity at 200 nM CB126. Moreover, GUS activities in the MLP plants treated with other PCB congeners were significantly higher than those in the plants given the mock treatment. As GUS activities in the aerial parts of the plants were significantly correlated with the accumulation level of PCBs, these results strongly suggest that zucchini MLPs are related to the translocation of hydrophobic pollutants from the roots to the aerial parts through their binding affinity.

Keywords:

Aryl hydrocarbon receptor, major latex-like protein, 3-methylcholanthrene, persistent organic pollutant, polychlorinated biphenyl, transgenic tobacco plant

Abbreviations: AhR, aryl hydrocarbon receptor; BCF, bioconcentration factor; CB118, 2,3',4,4',5-pentachlorobiphenyl; CB126, 3,3',4,4',5-pentachlorobiphenyl; CB77, 3,3',4,4'-tetrachlorobiphenyl; DMSO, dimethyl sulfoxide; GUS, β-glucuronidase; IAA, indole-3-acetic acid; MC, 3-methylcholanthrene; MLP, major-latex like protein; MS, Murashige and Skoog; PCB, polychlorinated

29 biphenyl; POP, persistent organic pollutant; RRP, ripening-related protein; TEF, toxic equivalency
30 factor;
31

1. Introduction

Organic pollution is a major global concern. Of these, persistent organic pollutants (POPs) have been receiving attention as they are anthropogenic compounds with chemical stability and hydrophobicity, making them resistant to environmental degradation. Some compounds belonging to the polychlorinated biphenyls (PCBs) group are widely distributed in the environment. PCBs are used as heat exchange fluids in electric transformers and capacitors and as additives in substances such as paints, flame retardants, household sealants, and light ballasts. They are mainly released into the environment through discarded industrial products; also, they are unintentionally produced during waste combustion. The route of exposure is mainly through the consumption of contaminated crops, fish, and other seafood. Several PCB congeners, known as dioxin-like PCBs, have harmful effects such as immunosuppressive (Harper et al., 1995), obesogenic, and diabetogenic (Langer et al., 2014) effects, as well as the ability to cause hormonal (Plísková et al., 2005) and neurological (Jacobson and Jacobson, 1996) disorders.

Once released into the environment, PCBs and other POPs strongly adsorb to soil particles, especially to soil organic matter, due to their hydrophobicity. Thus, reverse desorption occurs with a very low efficiency, resulting in its low bioavailability. However, higher levels of POPs accumulated in the aerial parts of plants have been detected in some plant species belonging to the Cucurbitaceae family, such as zucchini, winter squash, cucumber, and watermelon (Otani et al., 2007). Moreover, it was shown that zucchini cultivars may differ in terms of their potential to accumulate PCBs in their green parts, and based on this potential, they are then classified as higher and lower accumulators (Matsuo et al., 2011). Evidence showing that high concentrations of PCBs in the aerial part of *Cucurbita pepo* were accumulated as a result of uptake from the roots and translocation to the aboveground parts has been provided (Whitfield et al., 2008). A general factor responsible for the translocation of POPs from the roots to the aerial parts of the cucurbit species is the major latex-like protein (MLP) (Inui et al., 2013; Goto et al., 2019). Since their discovery in opium poppy (Nessler et al., 1990), MLPs have been identified in a number of species such as cotton (Yang et al., 2015), grapevine (Zhang et al., 2018), mulberry (Gai et al., 2017), ginseng (Sun et al., 2010), and

Arabidopsis (Wang et al., 2016). MLPs are members of the major latex protein/ripening-related protein (MLP/RRP) subfamily belonging to the Bet v 1 superfamily and are involved in multiple biological processes in plants such as induction during abiotic (Chen and Dai, 2010; Wang et al., 2016) and biotic stress responses (Yang et al., 2015), plant development (Guo et al., 2011; Litholdo et al., 2016), and fruit ripening (Ruperti et al., 2002; Chruszcz et al., 2013). The similar characteristics of MLPs are determined by the common structure of the internal cavity that selectively binds hydrophobic molecules (Radauer et al., 2008). Additionally, it was shown that zucchini MLPs can bind hydrophobic molecules with aromatic structures such as PCBs and translocate them into upper plant parts (Inui et al., 2013; Goto et al., 2019). Based on this knowledge, it was hypothesized that exogenous expression of zucchini *MLP* genes in transgenic plants can lead to high accumulation of hydrophobic compounds in the aerial parts.

In this study, we present the transport enhancement of hydrophobic pollutants by expressing zucchini *MLP* genes in transgenic tobacco plants. We employed a dioxin bioassay system using transgenic tobacco plants expressing an aryl hydrocarbon receptor (*AhR*) gene to evaluate MLP functions in plants. AhR is a ligand-responsive transcription factor that represents a general intermediate in chemical communication between the host organism and the environment (Fernandez-Salguero et al., 1996; Rowlands and Gustafsson, 1997; Shimizu et al., 2000; Cella and Colonna, 2015). It has already been established that AhR could also bind exogenous and endogenous compounds and thus plays a critical role in cell processes and homeostasis (Denison and Nagy, 2003; Kadow et al., 2011; Mulero-Navarro and Fernandez-Salguero, 2016; Chitrala et al., 2018). AhR-ligand complexes induce gene expression by binding to dioxin-responsive elements on their promoters (Matthews and Gustafsson, 2006; Beischlag et al., 2008). For bioassays analyzing dioxin pollution, transgenic tobacco plants have been developed using a combination of a recombinant *AhR* gene and an inducible reporter β-glucuronidase (*GUS*) gene (Kodama et al., 2007, 2009). It was reported that transgenic *Arabidopsis* and tobacco plants expressing *AhR* gene with a GUS inducible expression system were able to detect 10 and 100 ng/mL 3,3',4,4',5-pentachlorobiphenyl (CB126), respectively, and 1 μg/mL 3-methylcholanthrene (MC) (Gion et al., 2012). By introducing one of the three *MLP* genes cloned from zucchini cultivars into AhR transgenic tobacco plants, the involvement

of MLPs in the high accumulation of hydrophobic pollutants in the aerial parts of plants was revealed. Furthermore, candidates for endogenous ligands of MLPs were also discussed.

2. Materials and Methods

2.1. Production of transgenic tobacco plants expressing the MLP genes

The plasmids pTMLP-PG1-2, pTMLP-GR1-4, and pTMLP-GR3-3 containing *MLP-PG1* (AB753855), *MLP-GR-1* (AB753856), and *MLP-GR3* (AB753857) genes (Inui et al., 2013), respectively, were digested with *Sal*I and *Not*I prior to insertion into the pENTR1A vector (Thermo Fisher Scientific Inc., Waltham, MA, USA) digested by both restriction enzymes. The resulting plasmids were then reacted with the plant expression vector pGWB502Ω (Nakagawa et al., 2007) using LR Clonase Enzyme Mix (Thermo Fisher Scientific Inc.). Finally, pGWB-MLP-PG1, pGWB-MLP-GR1, and pGWB-MLP-GR3 were constructed (Fig. S1). The vector control plasmid pGWB-CC-11 was also constructed without the *MLP* genes. The transgenic tobacco line XD4V-26 expressing recombinant mouse *AhR* gene combined with the GUS inducible expression system was subjected to a second transformation (Fig. S1) (Kodama et al., 2009). Transformation of tobacco plants was carried out as previously described (Kodama et al., 2007). Briefly, each plasmid was introduced into *Rhizobium radiobacter* LBA4404. Leaf discs prepared from XD4V-26 were incubated with recombinant *Rhizobium*. Regenerated shoots (T_0 transgenic plants) resistant to hygromycin were then selected. Plants transformed with pGWB-CC-11, pGWB-MLP-PG1, pGWB-MLP-GR1, and pGWB-MLP-GR3 were named VC, PG1, GR1, and GR3, respectively.

