Establishment of Transgenic *Drosophila* Bearing Metallothionein-*lacZ* Fusion Gene and Its Practical Use

Young-Shin Kim, Hyun-Sook Park[1], Mi-Ae Yoo[1], and Won-Ho Lee*

Department of Biology and [1]Department of Molecular Biology, College of Natural Sciences, Pusan National University, Pusan 609-735, Korea

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The reporter plasmid pMT-*lacZ* containing the metallothionein (MT) promoter region (-320～+58 with respect to the transcription initiation site) fused to the *lacZ* gene in a P-element vector was constructed. Transgenic *Drosophila* bearing the MT-*lacZ* fusion gene were established by P-element mediated transformation. Expression of the MT-*lacZ* fusion gene in transformants was examined during development. By treatment with low concentration of cadmium (≥10 μM) or paraquat (≥50 μM), increased expression of β-galactosidase was shown in fat body, brain lobe, and ganglion transgenic larval tissues. The results show that transformants bearing the MT-*lacZ* fusion gene are useful for further studies on the mechanism of regulation of MT gene expression and for monitoring toxic metals.

Metallothioneins (MTs) are small, cystein-rich metal-binding proteins that are expressed ubiquitously in eukaryotes (Kagi and Kojima, 1979; Hamer, 1986). Overexpression of MT can protect against heavy metal toxicity such that MT has been considered to play important roles in metal homeostasis (Kagi and Schaffer, 1988; Andrews, 1990; Masters et al., 1994). MT is an efficient scavenger of hydroxyl radicals (Abel and Ruiter, 1989; Dalton et al., 1994; Dalton et al., 1996), and it has been shown that yeast and mammalian MTs can functionally substitute for superoxide dismutase in protecting cells from oxidative stress (Tamai et al., 1993; Dalton et al., 1994).

The biosynthesis of MTs is enhanced both *in vivo* and in cultured cells by metal ions, glucocorticoids, cytokines, growth factors, and many other chemicals (Durnam and Palmiter, 1981; Hager and Palmiter, 1981; Palmiter, 1987; Lazo et al., 1995). The proximal regulatory region, termed metal response elements (MREs), of the MT genes has been shown to induce transcription in the presence of heavy metals (Stuart et al., 1984; Silar et al., 1990).

In *Drosophila*, two MT homologue genes, *Mto* and *Mtn*, have been identified (Maroni et al., 1986), and characterized to show increased expression by the various metals (Lastowski-Perry et al., 1985; Otto et al., 1987; Silar et al., 1990; Bonneton et al., 1996). However, the regulatory mechanism of this gene expression remains to be clarified.

In this study, to obtain an *in vivo* model system for the investigation of regulation mechanisms of the MT gene (*Mtn*) expression and for the monitoring of toxic metals, we established transgenic *Drosophila* carrying the MT promoter region (-320～+58 with respect to the transcription initiation site) fused to *E. coli lacZ* gene. Expression patterns of the MT-*lacZ* gene in transgenic *Drosophila* were examined at various developmental stages. Induction of the MT-*lacZ* gene by cadmium or paraquat in both cultured cells and transgenic flies was also examined.

* To whom correspondence should be addressed.
Tel: 82-51-510-2257, Fax: 82-51-513-9258

Materials and Methods

Construction of the plasmid pMT-lacZ

A 378 bp DNA fragment containing MT promoter region (-320～+58 with respect to the transcription initiation site) was isolated from the plasmid pMT-CAT by digesting with *Sma*I and *Pst*I. The fragment was blunt-ended with T4 DNA polymerase and then subcloned into *Sma*I site of the plasmid pGEM-3. The resultant plasmid was named pGEM-MT. The 378 bp promoter fragment isolated from the plasmid pGEM-MT by digestion with *Bam*HI was inserted into *Bam*HI site of the plasmid pCaSpeR-AUG-βgal containing a P-transposable element. The resultant plasmid was named pMT-*lacZ*.

