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Antagonistic effects of ethyl methanesulfonate and maleic hydrazide in inducing somatic mutations in the stamen hairs of *Tradescantia* clone BNL 4430

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Mutagenic interaction between ethyl methanesulfonate (EMS; a monofunctional alkylating agent) and maleic hydrazide (MH; a promutagen activated into a mutagen in plants highly likely by peroxidase) was studied in the stamen hairs of *Tradescantia* clone BNL 4430, a blue/pink heterozygote. Since EMS has been shown to act synergistically with X rays in inducing mutations, and mutagenic synergisms have also been observed between X rays and MH by exposing to X rays before MH treatments, EMS and MH were expected to act synergistically at least by exposing to EMS before MH treatment. Three different combined treatments were conducted, i.e., by exposing for 4 h to 18.8 mM EMS 44 or 20 h before starting or 20 h after completing 1 mM MH treatments for 4 h. Unexpectedly, however, clear antagonistic effects in inducing somatic pink mutations were detected after all these combined treatments. Especially, the induced mutation frequency by exposing to EMS 44 h before the MH treatment was significantly lower than that induced by MH alone. The clear mutagenic antagonisms observed were thought to have resulted from EMS-caused inhibition of activation of MH by peroxidase, EMS ethylating and thus inactivating this enzyme or its precursors. Decreased peroxidase activities than those after treatments with MH alone were measured after two combined treatments, i.e., 12 h after the one exposing to EMS 44 h before MH and 24 h after the other exposing to EMS 20 h after MH, but the decreases were not large enough or their fluctuations were too large to judge them to be statistically significant. Comparisons of mutation frequencies induced by the combined treatments exposing to EMS before MH with those by MH alone suggest that there are some mechanisms (other than ethylation of peroxidase or its precursors) by which EMS suppresses the activation of MH.

INTRODUCTION

Surveys of mutagenic interactions among different mutagens and promutagens have become increasingly important because various kinds of mutagens and promutagens coexist in our environment. In fact, mutagenic synergisms among different mutagens have clearly been detected with *Tradescantia* stamen-hair system (Cebulska-Wasilewska et al., 1981; Ichikawa, 1992; Ichikawa et al., 1993; Shima and Ichikawa, 1994, 1995, 1997). The mutagenic synergisms detected so far are those among four monofunctional alkylating agents and X rays, i.e., between ethyl methanesulfonate (EMS) and X rays (Cebulska-Wasilewska et al., 1981; Shima and Ichikawa, 1994), methyl methanesulfonate (MMS) and X rays (Ichikawa, 1992; Ichikawa et al., 1993; Shima and Ichikawa, 1994), EMS and MMS (Shima and Ichikawa, 1994), dimethyl sulfate (DMS) and X rays (Shima and Ichikawa, 1995) and *N*-ethyl-*N*-nitrosourea (ENU) and X rays (Shima and Ichikawa, 1997). Differences in pattern of the synergisms have also been reported (Shima and Ichikawa, 1994, 1995, 1997).

Mutagenic interactions between X rays and three promutagens, maleic hydrazide (MH), *o*-phenylenediamine (PDA), and *N*-nitrosodimethylamine (DMN), have also been found to occur in *Tradescantia* stamen hairs (Xiao and Ichikawa, 1995, 1998a). Namely, both synergisms and antagonisms have been observed by X-raying before and after 4-h treatments with these promutagens, respectively, being clearly different from no antagonism found among above alkylating agents and X rays. When X-rayed during these promutagen treatments, however, synergistic,

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additive and antagonistic effects with X rays have been observed for DMN, PDA, and MH, respectively (Xiao and Ichikawa, 1995, 1998a). The involvement of peroxidase in activating MH into a mutagen in the floral tissues has been shown to be very likely, and the occurrences of such synergisms and antagonisms between MH and X rays have clearly been related to increases and decreases, respectively, in peroxidase activity (Xiao and Ichikawa, 1996). Mutagenic synergism has also been detected between X rays and 1,2-dibromoethane (EDB), a promutagenic and bifunctional alkylating agent (Xiao and Ichikawa, 1998b).