2.2. Selection of transgenic tobacco plants based on expression levels of MLP genes

Total RNA was isolated from the leaf tissues of the T_0 transgenic tobacco plants using TRIzol Reagent (Thermo Fisher Scientific Inc.). Approximately 1 μg of total RNA was used for cDNA synthesis using ReverTra Ace qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd., Osaka,

Japan) according to the manufacturer's instructions. Relative expression levels of the *MLP* genes in the T_0 plants were evaluated by qRT-PCR (Light Cycler 480 II, Roche Applied Science, Indianapolis, IN, USA) using a Thunderbird SYBR qPCR Mix (Toyobo) and specific primer pairs listed in Table S1. PCR was carried out under the following conditions: 1 min at 95°C; 40 cycles of 15 s at 95°C, 30 s at 60°C; 5 s at 95°C, and 1 min at 65°C. The tobacco *actin* gene was amplified as an internal standard under the same conditions. Relative expression levels were calculated using the $\Delta\Delta C_T$ method after confirming specific amplification based on the melting curves. Transgenic plants showing high expression of *MLP* genes were selected, and homozygous T_2 and T_3 seeds were used in subsequent experiments.

2.3. Treatment with MC and PCBs in solid and soil media

T_0 transgenic tobacco plants were incubated on Murashige and Skoog (MS) medium containing 306 nM CB126 dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in the contaminated medium was set to 0.1%. As control, the same concentration of DMSO in MS medium was used. Leaves were subjected to GUS activity measurements. T_2 and T_3 transgenic tobacco seeds were surface-sterilized and incubated on MS medium supplemented with different concentrations of MC or PCBs such as CB126, 3,3',4,4'-tetrachlorobiphenyl (CB77), and 2,3',4,4',5-pentachlorobiphenyl (CB118). Plants were grown at 25°C under a 16/8 h light/dark cycle for 2 weeks.

CB126 was dissolved in acetone, and different concentrations of CB126 were mixed with sterilized and sieved soil at a ratio of 1:2 (v/w). The control soil was mixed with only acetone at the same ratio. Acetone was then completely evaporated. Each well of a 24-well glass plate was filled with 1 g of soil, and 500 µL of tap water was initially added. Afterwards, 4–5 T_3 transgenic tobacco seeds per well were sown. The plates were placed in a clear plastic box with a lid to prevent the seedlings from drying, and the seeds were incubated at 24°C under a 16/8 h light/dark cycle for 2 weeks in a plant incubator.

2.4. GUS assay of transgenic tobacco plants

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145 Aerial parts including stem, cotyledon, and leaves, and roots of the treated seedlings were
146 separated and immediately frozen in liquid nitrogen. Three to five samples per treatment were
147 subjected to the GUS assay as previously described (Kodama et al., 2007). Measurement of the
148 fluorescent signal was performed at 360 nm excitation and 450 nm emission detection using a
149 microplate reader (SH-9000, Corona Electric Co. Ltd., Hitachinaka, Ibaraki, Japan). Total soluble
150 proteins were quantified using the Bradford method (Bradford, 1976).

151

152 *2.5. Statistical analysis*

153

154 Statistical analysis to determine significance was performed using the *t*-test on Microsoft Excel.
155 Grubb's test was also performed to identify the outliers from normal distributions.

156

157 **3. Results and discussion**

158

159 *3.1. Effects of the expressed MLP genes on the induction of GUS activity in transgenic tobacco plants*
160 *treated with CB126*

161

162 It has been reported that MLPs are responsible for the transport of hydrophobic pollutants such
163 as PCBs and dieldrin from the roots to the aerial parts of zucchini plants (Inui et al., 2013; Goto et al.,
164 2019). Therefore, it was expected that expression of recombinant *MLP* genes in transgenic AhR
165 tobacco plants would result in high accumulation of hydrophobic pollutants in their aerial parts. In
166 turn, this would contribute to the high induction of GUS activity through the AhR system (Kodama et
167 al., 2007; Inui et al., 2012). In other words, the function of MLPs to transport pollutants was
168 illustrated using the GUS reporter system. T_0 PG1 and GR3 transgenic tobacco plants showed
169 significant average fold induction of GUS activity compared to VC (Fig. 1). Although GR1 did not
170 show a significant difference, GUS activity was still higher than that in the VC. A previous study
171 clearly showed that high GUS activity was significantly positively correlated with high accumulation

of hydrophobic pollutants in the XD4V-26 line (Inui et al., 2012). Therefore, the high fold induction of GUS activity observed in MLP plants was due to the high transportation of CB126 by MLPs to the aerial parts and its subsequent accumulation.

3.2. Selection of transgenic tobacco plants expressing the MLP genes

In T_0 transgenic tobacco plants, two lines with high expression levels of each *MLP* gene were selected for further analyses (PG1: PG1-13 and PG1-28; GR1: GR1-8 and GR1-17; GR3: GR3-20 and GR3-30) (Fig. S2). Transgenic tobacco lines transformed with the vector control (VC-9 and VC-11) were used as controls. From these T_0 plants, the homozygous hygromycin-resistant T_2 and T_3 progenies were obtained.

3.3. Spatial distribution of GUS activity in MLP transgenic tobacco plants

All tested MLP transgenic plants showed induction of GUS activity in both roots and aerial parts after MC treatment (Figs. 2A and B). The increase was significant in the roots of VC, PG1, and GR3 and in the aerial parts of VC, PG1, and GR3-30-2. GR1 plants tended to show induction, but this was not significant because of its high GUS activity even when MC was not added. Since roots are the most sensitive to POP pollution due to their intensive contact with these compounds from the soil and thus their higher POP accumulation in root cells, roots showed relatively high GUS activities compared to aerial parts. Aerial parts of the MLP plants PG1-28-1, GR1-8-4, GR1-17-4 and GR3-20-1 displayed 2.7, 1.6, 2.0, and 2.8 times higher fold induction of GUS activity than that of the VC11-3 line, respectively (Fig. 2B). These results suggest a crucial role for MLPs in the transfer of MC into the aboveground parts of the plants. In zucchini, hydrophobic compounds such as PCBs, dieldrin, 4-*t*-octylphenol, and 17β-estradiol, are transported to the aerial parts because of their binding to native MLPs (Goto et al., 2019).

Non-treated transgenic plants also showed GUS activity, probably due to the affinity of AhR to endogenous compounds. The binding affinity of AhR to indole-3-acetic acid (IAA) was reported for

AhR transgenic *Arabidopsis* plants incubated on an MS medium supplemented with IAA (Shimazu et al., 2010). Similar observations concerning higher values of GUS basal activity in AhR transgenic *Arabidopsis* have also been reported (Gion et al., 2012). Since a high background GUS activity was observed in the aerial parts of PG1-28-1, GR1-8-4, GR1-17-4, and GR3-20-1, endogenous AhR-binding compounds, such as IAA, may also be transferred from the roots to the aerial parts by recombinant MLPs.

