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Analysis of Gene Expression upon DNA Damage in *Arabidopsis*

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To understand their responses to DNA damage, *Arabidopsis* plants were exposed to methyl methanesulfonate (MMS). Such treatment inhibited growth and decreased the leaf chlorophyll content. A concomitant change in the expression of cell cycle-related genes also occurred. *CYCB1* expression was slightly increased but that of *CYCD1* declined. To identify the genetic elements in these responses, genome-wide transcription profiling of *Arabidopsis* was performed following the MMS treatment, using a cDNA microarray. Expression was altered by more than two-fold for 3666 genes – i.e., 1657 genes showed an increase and 2009 genes had a decrease in transcripts. Five DNA repair-related genes were slightly enhanced, while several disease resistance-related and glutathione transferase genes were strongly up-regulated. Interestingly, 27 ubiquitin-related genes were also altered by more than two-fold, suggesting that protein degradation may have been involved in those damage responses. These microarray results were validated by RT-PCR.

Keywords: Arabidopsis, DNA damage, methyl methanesulfonate, microarray

Plants are constantly exposed to environmental stresses. Their DNA can be damaged by ionizing and solar radiation, the process of DNA replication, or genotoxic chemicals, e.g., methyl methanesulfonate, N-methyl-N-nitrosourea, mitomycin C, bleomycin, and maleic hydrazide (Menke et al., 2001). Unlike other organisms, plants cannot minimize their exposure to these harmful agents, and must rely instead on inherent and rapid responses to such damage. Although the expression of stress-responsive genes following biotic or abiotic stress (Maleck et al., 2000; Schenk et al., 2000; Desikan et al., 2001; Chen et al., 2002; Seki et al., 2002; Ashrafuzzaman et al., 2005) has been extensively investigated, a genome-wide analysis of those responses to genotoxins is only partial. Chen et al. (2003) have developed a high-density colony array that covers only approximately 40% of the *Arabidopsis* genes, and a complete global view of genotoxic effects on the transcriptome of *Arabidopsis* is not yet available. Therefore, the objective of this study was to use an Affymetrix ATH1 chip covering 24,000 genes to assess the expression profile when *Arabidopsis* seedlings were exposed to 100 ppm methyl methanesulfonate (MMS). Data were then confirmed via RT-PCR.

MATERIALS AND METHODS

Plant Materials and MMS Treatment

All *Arabidopsis thaliana* plants were of the Columbia (Col-0) genotype, unless otherwise specified. Seeds were germinated in a ½-strength Murashige and Skoog (MS) solid medium without sucrose, at 24-25°C under long-day conditions (16-h photoperiod). Ten-day-old seedlings were then transferred to ½-strength liquid sucrose-free MS media with or without a supplement of 40 to 200 ppm MMS. They were grown with shaking for 1 to 8 d. After harvesting, the seedlings were either used immediately for RNA isolation or frozen in liquid nitrogen and stored at -80°C.

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Measurement of Chlorophyll Content

Chlorophyll was extracted with 80% acetone, and its content determined spectrophotometrically [chlorophyll a (μg mL^{-1}) = 12.21 X A_{663} – 2.81 X A_{646}, chlorophyll b (μg mL^{-1}) = 20.13 X A_{646} – 5.03 X A_{663}] at 663 and 646 nm, respectively, according to the method of Lichtenthaler and Wellburn (1983).

Microarray Experiments

Ten micrograms of total RNA was used for each hybridization on Affymetrix ATH1 Chips (USA), representing 24,000 *Arabidopsis* genes. The data were first analyzed with microarray Suite 5.0 software (Affymetrix). For each microarray, an overall intensity normalization was performed for entire probe sets. Using the default parameters for GeneChip Suite 5.0, detection P-values and signal values were calculated for each probe set. The P-values generated by this analysis determined whether the transcripts were being reliably detected. For functional classification, Affymetrix ATH1 Chip annotation was used, based on data from the TIGR database.