Therefore, occurrences of such mutagenic interactions must be taken into consideration for evaluating real risks of environmental mutagens and promutagens. Mutagenic synergisms, which have most serious implications for current risk evaluations of individual mutagens and promutagens, are considered to occur between different mutagenic agents which have some interrelated or at least partly common mechanisms of action, and no synergism would appear between different agents which cause entirely different damages on DNA (Shima and Ichikawa, 1994).

In the present study, mutagenic interaction between EMS and MH was investigated in the stamen hairs of *Tradescantia* clone BNL 4430 which has been used in earlier related studies (Ichikawa et al., 1993; Shima and Ichikawa, 1994, 1995, 1997; Xiao and Ichikawa, 1995, 1996, 1998a, 1998b). Since EMS has been shown to act synergistically with X rays (Cebulska-Wasilewska et al., 1981; Shima and Ichikawa, 1994), and MH has also been shown to act synergistically with X rays when exposed to X rays before MH treatments (Xiao and Ichikawa, 1995), EMS and MH were expected to act synergistically at least by exposing to EMS before MH treatment.

Peroxidase activities were also assayed in the inflorescences treated with EMS and MH in combinations, and were compared with those after treating with either EMS or MH alone and those in controls, in order to clarify the role of this enzyme in the mechanisms of mutagenic interactions, if occurred, of EMS and MH.

MATERIALS AND METHODS

Tester plants used. Young inflorescence-bearing shoots with roots of clone BNL 4430, the best *Tradescantia* mutagenicity tester plants as described earlier (Shima and Ichikawa, 1994; Ichikawa et al., 1995; Shima et al., 1997), were used in the present study. This clone is a diploid hybrid (2n = 12) between a blue-flowered *Tradescantia hirsutiflora* Bush and a pink-flowered *T. subacaulis* Bush (Emmerling-Thompson and Nawrocky, 1980), thus is a blue/pink heterozygote. This clone has been frequently used in studies of somatic mutations in the stamen hairs induced by ionizing radiations and various chemicals as reviewed earlier (Schairer and Sautkulis, 1982; Ichikawa, 1992), and has been shown to be more sensitive to alkylating agents than other clones (Sparrow et al., 1974; Nauman et al., 1976; Ichikawa et al., 1993).

Growing conditions. The young inflorescence-bearing shoots with roots were cultivated in a nutrient solution circulating (NSC) growth chamber as described earlier in detail (Shima et al., 1997). The environmental conditions in the NSC growth chamber were 22.0 ± 0.5°C during the 16-h day with a light intensity of 7,500 lux from white fluorescent tubes and 20.0 ± 0.5°C at night. The nutrient solution used was a 1/2,500 Hyponex solution.

Treatments with EMS or MH. Young inflorescences just before initiating flowering were treated with 18.8 mM EMS (Cas no. 62-50-0; Nacalai Tesque) or 1 mM MH (Cas no. 123-33-1; Wako Pure Chem. Ind., Ltd.) dissolved in 5 mM phosphate buffer (pH 7.0), for 4 h at 23.0 ± 0.5°C. Control inflorescences were treated with the phosphate buffer. Immediately after the treatments, inflorescences were washed with tap water for 2 min. The methods used for these treatments have been described elsewhere (Ichikawa and Takahashi, 1978; Ichikawa et al., 1990, 1993; Sanda-Kamigawara et al., 1991; Ichikawa, 1992; Shima and Ichikawa, 1994; Xiao and Ichikawa, 1995).

Combined treatments with EMS and MH. Young inflorescences were treated for 4 h with 18.8 mM EMS 44 or 20 h before starting or 20 h after completing 1 mM MH treatments for 4 h. These three combined treatments were conducted in Experiments 1, 2, and 3, respectively, and in each experiment, two positive controls treated with 18.8 mM EMS alone and 1 mM MH alone were set up for comparison, besides the control treated with 5 mM phosphate buffer.