3.4. GUS activity of transgenic tobacco plants in a concentration series of CB126

The dose-dependency toward CB126 of the T_3 progeny of lines PG1-28-1-1, GR1-8-4-1, and GR3-20-1-1 was subjected to further assessment. All the MLP plants grown on different concentrations of CB126 showed a dose-dependent induction of GUS activity and a significant increase at higher doses of CB126 (40 and 200 nM) (Fig. 3). Furthermore, PG1-28-1-1 and GR3-20-1-1 were sensitive to 8 nM CB126, causing a significant increase in GUS activity compared to the DMSO-treated plants. PG1-28-1-1 showed induction even at the lowest concentration of 0.32 nM CB126. Significantly increased GUS activities in AhR transgenic *Arabidopsis* and in tobacco plants treated on MS medium supplemented with 3 and 306 nM CB126, respectively (Gion et al., 2012; Inui et al., 2012), were observed. VC-9-1-1 also showed dose-dependent GUS activity, and induction of GUS activity was significant at 200 nM CB126. However, activities at all concentrations were extremely low compared to the MLP plants. Increased sensitivity in MLP plants strongly indicated the participation of MLPs in the transport of these hydrophobic compounds. DMSO-treated MLP plants showed higher background GUS activity than DMSO-treated VC plants, which may represent the interference of endogenous hydrophobic ligands and the promotion of their availability toward AhR.

3.5. Phenotypic changes in MLP transgenic tobacco plants germinated in a concentration series of CB126

227 The T_3 transgenic tobacco line GR3-20-1-1 exhibited distinct phenotypic alterations compared to
228 VC plants when incubated in medium containing 40 and 200 nM CB126 (Fig. 4). These plants had
229 first leaves that were pale green in color and had inhibited growth. In contrast, VC showed little to no
230 phenotypic changes. These results revealed that GR3-20-1-1 line was more sensitive to CB126 than
231 the VC plants, and MLP-GR3 contributed to the promotion of phenotypic changes. As MLP-GR3 has
232 a binding activity toward hydrophobic compounds (Goto et al., 2019), these results suggest that the
233 functions of MLP-GR3 occurred by binding plant endogenous compounds were inhibited by binding
234 CB126. This proposes further studies for identifying the endogenous ligands of MLPs and for
235 clarifications regarding the functions of MLP in plants. Interestingly, the GR3-20-1-1 line grown on
236 200 nM CB126 retained the distorted phenotype during the incubation period and did not exhibit
237 further growth, whereas some of the plants grown on 40 nM CB126 seemed to overcome the toxic
238 effect of CB126 under the prolonged incubation; it was observed that newly emerging leaves of this
239 plant were green and were of normal size although not the all plants recovered, probably due to the
240 physiological fluctuations (Fig. S3). It has been reported that plants accumulate higher concentrations
241 of xenobiotic compounds in cotyledons than in other leaves (Aajoud et al., 2006). Therefore, it may
242 be suggested that the phenotype of the emerging leaves was not affected by lower CB126
243 accumulation. Furthermore, it is possible that CB126 is metabolized by plant enzymes to lower toxic
244 metabolites (Aken et al., 2010).
245
246 *3.6. GUS activity of transgenic tobacco plants treated with PCB congeners and MC*
247
248 GUS activities in VC-9-1-1 and GR3-20-1-1 lines were significantly induced after treatment with
249 500 nM CB77 (Fig. 5). In contrast, GUS activity in the CB118-treated plants was significantly
250 increased only in PG1-28-1-1 when incubated with 500 nM CB118 (Fig. 5). The GUS enzyme
251 activity was influenced by two major factors, namely, the specific binding affinities of MLPs and
252 AhR toward different PCBs, and thus the level of GUS response induction would reflect both the
253 binding actions of CB77 or CB118 as ligand molecules to MLP and to AhR. Bioconcentration factor
254 (BCF) represents the ease of accumulation of compounds in the aerial parts. In previous studies, it

255 was shown that the BCF of PCBs in plants differed depending on plant species or genotype. CB77
256 showed the highest BCF for *C. pepo* cultivar Patty Green among the 14 other PCBs, while CB118 had
257 the highest value for *C. pepo* cv. Gold Rush (Inui et al., 2008). In both cultivars, CB126 had low BCF,
258 suggesting that the binding affinity of MLPs toward CB77 and CB118 was higher than that toward
259 CB126. The XD4V-26 transgenic tobacco line also showed much higher BCF values for CB77 and
260 CB118 than CB126 (Inui et al., 2012). These results suggested that CB77 and CB118 accumulated
261 more compounds in the aerial parts than CB126. However, sensitivity toward CB126 of the transgenic
262 tobacco plants was much higher than that toward the other PCBs. As previously suggested (Gion et
263 al., 2012), the important factor determining GUS activity induction was the toxic equivalency factor
264 (TEF) of the PCB congeners. CB126 has the highest TEF of 0.1 among PCB congeners, whereas
265 CB77 and CB118 have lower TEF values of 0.0001 and 0.00003, respectively (Van den Berg et al.,
266 2006). Compounds with high TEF values usually show high toxicity because of their high binding
267 affinities toward AhR. In contrast, the binding affinity of MLPs also appeared to be crucial at
268 critically low concentrations of PCBs (Fig. 5). MLPs possess different binding affinities to PCB
269 congeners depending on their 3D structures (Iwabuchi et al., 2020). In the hydrophobic cavity
270 associated with binding of ligands such as steroid hormones, phospholipids, and flavonoids (Lytle et
271 al., 2009; Choi et al., 2015), MLPs exhibit higher binding affinity to PCBs with chlorine residues at
272 the 2-position (Goto et al., 2019). No changes in the phenotype of the treated plants were observed at
273 any concentration, probably because these PCB congeners do not have high binding affinities toward
274 AhR.
275 Lines VC-9-1-1, PG1-28-1-1, and GR3-20-1-1 were treated with different concentrations of MC
276 for 2 weeks (Fig. 2C). Similar to the treatments with PCBs, MLP tobacco plants showed dose-
277 dependent increases in GUS activity and higher sensitivity than VC. Significant induction was
278 observed in PG1-28-1 at 1.6 and 5000 nM MC. A significant increase in GUS activity was measured
279 in line GR3-20-1 treated with 200 and 5000 nM MC, whereas a significant decrease was observed in
280 GR3-20-1 at 1.6 nM MC. These results indicated that the enhanced transfer of hydrophobic
281 compounds with a different chemical structure from PCBs was achieved by the MLPs.
282

3.7. GUS activity of transgenic tobacco plants on soil supplemented with CB126

Regarding the strong correlation between the type and structure of the soil and the level of adsorption of organic pollutants to the soil particles (Ahmed et al., 2015), it is uncertain to what extent the plants would be able to desorb these compounds from soil organic matter and uptake them by the root cells; this uncertainty also serves as a limiting factor. GR3-20-1-1, showing the highest sensitivity to the tested compounds, and VC-9-1-1 were cultivated on soil contaminated with different concentrations of CB126 (Fig. S4). Although the CB126 concentration was higher than that used in the MS medium, no significant induction and dose-dependent correlation were observed in VC-9-1-1. This may be due to the strong adsorption of the hydrophobic CB126 to the soil particles, making desorption inefficient. The bioavailability of CB126 in MS medium was found to be higher than that in the soil, which contributed to an increase in GUS activity. In general, highly chlorinated PCBs show higher hydrophobicity than low chlorinated ones (Jing et al., 2018), and these enter the root cells by diffusing in a passive manner. Moreover, hydrophobicity is inversely correlated with the bioavailability of POPs since highly hydrophobic molecules strongly adsorb to the soil particles (Delle Site, 2001; Ahmed et al., 2015). These are indicated by the results of positive correlation between the root concentrations of POPs and their hydrophobicity (Liu and Schnoor, 2008; Namiki et al., 2018). In contrast, GR3-20-1-1 showed significantly higher GUS activities when cultivated at 62.5 nM CB126 than when it was cultivated at higher concentrations. Elucidation of the causes behind this phenomenon in further studies would be valuable to the understanding of *in planta* mechanisms related to responses to xenobiotic molecules. Nevertheless, it was demonstrated that sensitivity of transgenic tobacco plants toward CB126 was conferred by the expression of zucchini *MLP* genes.