Cell culture, DNA transfection, X-gal staining and CAT assay

Drosophila Kc cells were grown at 25℃ in M3 (BF) medium (Sigma) supplemented with 2% fetal bovine serum (FBS) and 0.5% Penicillin-Streptomycin (PS, GIBCO). *Drosophila* S2 cells were grown in M3 (BF) medium supplemented with 10% FBS and 1% PS. Cells were plated at 5×10^6 cells per 60-mm dish at 16 h before DNA transfection. Ten μg DNA was transfected into cells by the calcium phosphate copre-

cipitation method described elsewhere (Di Nocera et al. 1983).

Cells were rinsed three times with 1 X PBS (130 mM NaCl, 7 mM $Na_2HPO_42H_2O$, 3 mM $NaH_2PO_42H_2O$) at 48 h after transfection and then fixed with 0.2% glutaraldehyde in PBS for 5 min at 4℃. Fixed cells were rinsed three times with 1XPBS and then replaced with X-gal staining solution containing 10 mM sodium phosphate (pH 7.2), 150 mM NaCl, 1 mM $MgCl_2$, 6.1 mM potassium ferricyanide, 6.1 mM potassium ferrocyanide and 0.2% X-gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside). The cells were incubated in the dark at 37℃ overnight. Stained cells were rinsed with 1 X PBS and then photographed.

The CAT assay was carried out as described elsewhere (Yamaguchi et al., 1988) after preparation of cell extracts from transfected cells. The CAT activities were quantified by an image analyzer BAS1500 (Fuji Film).

Establishment of transgenic flies

Germ line transformation was carried out essentially as described by Karess (1985). The embryos were injected with 0.5 μg/μl of DNA containing the plasmid pMT-*lacZ* and helper plasmid pπ25.7wc (Rubin and Spradling, 1983). The phenotype of the G1 offspring was facilitated by the w^+ marker with variable yellow to red eye color on vector the pCaSpeR-AUG-βgal (Thummel et al., 1988). The *w* strain for host strain of P-element mediated transformation and the *w; Pm/SM1; Pre/TM3* strain for balancer chromosomes were used.

Drosophila cultures and treatments

Flies were maintained at 25℃ on standard cornmeal agar medium. Third instar larvae and 3-day-adults of transgenic and wild type flies were cultured on medium containing various concentrations of cadmium for 48 h. In the case of paraquat, third instar larvae were dissected on ice and the larval tissues were incubated in Ringer's solution containing various concentrations of paraquat for 5 h (Keyse and Tyrrell, 1987; Keyse et al., 1990; Dalton et al., 1994).

Analysis of expression of the MT-lacZ gene in transgenic flies

Larval tissues dissected from the third instar larvae were incubated in fixative (12 mM sodium cacodylate buffer, pH 7.3/10% glutaraldehyde) for 10 min at room temperature. The fixed tissues were then incubated with a staining solution containing 0.2% X-gal in the dark at 37℃ for 5 to 16 h. For photography, tissues were immersed in glycerol, mounted on slides, and photographed with an Olympus microscope (BX-40).

For quantitative measurement of β-galactosidase activity, groups of 20 to 100 individuals of dechorinated embryos, larvae, pupae and adult flies were homogenized in 200 μl of ice-cold assay buffer (50 mM potassium phosphate, pH 7.5/1 mM $MgCl_2$). Homogenates were centrifuged at 10,000×g at 4℃ for 10 min. For each assay, 200 to 300 μl of the supernatant was added to give 1 ml of assay buffer containing 1 mM chlorophenol red-β-D-galactopyranoside substrate (CPRG: Boehringer Mannheim). Reaction incubations were carried out at 37℃ in the dark. Substrate conversion was measured at 574 nm using a spectrophotometer 0.5, 1, 2, and 3 h after addition of the extract, and the rate of color development was linear. The β-galactosidase activity was defined as absorbance