Scoring mutations. The methods used for scoring pink mutations in the stamen hairs were identical to those described elsewhere in detail (Ichikawa, 1992). Based on the 3-week daily scorings of the numbers of hairs and pink mutants events (PMEs) as well as the average number of cells per hair, daily somatic mutation frequencies as the numbers of PMEs per 10^4 hair-cell divisions were calculated for each treatment. The data were pooled for the 4-day peak period of mutation frequency for each treatment as in earlier studies (Ichikawa et al., 1993; Shima and Ichikawa, 1994, 1995, 1997; Xiao and Ichikawa, 1995, 1998a, 1998b).

Statistical examinations. Chi-square test was used for determining the occurrence of a synergistic or an antagonistic effect, examining whether or not an induced mutation frequency obtained after a combined treatment with EMS and MH was significantly higher or lower than that expected from an additive effect of EMS and MH. The

expected value was obtained by simply summing induced mutation frequencies in the two positive controls in the same experiment.

Peroxidase assays. Changes in peroxidase activity were assayed using the same methods as those described earlier in detail (Xiao and Ichikawa, 1996), i.e., following the methods described by Gichner et al. (1994) except for minor modifications. For the peroxidase assays, five young inflorescences each treated with EMS alone, MH alone, EMS and MH in the above three different combinations, or with the phosphate buffer (controls) were harvested 0 (immediately), 12, and 24 h after completing the combined treatment in each experiment. Each measurement of the absorbance at 470 nm was made on five samples (each from one inflorescence) every 30 sec for 5 min using a Beckman DU 640 spectrophotometer, and the intermediate three out of the five values obtained were averaged to determine the peroxidase activity. Differences between the peroxidase activities assayed were examined by *t*-test.

RESULTS

EMS- and MH-induced mutation frequencies. The mutation frequencies per 10^4 hair-cell divisions induced by 18.8 mM EMS in Experiments 1 to 3 are presented in Table 1 (the second line in each experiment). The values obtained are consistent with the dose-response curve with a slope of 1.293 on a log-log graph determined earlier for EMS by Shima and Ichikawa (1994).

The mutation frequencies induced by 1 mM MH in Experiments 1 to 3 are also listed in Table 1 (the third line in each experiment). These frequencies are also consistent with the dose-response curve with a slope of 1.031 on a log-log graph determined earlier for MH (Xiao and Ichikawa, 1995).

Effects of combined treatments. The induced mutation frequencies per 10^4 hair-cell divisions determined after three combined treatments with 18.8 mM EMS and 1 mM MH are also presented in Table 1 (the fourth line in each experiment). Any of the three combined treatments, i.e., exposing to EMS 44 or 20 h before starting or 20 h after completing the MH treatments, produced significantly lower mutation frequencies (at 0.1% level) than those expected from their additive effects, showing that EMS and MH acted antagonistically. Especially, the mutation frequency obtained by exposing to EMS 44 h before the MH treatment was significantly lower (at 0.1% level) than that induced by MH alone (Experiment 1), and the frequency induced by exposing to EMS 20 h before the MH treatment was only comparable to that by MH alone (Experiment 2).

Peroxidase activities. The highest peroxidase activities assayed during the 5-min measurements (mostly obtained at 5 min) in the samples harvested 0, 12, and 24 h after completing all the treatments in each experiment were converted into the relative peroxidase activities (RPAs) against each control for easier comparison, and all such RPA values obtained are listed in Table 2.

As seen in Table 2, the RPAs obtained after exposing to EMS 44 h before starting the MH treatment (Experiment 1) were not different significantly from each control, although the value of 0.981 ± 0.022 obtained 12 h after the combined treatment (60 and 12 h after EMS and MH

Table 1. Antagonistic effects observed after combined treatments with 18.8 mM EMS and 1 mM MH in inducing somatic pink mutations in the stamen hairs of *Tradescantia* clone BNL 4430