4. Conclusion

By introducing *MLP* genes from zucchini to AhR transgenic tobacco plants, it was confirmed that MLPs were involved in the accumulation of hydrophobic compounds such as PCB congeners and MC in aerial parts. MLP transgenic tobacco plants were able to respond and therefore detect lower

concentrations of PCB congeners and MC, whereas AhR transgenic tobacco plants detected only high concentrations, indicating that the amounts of PCBs and MC were translocated by MLPs from the roots, thereby increasing their concentrations in the aboveground tissues, leading to the significant induction of the GUS reporter system. Efficient phytoremediation of soils contaminated with hydrophobic pollutants can be achieved by the expression or activation of *MLP* genes in other plant species as well as in Cucurbitaceae plants. Further investigations addressing the abnormal growth of plants incubated at higher CB126 concentrations would reveal the physiological functions of MLPs and the mechanism of involvement of hydrophobic pollutants in plant physiology. This study also provided initial arguments regarding the role of zucchini MLPs in perceiving environmental stresses caused by pollutants.

Author contributions

PS and HI performed the experiments, collected and analyzed the data, and wrote the manuscript. HI conceptualized the overall work.

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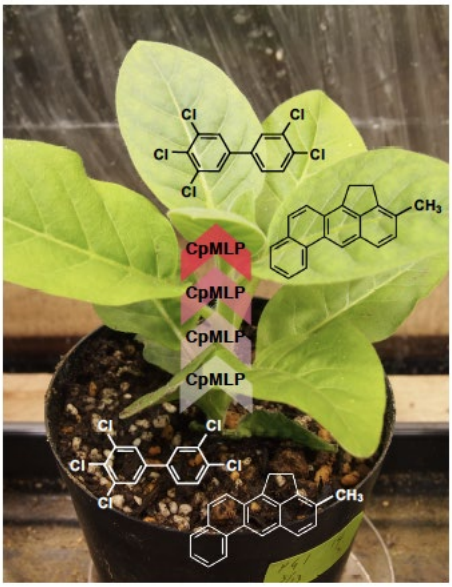
494 **Graphic abstract**

495



Transgenic tobacco plants expressing zucchini *MLP* genes

Figure legends

Fig. 1. Fold induction of β-glucuronidase (GUS) activities in the transgenic tobacco plants expressing major latex-like protein (*MLP*) genes under treatment of 3,3',4,4',5-pentachlorobiphenyl (CB126). T_0 transgenic tobacco plants were incubated in a solid medium containing 306 nM CB126 for 2 weeks. Leaves were subjected to GUS activity measurements. Fold induction was calculated by dividing GUS activity upon treatment with CB126 by GUS activity upon treatment with dimethyl sulfoxide (DMSO). Black and white circles indicate the fold induction of GUS activity toward 0.1% DMSO treatment in individual plants and average fold induction of GUS activities of each line (VC, n=11; PG1, n=24; GR1, n=12; GR3, n=57), respectively. The numbers indicate average fold induction. VC, PG1, GR1, and GR3 indicate the vector control plants and transgenic plants expressing *MLP-PG1*, *MLP-GR1*, and *MLP-GR3* genes, respectively. Asterisks indicate significant differences compared to the average fold induction in VC (* p<0.05; ** p<0.01; Student's t-test).

Fig. 2. β-glucuronidase (GUS) activities in transgenic tobacco plants expressing major latex-like protein (*MLP*) genes incubated in a solid medium containing 3-methylcholanthrene (MC). The seeds of T_2 homozygous transgenic tobacco plants were sowed and incubated in a solid medium containing 5 μM MC for 2 weeks. Roots (A) and aerial parts (B) of plants were subjected to GUS activity measurements. White and black bars indicate the treatment with 0.1% dimethyl sulfoxide (DMSO) and 5 μM MC, respectively. (C) Seeds of the T_2 homozygous transgenic tobacco plants were sowed and incubated in a solid medium containing MC for 2 weeks. Aerial parts of plants were subjected to GUS activity measurement. White and black bars indicate the treatment with 0.1% DMSO and concentration series of MC at 0.064, 0.32, 1.6, 8, 40, 200, 1000, and 5000 nM (from left to right), respectively. Error bars indicate standard deviation (n=3). VC, PG1, GR1, and GR3 indicate the vector control plants and transgenic plants expressing *MLP-PG1*, *MLP-GR1*, and *MLP-GR3* genes, respectively. Error bars indicate standard deviation (n=3). Asterisks indicate significant differences compared to the treatment with DMSO (* p<0.05; ** p<0.01; *** p<0.001; Student's t-test).

Fig. 3. β-glucuronidase (GUS) activities in the transgenic tobacco plants expressing major latex-like protein (*MLP*) genes incubated in a solid medium containing 3,3',4,4',5-pentachlorobiphenyl (CB126). The seeds of T_3 homozygous transgenic tobacco plants were sowed and incubated in a solid medium containing CB126 for 2 weeks. Aerial parts of the plants were subjected to GUS activity measurements. White and black bars indicate treatment with 0.1% dimethyl sulfoxide (DMSO) and a concentration series of CB126 at 0.32, 1.6, 8, 40, 200 nM (from left to right), respectively. VC, PG1, GR1, and GR3 indicate the vector control plants and transgenic plants expressing *MLP-PG1*, *MLP-GR1*, and *MLP-GR3* genes, respectively. Error bars indicate standard deviation (n=4–5). Asterisks indicate significant differences compared to the treatment with DMSO (* p<0.05; ** p<0.01; Student's t-test).

Fig. 4. Phenotypes of T_3 transgenic tobacco plants expressing major latex-like protein genes incubated in a solid medium containing 3,3',4,4',5-pentachlorobiphenyl (CB126).
Seeds of the T_3 homozygous transgenic tobacco plant lines VC-11-3-2 (A) and GR3-20-1-1 (B) were sowed and incubated in a solid medium containing different concentrations of CB126 for 2 weeks. All media contained 0.1% dimethyl sulfoxide.