RT-PCR Analysis

Total RNA was extracted with an RNeasy Plant mini kit, according to the manufacturer's protocol (Qiagen, Germany). For RT-PCR analysis, first-strand cDNA was synthesized, with Superscript reverse transcriptase (Invitrogen, USA), from 1 to 2 μg of total RNA in a 20 μL reaction volume. Afterward, 1 μL of the reaction mixture was subjected to PCR in a 20 μL reaction volume. RT-PCR runs consisted of 20 to 30 cycles, each comprising 94°C for 1 min, 50 to 60°C for 30 s, and 72°C for 1 min; followed by a final step of 72°C for 10 min to complete the polymerization. Primers included: for CycB1, 5'-gaatcattgg gagagcgatc-3' and 5'-gaattttgaa tcagagagag at-3'; CycD1, 5'-caaggggaaggaataagagt-3'and 5'-aaacaaacacgtgagacaca-3'; at3g60140, 5'-acggtacaaaaccaagagaa-3' and 5'-catgtccaaccataaggaat-3'; at1g05680, 5'-agatcaaatgttggaactcg-3' and 5'-cctccataacttcttccaca-3'; at4g37370, 5'-atggaaaccaaaaccctaat-3' and 5'-gcttctcgtttctcatcaac-3'; at5g13080, 5'-cttcttttggaattcaggtg-3' and 5'-gcatggtttttcttttcaac-3'; and TUB, 5'- ctcaagaggttctcagcagta-3'

and 5'- tcaccttcttcatccgcagtt-3'.

RESULTS AND DISCUSSION

To study the effect of DNA damage on their growth, liquid-cultured *Arabidopsis* plants were treated with the alkylating agent MMS. At a concentration of 60 ppm, the plants turned white (Fig. 1A) and stopped growing due to cell death, while untreated plants continued their development.

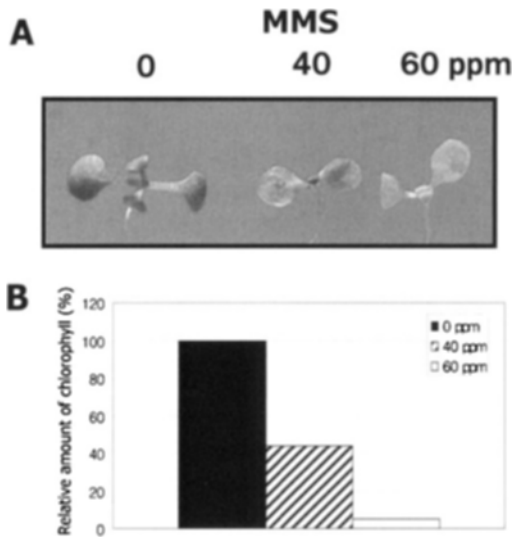

Figure 1. Effects of methyl methanesulfonate on growth of *Arabidopsis*. **A**, Seedlings were treated with various concentrations of MMS. Photographs taken after 8 d. **B**, Chlorophyll content of MMS-treated and untreated seedlings, presented as percentage compared with untreated seedlings.

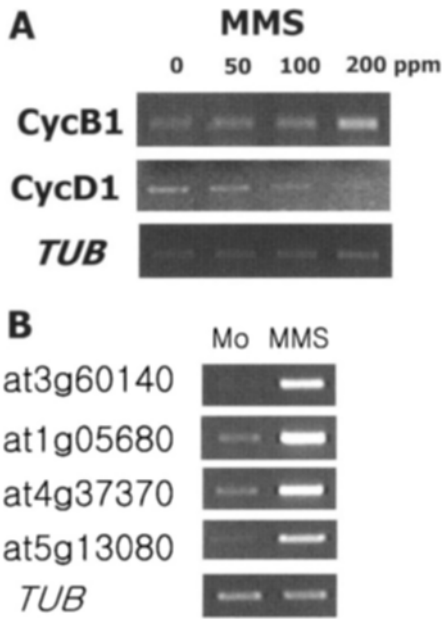

Figure 2. Reverse transcription-PCR analysis. **A**, Cyclin gene expression after MMS exposure. Seedlings were treated with indicated amount of MMS for 24 h. **B**, Verification of microarray data. Four genes were selected and their expression was tested after treatment with 100 ppm MMS for 24 h.