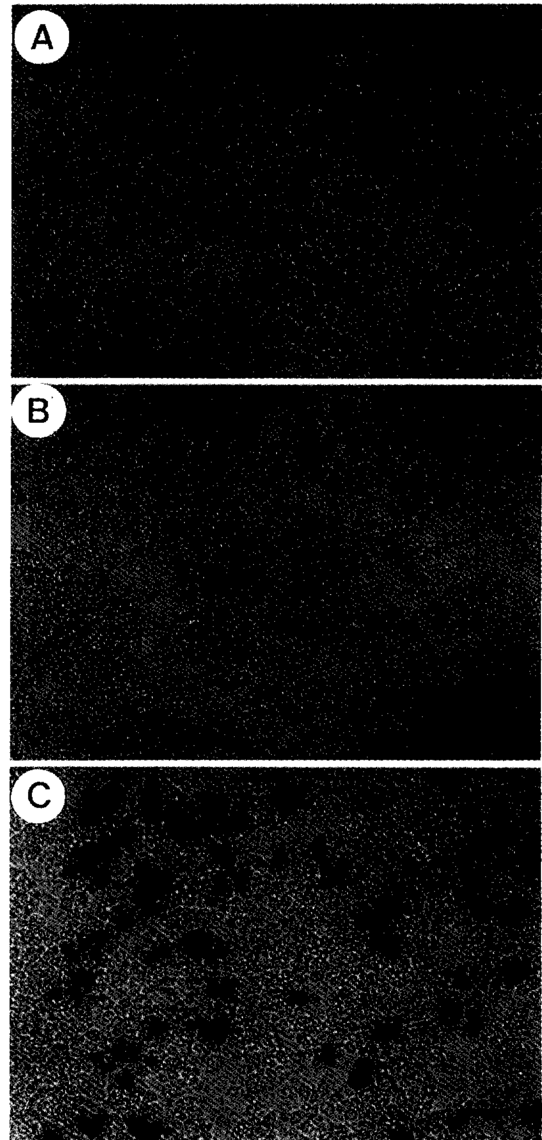

Fig. 1. Expression of the *lacZ* in Kc cells transfected with the plasmid pMT-*lacZ*. A, Cells transfected with the control plasmid, pCaSpeR-AUG-βgal. B, Nontreated cells transfected with the pMT-*lacZ*. C, 60 μM cadmium-treated cells transfected with the pMT-*lacZ*. Scale bars=300 μm.

Fig. 2. Expression of the MT-*lacZ* in transgenic tissues. Third instar larvae of host strain (A) and transgenic flies (B) were cultured on medium. In transgenic larval tissues, expression of the MT-*lacZ* fusion gene was detected in brain, ganglion and fat body. In transgenic ovaries high expression of the MT-*lacZ* in oocyte during oogenesis, but slight expression was observed in the transgenic male reproductive system. b, brain lobe. g, ganglion. f, fat body. gt, gut. o, oocyte. t, testis. aed, anterior ejaculatory duct. Scale bars=300 μm.

per mg of protein. To correct for endogenous β-galactosidase activity, extracts from the wild type strain were included in each experiment and endogenous reading was subtracted from readings obtained with each transformant line.

Results

To establish transgenic flies useful for studying on regulation of the MT gene expression, we constructed the plasmid pMT-*lacZ* containing the MT promoter region (-320~+58 with respect to the transcription initiation site) fused with the *lacZ* gene in a P-element vector as described in materials and methods. Expression of the *lacZ* gene under the MT promoter fragment of the plasmid pMT-*lacZ* was examined in Kc cells by transient expression assay and X-gal staining. When cells transfected with pMT-*lacZ* gene were treated with cadmium, expression of the *lacZ* gene was strongly induced (Fig. 1).

The reporter plasmid pMT-*lacZ* was injected into embryos of *Drosophila* strain *w* together with helper plasmid pπ25.7wc. Selection of transgenic flies bearing MT-*lacZ* fusion gene was facilitated by the *w+* on the vector pCaSpeR-AUG-βgal. Five independent transformant lines were established, and their chromosomal linkages were determined (Table 1). β-Galactosidase activities in adult males of these transformant lines were examined. Two strains (#20 and #42) showed higher expression of the MT-*lacZ* gene than the other three strains (#60, #89, and #91) (Table 1). These results seem to be due to a position effect on the location of P-element construct insertion.