Fig. 5. The β-glucuronidase (GUS) activities in the transgenic tobacco plants expressing major latex-like protein (*MLP*) genes incubated in a solid medium containing polychlorinated biphenyls (PCBs). The seeds of T_3 homozygous transgenic tobacco plants were sowed and incubated in a solid medium containing 3,3',4,4'-tetrachlorobiphenyl (CB77) and 2,3',4,4',5-pentachlorobiphenyl (CB118) for 2 weeks. Aerial parts of plants were subjected to measurement of GUS activity. White, grey, and black bars indicate treatment with 0.1% dimethyl sulfoxide (DMSO) and the concentration series of CB77 and CB118 at 200 nM (grey bar) and 500 nM (black bar), respectively. Error bars indicate standard deviation (n=5). VC, PG1, and GR3 indicate the vector control plants and transgenic plants

549 expressing *MLP-PG1*, and *MLP-GR3* genes, respectively. Asterisks indicate significant differences
550 compared to the treatment with DMSO (* $p<0.05$; Student's *t*-test).
551
552 **Fig. S1.** Plasmids for the expression of major latex-like protein (*MLP*) genes in the transgenic tobacco
553 line XD4V-26.
554 AhR, mouse arylhydrocarbon receptor gene; GUS, β-glucuronidase gene; His, hexahistidine tag;
555 HPT, the expression unit for hygromycin resistance gene; LB, left border; LexA, DNA-binding
556 domain of *E. coli* LexA; 8xLexA-46, 8 copies of LexA binding domain combined with cauliflower
557 mosaic virus (CaMV) 35S minimal promoter; NLS, SV40 large T-antigen nuclear localization signal;
558 NPT II, the expression unit for kanamycin resistance gene; Ω, tobacco mosaic virus 5'-untranslated
559 leader sequence; P, promoter; RB, right border; 35S-P, CaMV 35S promoter; T, terminator; VP16,
560 transactivating domain of *Herpes simplex* VP16.
561
562 **Fig. S2.** Relative expression levels of major latex-like protein (*MLP*) genes in the T_0 transgenic
563 tobacco plants expressing *MLP-PG1* (A), *MLP-GR1* (B), and *MLP-GR3* (C) genes.
564
565 **Fig. S3.** Phenotypes of the GR3-20-1-1 expressing major latex-like protein genes incubated in a solid
566 medium containing 3,3',4,4',5-pentachlorobiphenyl (CB126).
567 The seeds of the T_3 homozygous transgenic tobacco plant line GR3-20-1-1 were sowed and incubated
568 in a solid medium containing different concentrations of CB126 for 3 weeks. All media contained
569 0.1% dimethyl sulfoxide.
570
571 **Fig. S4.** β-glucuronidase (GUS) activities in the transgenic tobacco plants expressing major latex-like
572 protein (*MLP*) genes incubated in soil containing 3,3',4,4',5-pentachlorobiphenyl (CB126).
573 Seeds of T_3 transgenic tobacco plants were sowed and incubated in soil containing CB126 for 2
574 weeks. Aerial parts of plants were subjected to GUS activity measurements. White and black bars
575 indicate the treatment with acetone and CB126 concentration series at 31.3, 62.5, 125, 250, and 500

576 nM (from left to right), respectively. VC and GR3 indicate the vector control plants and transgenic
577 plants expressing the *MLP-GR3* gene, respectively. Error bars indicate standard deviation (n=4–5).
578 Asterisk indicates significant differences compared to the treatment with acetone (* p<0.05; Student's
579 t-test).
580

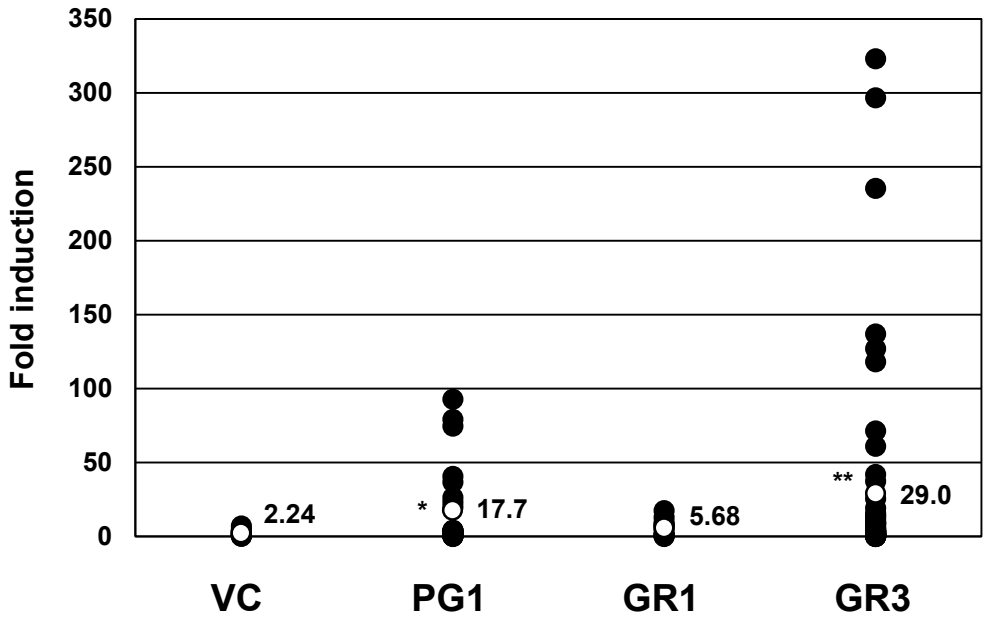

Fold induction
350
300
250
200
150
100
50
0
2.24
*
17.7
5.68
**
29.0
VC
PG1
GR1
GR3

Fig. 1.