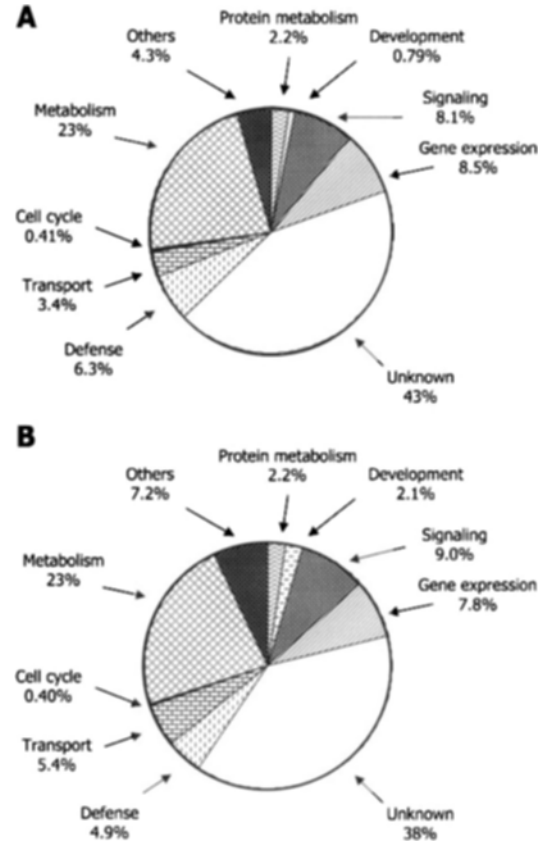

Figure 3. Functional classification of genes in which expression was changed >2-fold after MMS treatment. **A**, Induced genes. **B**, Repressed genes.

MMS treatment caused an almost entire loss of chlorophyll (Fig. 1B).

DNA damage can lead to cell cycle arrest (Wilson, 2004), which can affect gene expression. To test this, RT-PCR was performed after the MMS treatment. Expression of *CYCB1* was slightly increased while that of *CYCD1* was greatly decreased, suggesting that DNA damage may have influenced the cell cycle through the latter gene (Fig. 2A). Chen et al. (2003) also have found altered expression of the genes that regulate the cell cycle in *Arabidopsis* seedlings treated with several genotoxic reagents. There, transcript levels of *CYCB1* were greatly increased (in accordance with these current results), while those of *CYCD3*, *CYCB2*, *RB*, and *KRP1* were unchanged. Furthermore, *WEE1* was up-regulated by their treatment. Based on their results and those reported here, one can conclude that DNA damage may inhibit the cell cycle through the down-regulation of *CYCD1*, a positive regulator, as well as via the up-regulation of *WEE1*, a negative regulator of that cycle (Sorrell et al., 2002).

To further characterize the plant response, genome-wide transcription profiling was performed using a cDNA microarray. In previous research, Chen et al. (2003) studied an array covering about 10,000 genes (corresponding to at least 40% of the *Arabidopsis* genes). Here, an Affymetrix ATH1 chip covering approximately 24,000 genes was utilized. When seedlings were treated with 100 ppm MMS for 24 h, the expression of 3666 genes was changed by more than two-fold. Among them, 1657 were up-regulated while 2009

Table 1. Genes induced by more than 10-fold upon MMS treatment.