Table 1. Transformants carrying the MT-*lacZ* fusion gene obtained in this study and β-galactosidase activities in these lines

Strains	Chromosomal linkage	β-Galactosidase[1] activity (mean ± SE)
Control (w)		0.52 ± 0.15
#20	II	2.28 ± 0.11
#42	II	1.64 ± 0.47
#60	X	0.74 ± 0.05
#89	II	0.85 ± 0.07
#91	III	0.81 ± 0.08

[1]β-Glactosidase activities in whole-body homogenates of adult males were measured. For each assay, 200 to 300 μl of the supernatant was added to make 1 ml of assay buffer containing 1 mM chlorophenol-red-β-D-galactopyranoside substrate (CPRG). Reaction incubations were carried out at 37℃ in the dark. Substrate conversion was measured at 574 nm using a spectrophotometer 0.5, 1, 2, and 3 h after addition of the extract, and the rate of color development was linear.

Regulation of the MT promoter activity during *Drosophila* development was examined. Male transgenic flies were crossed with wild type females to examine zygotic MT-*lacZ* expression and single copy gene expression. Expression of the MT-*lacZ* fusion gene at various developmental stages of transformants was examined with X-gal staining. In transgenic larval tissues expression of the MT-*lacZ* was detected in brain, ganglion, fat body and gut (Fig. 2). In the ovaries of adult transgenic females, high expression of the MT-*lacZ* fusion gene was detected in oocyte and follicle cells during oogenesis. In the male repro-

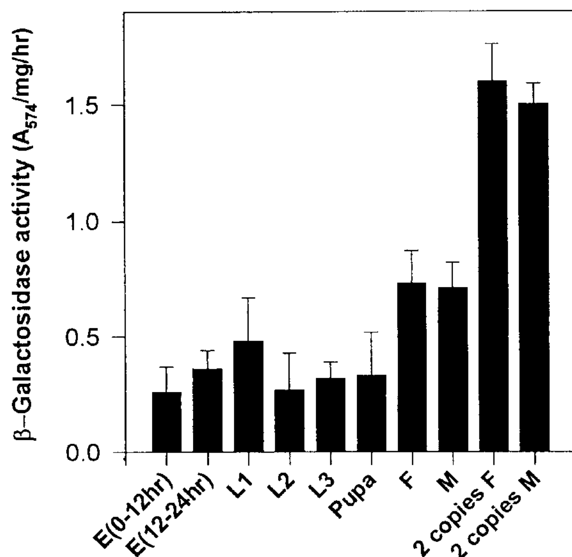

Fig. 3. β-Galactosidase activity in transgenic flies bearing pMT-*lacZ* fusion gene at various developmental stages. Male transgenic flies were crossed with wild type females to examine zygotic expression of the *lacZ*. Extracts of individuals at various stages of development were prepared. Extracts of transgenic females and males containing two copies of the MT-*lacZ* gene were also examined. To correct for endogenous β-galactosidase activity, the host strain was included in each experiment, and the value of this background was subtracted from values obtained with transformants. The β-galactosidase activity was defined as absorbance per h units per mg of protein. E, embryo. L1, 1st instar larvae. L2, 2nd instar larvae. L3, 3rd instar larvae. F, female. M, male. Data shown are mean ±SE of four independent experiments.

ductive system, only weak expression was detected in testis and anterior ejaculatory duct (Fig. 2). Five independent transformant strains showed the same expression pattern of MT-*lacZ* in all tissues examined although a quantitative difference in the activity of β-galactosidase was occasionally detected. Thus, we have used the strain #20 in the following experiments.

β-Galactosidase activities in transgenic flies at various developmental stages were quantified in Fig. 3. Higher expression of the *lacZ* was observed at adult stage in females and males than at other stages of development. β-Galactosidase activities in transgenic females and males containing two copies of the MT-*lacZ* gene were about two times higher than that in the flies containing single copy gene (Fig. 3).