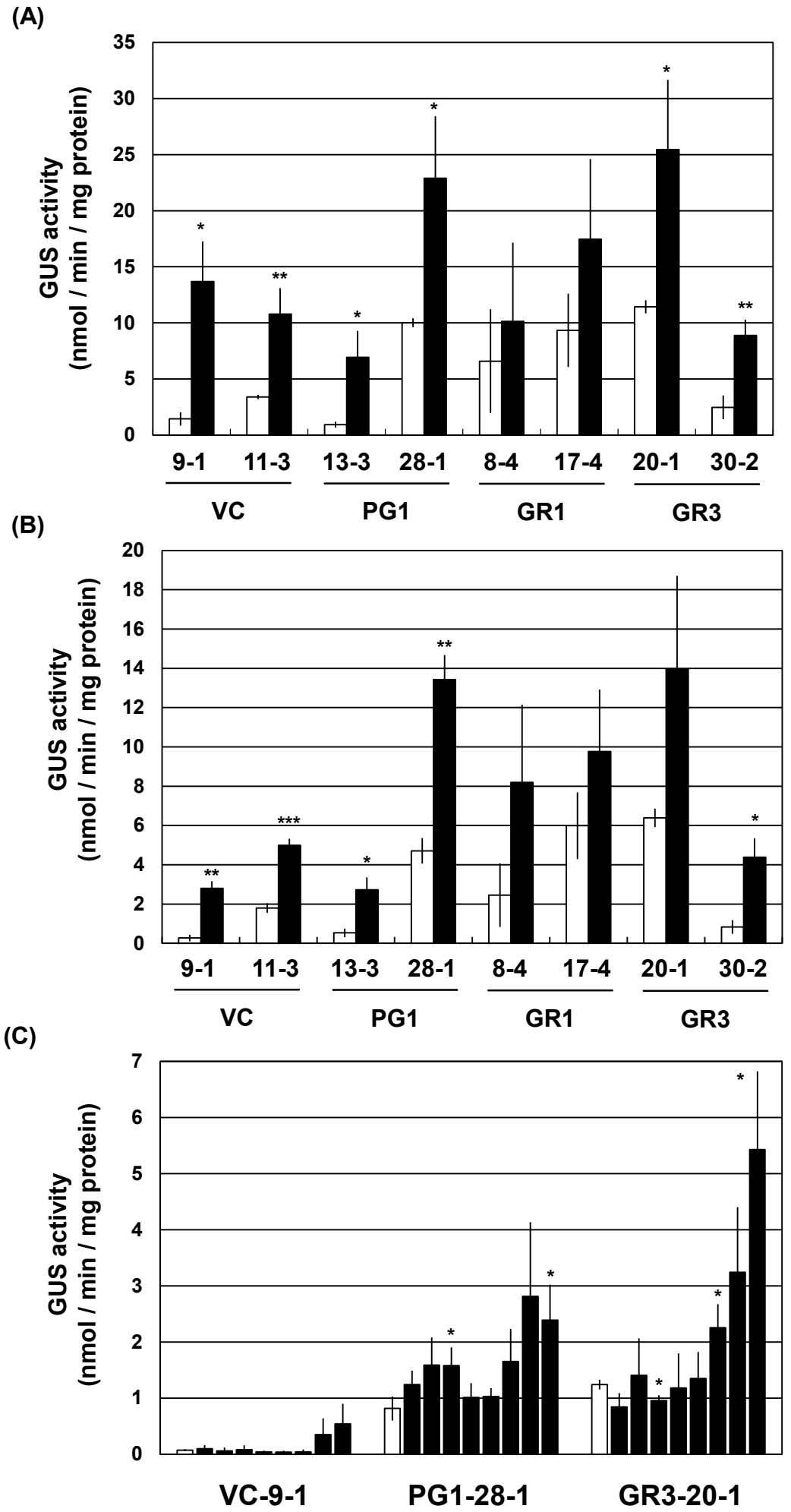

(A)
GUS activity
(nmol / min / mg protein)
35
30
25
20
15
10
5
0
9-1
11-3
13-3
28-1
8-4
17-4
20-1
30-2
VC
PG1
GR1
GR3
(B)
GUS activity
(nmol / min / mg protein)
20
18
16
14
12
10
8
6
4
2
0
9-1
11-3
13-3
28-1
8-4
17-4
20-1
30-2
VC
PG1
GR1
GR3
(C)
GUS activity
(nmol / min / mg protein)
7
6
5
4
3
2
1
0
VC-9-1
PG1-28-1
GR3-20-1

Fig. 2.

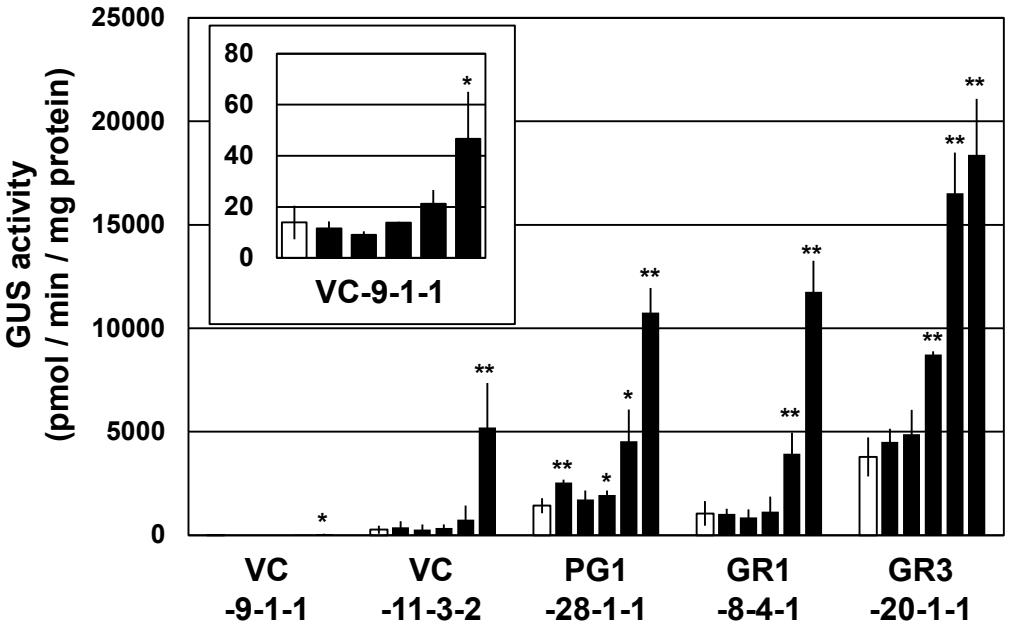

GUS activity
(pmol / min / mg protein)
25000
20000
15000
10000
5000
0
80
60
40
20
0
VC-9-1-1
VC
-9-1-1
VC
-11-3-2
PG1
-28-1-1
GR1
-8-4-1
GR3
-20-1-1

Fig. 3.