EMS treatment	MH treatment	No. of hairs scored	No. of PMEs[a] scored	Average no. of cells/hair	No. of PMEs /10^4 cell divisions (± SE)	Minus control (± SE)	Expected[b]	*P*
Experiment 1 (EMS 44 h before MH)								
–	–	19,814	60	24.51	1.29 ± 0.17	—		
+	–	11,219	305	23.40	12.1 ± 0.7	10.9 ± 0.7		
–	+	8,654	388	20.82	22.6 ± 1.1	21.3 ± 1.2		
+	+	9,063	291	22.71	14.8 ± 0.9	13.5 ± 0.9	32.2	< 0.001
Experiment 2 (EMS 20 h before MH)								
–	–	19,103	72	24.03	1.64 ± 0.19	–		
+	–	18,091	451	22.85	11.4 ± 0.5	9.77 ± 0.57		
–	+	9,680	426	22.78	20.2 ± 1.0	18.6 ± 1.0		
+	+	18,962	766	21.56	19.6 ± 0.7	18.0 ± 0.7	28.3	< 0.001
Experiment 3 (EMS 20 h after MH)								
–	–	27,343	101	23.57	1.64 ± 0.16	–		
+	–	9,591	270	22.15	13.3 ± 0.8	11.7 ± 0.8		
–	+	10,617	453	21.63	20.7 ± 1.0	19.1 ± 1.0		
+	+	8,447	378	18.43	25.7 ± 1.3	24.0 ± 1.3	30.7	< 0.001

[a] Pink mutant events.
[b] Based on the additive effects of EMS and MH in the same experiment.

Table 2. The relative peroxidase activities (RPAs) against each control determined in the inflorescences treated with MH alone, EMS alone, or in combinations

Hours after MH	Hours after EMS	RPA ± SD by MH alone	RPA ± SD by EMS alone	RPA ± SD by MH + EMS	SD in control (1.000)
Experiment 1 (EMS 44 h before MH)					
0	48	0.975 ± 0.018	0.960 ± 0.069	1.000 ± 0.069	0.152
12	60	1.032 ± 0.017	1.038 ± 0.150	0.981 ± 0.022[a]	0.070
24	72	0.975 ± 0.027	0.988 ± 0.027	0.989 ± 0.076	0.149
Experiment 2 (EMS 20 h before MH)					
0	24	0.922 ± 0.002[b]	1.077 ± 0.092	1.039 ± 0.078	0.041
12	36	1.143 ± 0.066[c]	1.048 ± 0.052	1.136 ± 0.066[c]	0.077
24	48	1.076 ± 0.088	1.093 ± 0.038[d]	1.085 ± 0.077	0.022
Experiment 3 (EMS 20 h after MH)					
24	0	1.043 ± 0.095	0.944 ± 0.036	1.037 ± 0.077	0.103
36	12	1.075 ± 0.020[e]	0.975 ± 0.027	1.069 ± 0.011[e,f]	0.087
48	24	1.077 ± 0.050	1.002 ± 0.077	0.943 ± 0.099[g]	0.053

[a] Not but nearly significantly lower than MH alone ($0.10 < p < 0.05$).
[b] Not but nearly significantly lower than control ($0.10 < p < 0.05$).
[c] Not but nearly significantly higher than control ($0.10 < p < 0.05$).
[d] Significantly higher than control ($p < 0.05$).
[e] Not significantly but tended to be higher than control ($0.20 < p < 0.10$).
[f] Significantly higher than EMS alone ($p < 0.05$).
[g] Not significantly but tended to be lower than MH alone ($0.20 < p < 0.10$).

treatments, respectively) was nearly significantly lower than 1.032 ± 0.017 obtained 12 h after the MH treatment alone ($0.05 < p < 0.10$).

The RPAs in the samples exposed to EMS 20 h before starting the MH treatment (Experiment 2) were not different significantly from each control (Table 2), but the RPA of 1.136 ± 0.066 obtained 12 h after the combined treatment (36 and 12 h after EMS and MH treatments, respectively) was nearly significantly higher than its control ($0.05 < p < 0.10$). The values of 0.922 ± 0.002 and 1.143 ± 0.066 at 0 and 12 h after the MH treatment alone were nearly significantly lower and higher than each control ($0.05 < p < 0.10$), respectively, and 1.093 ± 0.038 obtained 48 h after the EMS treatment alone was significantly higher (at 5% level) than its control (Table 2).