Whether the MT gene expression in transgenic flies is induced by cadmium or paraquat was examined and its sensitivity was compared with that of S2 cells. CAT activities of S2 cells transfected with the plasmid pMT-CAT carrying MT promoter placed upstream of the CAT gene were increased by treatments of 10 to 100 μM cadmium or 100 to 1,000 μM paraquat (Fig. 4). The *lacZ* expression in transgenic flies was also induced by both cadmium and paraquat (Fig. 5). Induction of β-galactosidase activities in MT-*lacZ* transgenic flies was detected by treatment of 10 μM cadmium and by treatment of 50 μM paraquat. The endogenous *lacZ* expression in nontrans-

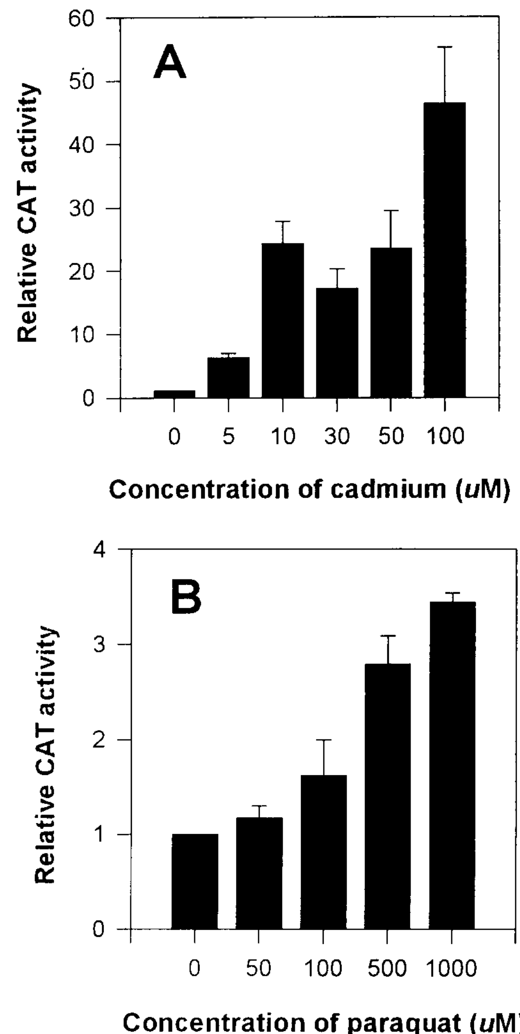

Fig. 4. Transient expression of the plasmid MT-CAT in S2 cells treated with cadmium or paraquat. Cadmium was added to the growth medium of the transfected cells 24 h after transfection. Cells were harvested after cadmium treatment for 24 h. In case of paraquat, various concentrations were added to the growth medium of the transfected cells 5 h after transfection. Cells were harvested after paraquat treatment for 48 h. The CAT activities were quantified by imaging analyzer BAS1500 (Fuji Film). The values are given as CAT activity relative to that of nontreated cells. A, Cadmium-treated cells transfected with the pMT-CAT. B, Paraquat-treated cells transfected with the pMT-CAT. Data shown are mean ±SE of three independent experiments.

genic flies was not induced under this condition. Treatment with ≥50 μM cadmium or ≥500 μM paraquat, expression of β-galactosidase in transgenic flies was observed at the basal level. It was postulated that proper treatment time and stress concentration can induce *lacZ* expression by the MT gene. We then compared CPRG and Galacto-Light activity. In the Galacto-Light assay, 10~20 μl crude extracts were used for detecting induction of MT-*lacZ* gene by ≥ 10 μM cadmium, showing that it is about 10~20-fold more efficient than CPRG. β-Galactosidase activities in dissected larval tissues were also examined by