Gene ID	Fold change	Name
At4g04610	36.2	5-Adenylsulfate reductase
At3g60140	30.1	Beta-glucosidase
At1g17170	30.0	Glutathione transferase, putative
At1g10585	26.4	Unknown
At1g05680	19.0	Indole3-acetate beta-glucosyltransferase
At3g05360	19.0	Disease resistance protein family (LRR)
At1g26380	18.7	FAD-linked oxidoreductase family
At1g72920	18.6	Disease resistance protein (TIR-NBS), putative
At5g51440	17.4	Mitochondrial heat shock 22kd protein-like
At5g22300	17.1	Nitrilase 4
At1g17180	15.7	Glutathione transferase, putative
At2g21640	15.4	Unknown
At1g72940	14.6	Disease resistance protein (TIR-NBS), putative
At4g21990	14.5	PRH26 protein
At4g37370	13.8	Cytochrome P450 family
At3g50930	13.8	BCS1 protein-like protein
At2g29460	13.6	Glutathione transferase, putative
At1g72900	13.2	Disease resistance protein (TIR-NBS), putative
At5g67080	12.6	Protein kinase-like
At5g62480	12.3	Glutathione transferase, putative
At2g15490	12.1	Putative glucosyltransferase
At2g15480	11.7	Putative glucosyltransferase
At1g69920	11.3	Glutathione transferase, putative
At1g23550	11.1	Unknown
At1g57630	10.9	Disease resistance protein (TIR), putative
At3g60120	10.8	Unknown
At3g04210	10.6	Disease resistance protein (TIR-NBS), putative
At4g21390	10.5	Serine/threonine kinase-like
At4g39940	10.4	Adenosine-5-phosphosulfate kinase
At4g37290	10.3	Unknown

were down-regulated.

Of the induced genes, 43% had unknown functions (Fig. 3A), whereas the rest were primarily involved in metabolism (23.0%), gene expression (8.5%), and signaling (8.1%); only a relatively low percentage functioned in the cell cycle (0.41%) and plant development (0.79%). A similar classification for repressed genes was noted here, where the functioning of a relatively large fraction (38%) was unknown and 23% of the remainder were involved in metabolism (Fig. 3B). Defense-related roles could be assigned to 6.3% and 4.9% of the induced and repressed genes, respectively. This result suggests that DNA damage and other biotic and abiotic stresses share common signaling pathways. Overall, this microarray analysis demonstrated that DNA damage can lead to extensive changes in plant metabolism and gene expression.

The expression of 30 genes was enhanced more than 10-fold (Table 1), with the most pronounced increase (>30-fold) being identified with adenylsulfate reductase, glucosidase, and glutathione S-transferase (GST). Among those 30 with elevated expression, 5 were associated with GST, while 6 were related to disease resistance (Table 1). In addition, the expression of 94 genes was decreased more than 10-fold by MMS treatment (Table 2), the most prominent being peroxidase, lipid transfer protein (LTP)/protease inhibitor/seed storage, and myrosinase-associated protein genes. In addition, eight different LTP family genes were down-regulated more than 10-fold, including three different genes for chlorophyll A-B binding proteins (LHCB). These results helped to explain the damage-induced loss of chlorophyll content seen in treated seedlings (Fig. 1B).

The 1657 up-regulated genes included 5 DNA repair-related genes, e.g., RAD54-like, RAD51-like, 2 DNA repair proteins, and the DNA mismatch repair protein MutS homolog 7. Although Chen et al. (2003) have reported that the puta-

Table 2. Genes decreased by more than 10-fold upon MMS treatment.