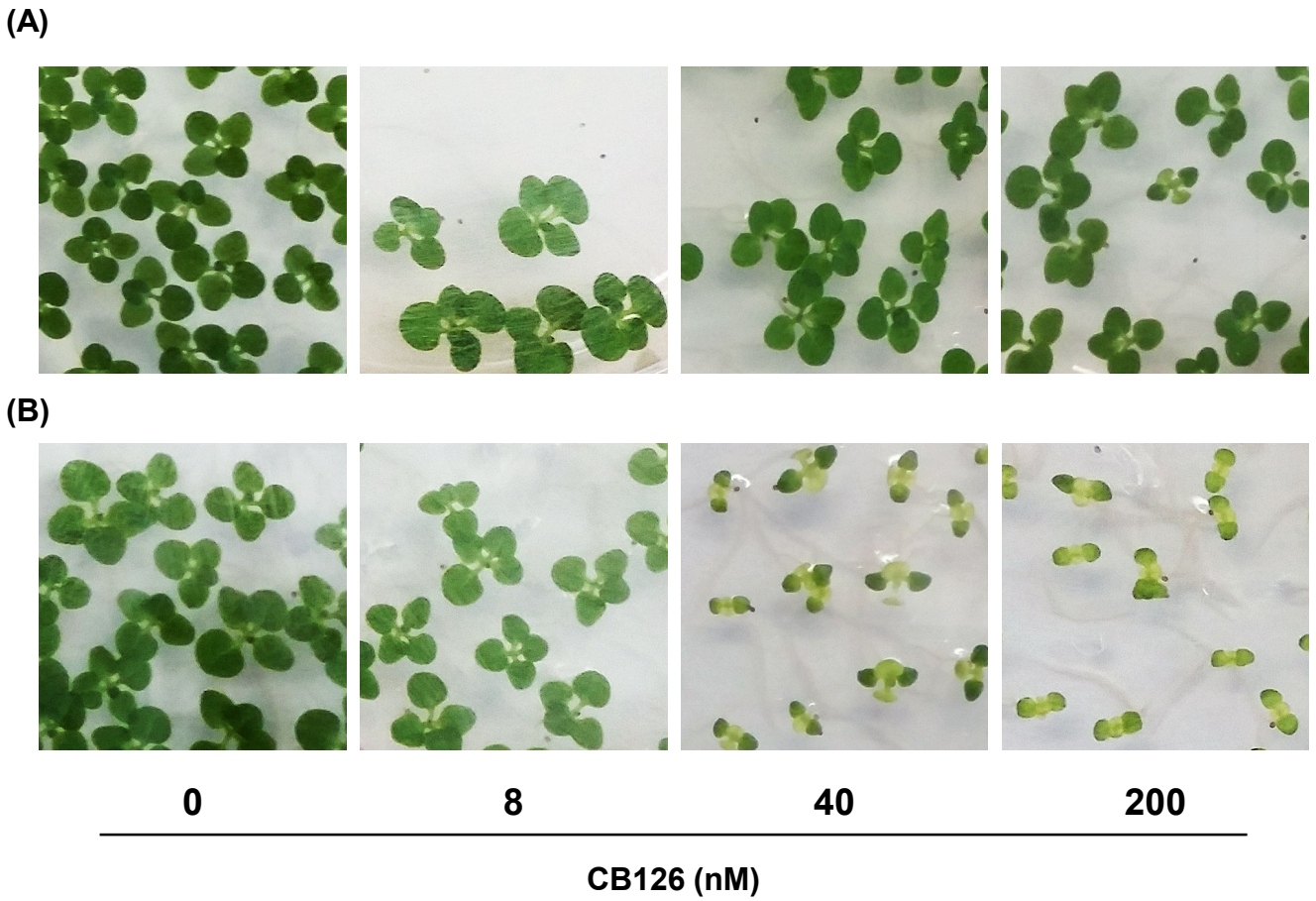


Fig. 4.

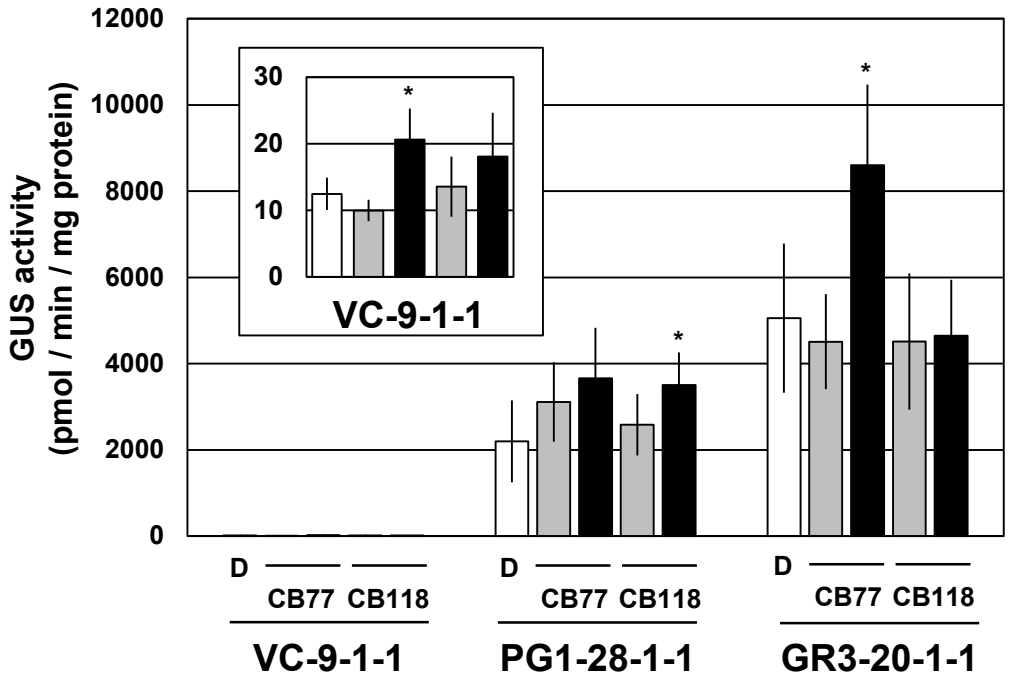

12000
10000
8000
6000
4000
2000
0
GUS activity
(pmol / min / mg protein)
30
20
10
0
VC-9-1-1
*
*
*
D
CB77
CB118
VC-9-1-1
PG1-28-1-1
GR3-20-1-1

Fig. 5.

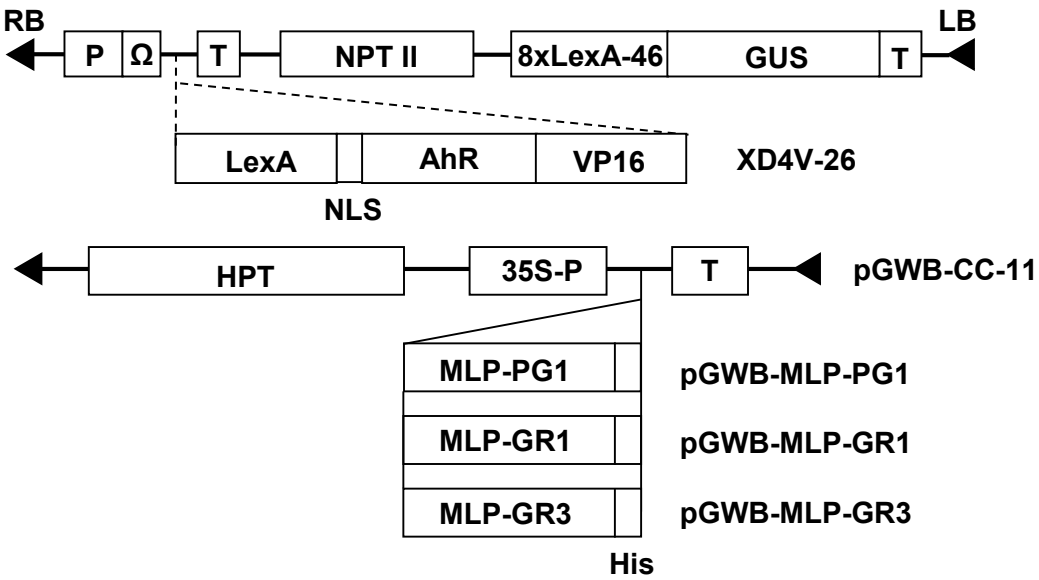

RB
P
Ω
T
NPT II
8xLexA-46
GUS
T
LB
LexA
AhR
VP16
XD4V-26
NLS
HPT
35S-P
T
pGWB-CC-11
MLP-PG1
pGWB-MLP-PG1
MLP-GR1
pGWB-MLP-GR1
MLP-GR3
pGWB-MLP-GR3
His

Fig. S1.