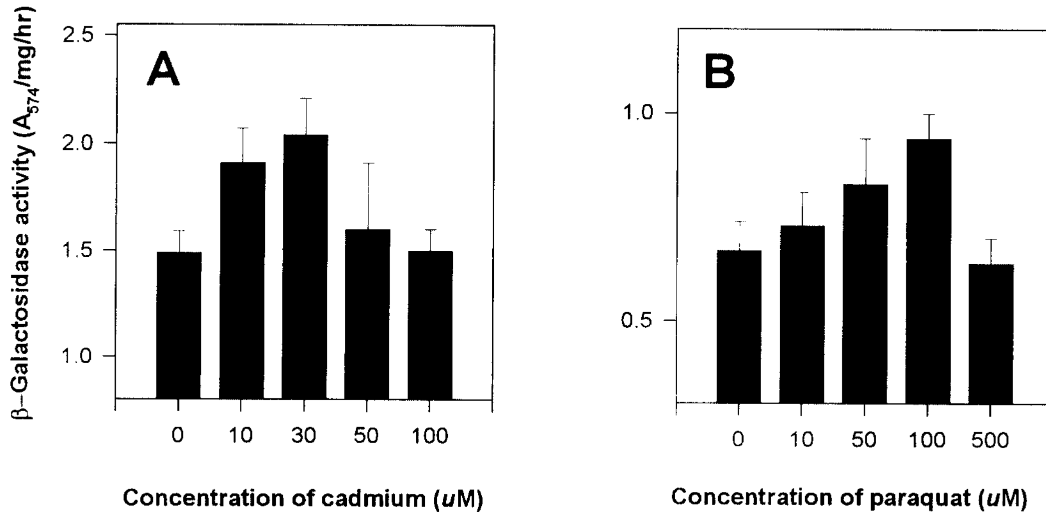

Fig. 5. Induction of β-galactosidase activity in transgenic flies bearing the MT-*lacZ* fusion gene by cadmium (A) or paraquat (B). Three-day-adults of host strain and transgenic flies were fed medium containing various concentrations of cadmium for 48 h. Third instar larvae of host strain and transgenic flies were dissected on ice and incubated in a *Drosophila* Ringer's solution with various concentrations of paraquat for 5 h. To correct for endogenous β-galactosidase activity, the host strain was included in each experiment, and value of this background was subtracted from values obtained with transformants. The β-galactosidase activity was defined as absorbance units per hr per mg of protein. Data shown are mean ±SE of seven independent experiments.

X-gal staining. As shown in Fig. 6, cadmium and paraquat induced the MT-*lacZ* gene expression in brain lobe, ganglion and in fat body, which is analogous to the vertebrate liver, of the transgenic line. The endogenous *lacZ* expression in nontransgenic tissues was not induced by cadmium or paraquat (data not shown).

Discussion

MT genes encode low molecular weight, cystein-rich metal binding proteins found in a wide variety of organisms including bacteria, fungi, insects, plants, fish, rodents and humans (Kagi and Kojima, 1979), and are thought to play several important roles in metal homeostasis (Kagi and Schaffer, 1988) and in protecting against metal toxicities (Beach and Palmiter, 1981; Karin et al., 1983; Karin et al., 1985).

Using transgenic flies bearing the MT-*lacZ* fusion gene, the regulation of the MT promoter activity during *Drosophila* development was examined. β-Galactosidase activity in transformants was higher in adults than in other stages (Fig. 3). Expression of the MT-*lacZ* gene was observed in brain, ganglion and fat body of larval tissues by X-gal staining (Fig. 2). In the reproductive system, β-galactosidase activity was expressed in oocytes and follicle cells during oogenesis of female ovaries (Fig. 2). This result coincides with expression of *Mtn* mRNA in larvae and adults by RNA analysis (Silar et al., 1990).

The synthesis of MT is homeostatically regulated in cells and organisms exposed to heavy metals. In mammals, induction of MT by metals is due to transcriptional activation of MT genes by heavy metal ions (Beach and Palmiter, 1981; Durnam and Palmiter, 1981; Karin et al., 1983; Karin et al., 1985). In this study, CAT activity in *Drosophila* S2 cells transfected with the plasmid MT-CAT was induced by 10~100 μM cadmium. The activities of the cadmium-treated cells were 6~46-fold higher than that of the nontreated cells (Fig. 4A). This is similar to the results in *Drosophila* S2 cells showing induction of MT mRNA levels by treatment of copper and cadmium (Bunch et al., 1988). The MT-*lacZ* gene expression in transformants was also induced by 10 μM cadmium (Fig. 5A). The MT-*lacZ* expression in transgenic third larval tissues treated with cadmium was induced especially in the fat body, which is analogous to the vertebrate liver and serves as storage point for food reserves. Increased *lacZ* expression by cadmium was also detected in brain lobe and ganglion (Fig. 6B).