The RPAs in the samples exposed to EMS 20 h after completing the MH treatment (Experiment 3) were not different significantly from each control, but the value of 1.069 ± 0.011 at 12 h after the combined treatment (36 and 12 h after MH and EMS treatments, respectively) tended to be higher than its control ($0.10 < p < 0.20$) and was significantly higher (at 5% level) than 0.975 ± 0.027 obtained 12 h after the EMS treatment alone, as shown in Table 2. The value of 1.075 ± 0.020 obtained 36 h after the MH treatment alone also tended to be higher than its control ($0.10 < p < 0.20$). On the other hand, the value of 0.943 ± 0.099 at 24 h after the combined treatment (48 and 24 h after MH and EMS treatments, respectively) tended to be lower than 1.077 ± 0.050 obtained 48 h after the MH treatment alone ($0.10 < p < 0.20$).

The RPA values listed in Table 2 show that both MH and EMS tended to cause increases in peroxidase activity (12 to 48 and 24 to 60 h after MH and EMS treatments, respectively), the increments with MH tending to be larger.

DISCUSSION

Antagonistic effects observed. It has been considered that mutagenic synergisms occur between different mutagens which have some interrelated or at least partly common mechanisms of action, and that no synergism would appear between different agents which cause entirely different damages on DNA (Shima and Ichikawa, 1994). It has also been thought most likely that DNA single-strand breaks produced commonly by X rays and several alkylating agents or promutagens are the major cause of the mutagenic synergisms observed earlier (Ichikawa et al., 1993; Shima and Ichikawa, 1995, 1997; Xiao and Ichikawa, 1995, 1998a, 1998b).

The action of MH on chromosomes has been described to be very similar to those of alkylating agents (Swietlinska and Zuk, 1978). Clear mutagenic synergisms have been observed between EMS and X rays (Cebulska-Wasilewska et al., 1981; Shima and Ichikawa, 1994) and between MH and X rays when X-rayed before MH treatments (Xiao and Ichikawa, 1995). Also, the slope values of the dose-response curves for EMS and X rays on log-log graphs have been confirmed to be comparable to each other (Shima and Ichikawa, 1994). Therefore, it was expected that synergistic effects must be observed between EMS and MH, at least by exposing to EMS before MH treatment.

Contrary to the expectation, however, clearly antagonistic effects of EMS and MH in inducing somatic mutations were observed after all the combined treatments conducted

(Table 1). The mutation frequencies induced by exposing to EMS before MH treatments (Experiments 1 and 2) were even lower than or only comparable to those induced by MH alone, and the frequency obtained by exposing to EMS 44 h before MH treatment (Experiment 2) was significantly lower than that by MH alone. It seemed therefore most likely that EMS caused strong inhibition of activation of MH, rather than that EMS- and MH-induced DNA strand breaks interacted to cause synergistic effects.

Inhibition of activation of MH. It is well known that MH is not only a potent clastogen (Darlington and McLeish, 1951; Evans and Scott, 1964; Swietlinska and Zuk, 1978), but also a promutagen which is activated in plant cells and becomes mutagenic (Gichner et al., 1982; Plewa and Gentile, 1982; Heindorff et al., 1984; Velemínsky and Gichner, 1988). That is, the activation of MH is necessary before MH shows its mutagenic effects.

It has been shown to be very likely that peroxidase is involved in activating MH into a mutagen in *Tradescantia* clone BNL 4430, and the occurrences of mutagenic synergisms and antagonisms have been found to be clearly related to the increases and decreases in peroxidase activity, respectively, after different combined treatments with MH and X rays (Xiao and Ichikawa, 1996). Alkylating agents including EMS are known to alkylate proteins as well as DNA (Vogel and Natarajan, 1982). Therefore, it seemed likely that the activation of MH was severely inhibited because of inactivation of peroxidase or its precursors caused by ethylation of them by EMS when exposed to EMS before MH treatments (Experiments 1 and 2), resulting in the obvious antagonistic effects observed (Table 1). The activation of MH also seemed to have been suppressed by inactivation of peroxidase by EMS when exposed to EMS after MH treatment (i.e., during the activation period of MH; Experiment 3), resulting also in the antagonistic effect observed (Table 1).