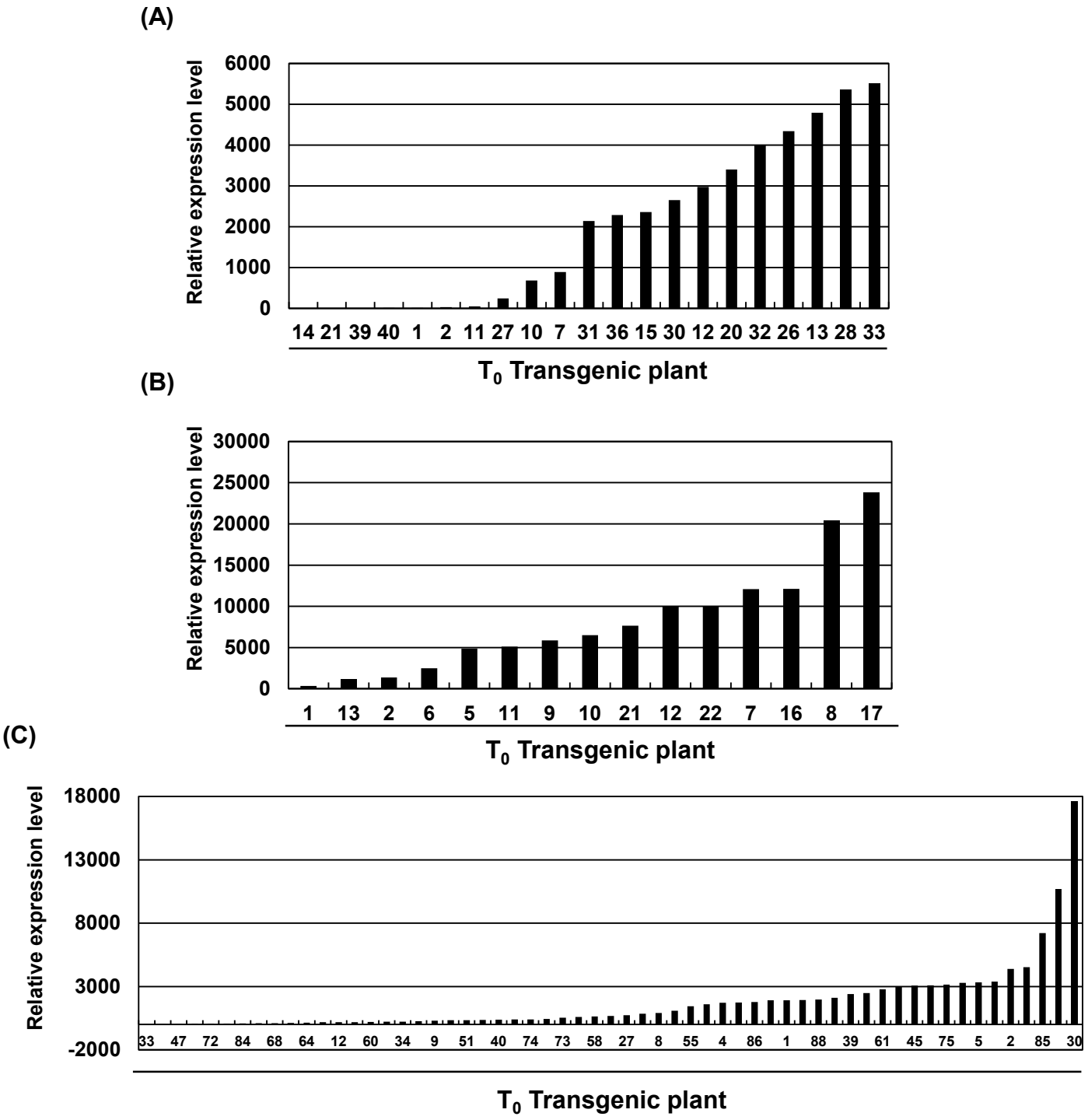

(A)
Relative expression level
6000
5000
4000
3000
2000
1000
0
14 21 39 40 1 2 11 27 10 7 31 36 15 30 12 20 32 26 13 28 33
T0 Transgenic plant
(B)
Relative expression level
30000
25000
20000
15000
10000
5000
0
1 13 2 6 5 11 9 10 21 12 22 7 16 8 17
T0 Transgenic plant
(C)
Relative expression level
18000
13000
8000
3000
-2000
33 47 72 84 68 64 12 60 34 9 51 40 74 73 58 27 8 55 4 86 1 88 39 61 45 75 5 2 85 30
T0 Transgenic plant

Fig. S2.

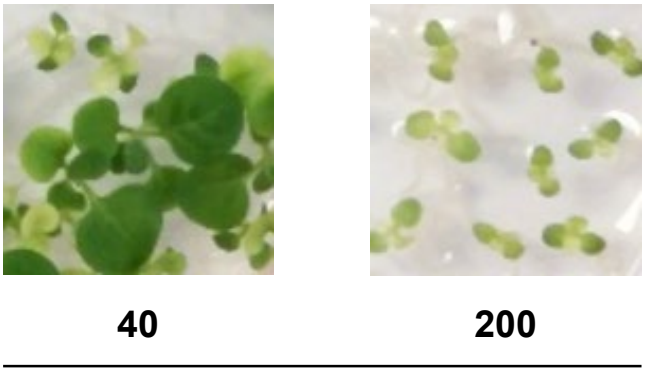


Fig. S3.

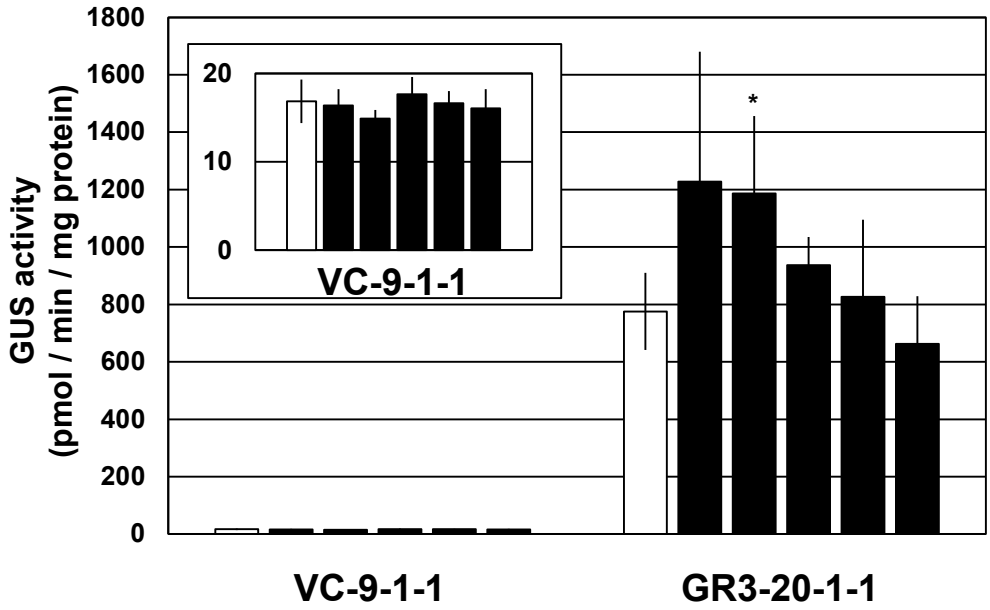

1800
1600
1400
1200
1000
800
600
400
200
0
GUS activity
(pmol / min / mg protein)
20
10
0
VC-9-1-1
*
VC-9-1-1
GR3-20-1-1

Fig. S4.

Supplementary Table 1 Primer sequences used in this study.

Primer name	Sequence
MLP-PG1-s	5′-TGAAGCTGTTGAAGGAGATC-3′
MLP-PG1-as	5′-CCTTTTCAAACTCAACAGTCCA-3′
MLP-GR3-s	5′-AATTCAAAGTGCTTAGAGCAAAGG-3′
MLP-GR3-as	5′-TGCCTTTTCAAACTCAATAGTCAA-3′
Actin-s	5′-ATTACCATCGGTGCTGAACG-3′
Actin-as	5′-CCAATCATTGAAGGTTGGAAA-3′