Gene ID	Fold change	Name	Gene ID	Fold change	Name
At1g04040	-10.1	Uknown	At1g01620	-11.9	PIP1C
At1g75800	-10.2	Thaumatin	At3g54700	-12.0	AtPT2
At1g55260	-10.2	Uknown	At1g70850	-12.1	Csf-2-related
At3g18280	-10.3	Lipid transfer protein	At3g19710	-12.1	Aminotransferase
At1g28290	-10.4	Proline-rich	At3g27690	-12.2	Lhcb2
At1g09750	-10.4	Uknown	At1g75500	-12.3	Lhcb2
At4g36540	-10.4	Uknown	At5g11420	-12.3	Uknown
At1g23090	-10.4	Sulfate transporter	At5g64100	-12.3	Peroxidase
At3g59930	-10.6	Defensin-like	At2g01520	-12.4	MLP-related
At4g37410	-10.7	Cytochrome P450	At2g05540	-12.5	Glycine-rich protein
At3g26450	-11.0	MLP-related	At2g17740	-12.6	DC1 domain protein
At3g15950	-11.2	DNA topoisomerase-related	At4g11650	-13.1	Osmotin-like
At1g68560	-11.2	Alpha-xylosidase	At4g01700	-13.1	Chitinase
At2g15050	-11.3	Lipid transfer protein	At4g33220	-13.2	Pectinesterase
At2g39200	-11.4	MLO family	At5g20630	-13.2	Germin-like
At5g54280	-11.4	Lhcb3	At1g52190	-13.3	POT family
At1g14280	-11.5	Phytochrome kinase substrate, putative	At1g30110	-13.4	Diadenosine tetraphosphate hydrolase
At1g22500	-11.7	RING finger protein	At1g62500	-13.4	LTP family
At5g62280	-11.7	Uknown	At3g59060	-13.5	bHLH protein
At4g37800	-11.8	Xyloglucanendo-transglycosylase	At1g33811	-13.7	Lipase
At1g58270	-11.8	MATH domain containing protein	At5g48485	-14.0	LTP family
At4g30190	-11.8	ATPase type 2	At2g29995	-14.1	Uknown
At5g65010	-11.8	ASN2	At1g19610	-14.1	PDF1.4, putative
At4g30170	-11.8	Peroxidase	At3g04720	-14.3	HEL protein

Table 2. Continued

Gene ID	Fold change	Name	Gene ID	Fold change	Name
At4g21960	-11.8	Peroxidase	At5g46050	-14.3	POT family
At4g14130	-14.5	Putative XTR7	At5g20700	-19.5	SAG102
At4g17870	-15.1	Expressed protein	At3g16370	-20.3	Hydrolase
At3g59060	-15.1	Uknown	At4g11320	-21.2	Cysteine proteinase
At5g44020	-15.3	Acid phosphatase	At5g64570	-21.3	Glycosyl hydrolase
At1g32450	-15.3	POT family	At1g68530	-21.4	CUT1
At2g06850	-15.4	EXGT-A1	At5g15230	-21.4	GASA4
At1g02640	-15.5	Glycosyl hydrolase	At4g02330	-22.6	Pectinesterase
At5g23010	-16.2	IMS3	At2g29980	-23.7	FAD3
At5g53880	-16.2	Uknown	At5g64120	-25.3	Peroxidase
At4g22212	-16.3	Defensin-like	At1g62510	-25.4	LTP family
At2g43620	-16.5	Chitinase	At1g12090	-27.7	LTP family
At4g30140	-16.7	Lipase	At1g69530	-29.8	Expansin-like
At2g41560	-17.1	Ca+ATPase	At4g12490	-32.2	LTP family
At2g36690	-17.2	Oxidoreductase	At4g12550	-33.3	LTP family
At1g76930	-18.1	Extension-like	At1g74670	-37.5	Gibberellin-regulated
At5g23020	-18.3	IMS2	At5g48490	-41.2	LTP family
At3g19850	-18.6	NPH family	At2g28630	-48.7	Ketoacyl-CoA synthase
At1g13300	-18.7	Myb family	At3g50740	-52.7	UDP-glucosyl transferase
At2g01530	-19.3	MLP-related	At1g29670	-58.4	Hydrolase
At2g10940	-19.4	LTP family	At3g14210	-70.7	Myrosinase-associated
At2g23600	-19.5	Hydrolase	At4g12500	-74.3	LTP family
At1g75750	-19.5	GASA1	at2g37130	-75.7	Peroxidase

Table 3. Changes in expression for DNA repair-related genes upon MMS treatment.