Changes in peroxidase activity in the floral tissues assayed after treating with MH alone showed that the RPA against each control tended to increase 12 to 48 h after the treatments (Table 2), roughly agreeing with earlier results (Xiao and Ichikawa, 1966). The RPA also tended to increase 24 to 60 h after treatments with EMS alone, suggesting that EMS also increased peroxidase activity, although the increases seemed smaller than with MH (Table 2).

Therefore, the relatively high RPA value obtained 12 h after the combined treatment in Experiment 3 (36 and 12 h after MH and EMS, respectively), which was significantly higher than that with EMS alone (Table 2), is considered to be solely due to MH. On the other hand, the decreased activity obtained 24 h after the same combined treatment (48 and 24 h after MH and EMS, respectively) tended to be lower than that with MH alone (their standard deviations were too large to judge them to be significantly different; Table 2), suggesting an EMS-caused suppression of peroxidase activity during the activation period of MH, which might have suppressed the activation of MH into a mutagen resulting in the antagonistic effect observed (Table 1).

The RPA value obtained 12 h after the combined treatment in Experiment 1 (60 and 12 h after EMS and MH, respectively) was nearly significantly lower than that with MH alone (Table 2), and it may also suggest an EMS-caused suppression of peroxidase activity. However, no other evident information could be obtained from the peroxidase assays in Experiments 1 and 2 exposing to EMS before MH treatments (Table 2), in spite of the clear antagonistic effects observed (Table 1). Almost identical RPAs in the samples treated with EMS alone, MH alone or in combination in Experiment 2 and harvested 24 h after the MH and the combined treatments (Table 2) seem to indicate even that no or little inhibition of peroxidase activity was caused by EMS.

On the other hand, as seen in Table 1, the somatic mutation frequencies induced by the combined treatments in Experiments 1 and 2 were obviously higher than those by EMS alone, whereas they were significantly lower than or only comparable to those by MH alone. These results indicate that the effects of MH were certainly suppressed strongly by EMS applied before the MH treatments. No evident changes in peroxidase activity detected in the samples exposed to EMS before the MH treatments (Table 2) may thus suggest that there are some mechanisms (other than ethylation of peroxidase or its precursors) by which EMS suppresses the activation of MH.

The relatively small increases in peroxidase activity by EMS (Table 2), which are much smaller than those by X rays reported earlier (Xiao and Ichikawa, 1996), may be related to the present unexpected results obtained by exposing to EMS before the MH treatments, being quite different from the synergistic effects detected earlier by X-raying before MH treatments (Xiao and Ichikawa, 1995). Anyway, however, no clear-cut clue was found from the peroxidase assays for the mechanisms of resulting in the obvious antagonisms observed when exposed to EMS before MH treatments.

The results obtained in the present and earlier studies (Xiao and Ichikawa, 1995, 1998a) indicate that the mechanisms of mutagenic interaction between EMS and MH are obviously different from those between X rays and three promutagens, MH (Xiao and Ichikawa, 1995), PDA, and DMN (Xiao and Ichikawa, 1998a). The interactions of EMS with MH are also quite different from those between X rays and four monofunctional alkylating agents, EMS (Cebulska-Wasilewska et al., 1981; Shima and Ichikawa, 1994), MMS (Ichikawa, 1992; Ichikawa et al., 1993; Shima and Ichikawa, 1994), DMS (Shima and Ichikawa, 1995), and ENU (Shima and Ichikawa, 1997), and between EMS and MMS (Shima and Ichikawa, 1994).

The mutagenic interactions between EMS and MH observed in the present study show more clearly than ever that the effects on the activation processes must be taken into consideration in cases of dealing with promutagens.

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