It was reported that MT is an efficient scavenger of hydroxyl radicals (Abel and de Ruiter, 1989; Dalton et al., 1994; Dalton et al., 1996). The free radicals cause severe damage to critical cellular macromolecules, including nucleic acids, proteins, and lipids (Halliwell and Gutteridge, 1984). Xenobiotics (e.g. paraquat, diethyl maleate, menadione) can induce MT, and each induces the formation of free radicals (Bauman et al., 1991; Min et al., 1991; Bauman et al., 1992). β-Galactosidase activity of transgenic larvae was also increased by treatment of 50 and 100 μM paraquat (Fig. 5B). In transgenic larval tissues, the induction of MT-*lacZ* was observed in fat body, brain lobe and ganglion (Fig. 6C). It has been reported that the effect of oxidative stress on MT promoter activity may be mediated by factors interacting with metal response elements (Stuart et al., 1984; Silar et al.,

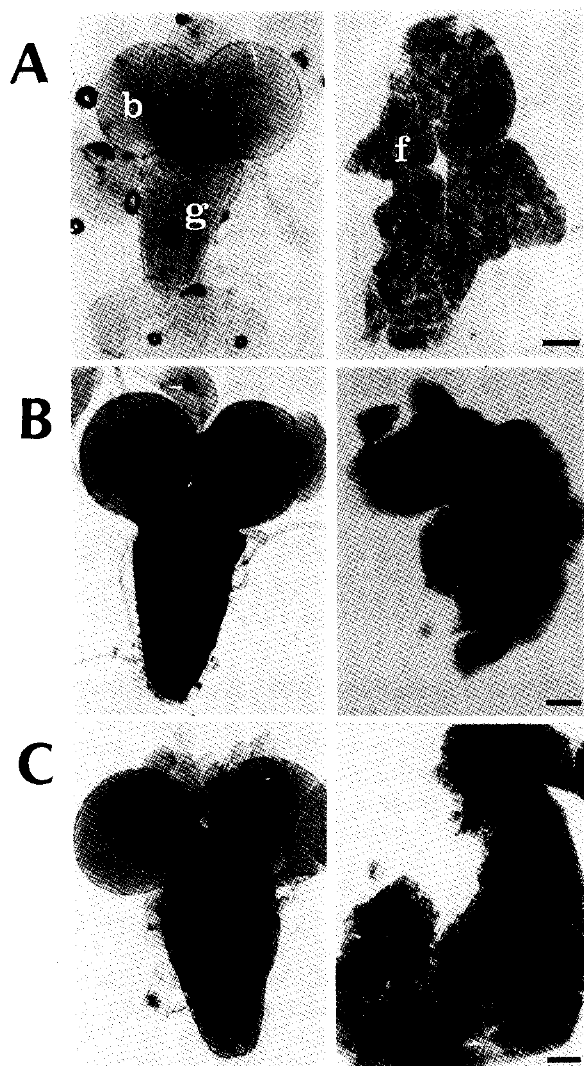

Fig. 6. Expression of the MT-*lacZ* in transgenic larval tissues treated with cadmium or paraquat. Seventy two-hour-larvae of host strain (A) and transgenic flies were cultured on medium containing 30 μM cadmium for 48 h (B) or 100 μM paraquat for 5 h (C). The larvae were dissected, fixed and incubated with a staining solution containing 0.2% X-gal in the dark at 37℃ for 5 to 16 h. b, brain lobe. g, ganglion. f, fat body. Scale bars = 300 μm.

1990) as well as antioxidant response elements (Dalton et al., 1994). However, the mechanism of regulation of MT gene expression remains to be clarified.

β-Galactosidase activity in transformants was increased by treatment with ≥10 μM cadmium or ≥50 μM paraquat. This result could indicate that transformants bearing the pMT-*lacZ* gene are highly sensitive against heavy metal and oxidative stress. Inductions of reporter gene or MT mRNA were detected by treatments with ≥500 μM $CdCl_2$ or 10 mM paraquat (Bonneton, 1996), and from 250 μM to 2.5 mM $CdCl_2$ (Silar et al., 1990) in *Drosophila*.

These results show that transformants bearing the MT-*lacZ* fusion gene established in this study are useful for further studies on the mechanism of regulation of the MT gene expression and for monitoring toxic metals.

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