Gene ID	Fold change	Name
At3g19210	3.3	RAD54, putative
At5g20850	3.5	Rad51-like
At1g03190	2.3	putative DNA repair protein
At1g30480	2.4	DNA damage repair protein, putative
At3g24492	2.4	DNA mismatch repair protein MutS homolog 7

tive ribonucleotide reductase small subunit gene (*RNR2*), associated with the damage response, is greatly increased by genotoxic treatment, this current investigation showed no up-regulation of *RNR2*. Other defense-related genes, such as the glutathione S-reductase and calmodulin-like genes, were found in both the current study and by Chen et al. (2003). Thus, the contents of those lists did not always overlap for several reasons. For example, in the previous research, *Arabidopsis* suspension cultures were treated with bleomycin and mitomycin C for 6 h (Chen et al., 2003). These differences in genotoxic agent, treatment time, and choice of plant materials may explain the discrepancy in results.

The ubiquitin (Ub)/26S proteasome pathway regulates a wide variety of processes, e.g., growth hormone-signaling and cell-cycling, by selectively removing specific proteins (Deshaies, 1999; Hellman and Estelle, 2002; Itoh et al., 2003; Risseeuw et al., 2003; Vierstra, 2003). In this pathway, proteins are modified with polymers of ubiquitin by an E1, E2, and E3 enzymatic cascade. The ubiquitinated proteins are then recognized and degraded by the 26S proteasome with the release of the undigested ubiquitins (Small and Vierstra, 2004). Members of the E3 family are the most

Table 4. Ubiquitin-related genes changed by at least two-fold upon MMS treatment.

Gene name	Fold change	Name
at4g29040	4.1	26S Proteasome AAA ATPase subunit RPT2a
at3g03060	2.6	26S Proteasome regulatory subunit, putative
at5g58290	2.5	26S Proteasome AAA ATPase subunit RPT3
at4g38630	2.1	26S Proteasome regulatory subunit S5A (RPN10)
at1g04810	2.0	26S Proteasome regulatory subunit (RPN2), putative
at1g45000	2.0	26S Proteasome AAA ATPase subunit 4, putative
at1g53750	2.8	26S Proteasome AAA ATPase subunit RPT1a
at5g45900	2.1	E1-Like protein
at1g75440	2.1	E2 (UBC16)
at3g08690	2.7	E2 UBC11
at1g69670	2.4	Cullin, putative
at2g25700	2.6	ASK1, putative
at1g68050	3.4	FKF1
at5g27750	2.1	F box protein
at1g25280	2.0	F box containing tubby family protein
at5g27420	2.6	RING H2 zinc finger-like
at1g74410	3.0	Putative RING protein
at3g16720	2.1	Putative RING protein
at3g05200	2.1	Putative RING protein ATL6
at3g61790	2.1	SINAT3a
at2g02350	2.9	SKP1 interacting protein3, putative
at2g21950	2.1	SKP1 interacting protein6, putative
at4g02890	2.2	UBQ14
at4g38930	2.1	Putative ubiquitin-dependent proteolytic protein
At1g76410	-3.2	Putative RING zinc finger
At3g23880	-3.4	F-box protein
At4g03190	-3.9	F-box protein
At3g61460	-4.6	RING finger protein

diverse components in the pathway; they include the RING finger protein, an SCF complex containing the F box protein, and HECT domain proteins. When *Arabidopsis* seedlings were challenged here with MMS, the expression of several subunits of 26S proteasome, E1, E2, F box protein, and RING finger proteins was changed (Table 4). This suggests that the responses to DNA damage involve extensive protein turnover.

To validate these results, RT-PCR was performed. Four of the most strongly induced genes were selected, and all again showed enhanced induction upon MMS treatment, thereby confirming the microarray data (Fig. 2B).

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