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The transcriptional response of *Arabidopsis* to genotoxic stress – a high-density colony array study (HDCA)

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Summary

A genome-wide transcription profiling of *Arabidopsis* upon genotoxic stress has been performed using a high-density colony array (HDCA). The array was based on a library of 27 000 cDNA clones derived from *Arabidopsis* cells challenged with bleomycin plus mitomycin C. The array covers more than 10 000 individual genes (corresponding to at least 40% of *Arabidopsis* genes). After hybridisation of the HDCA with labelled cDNA probes obtained from genotoxin-treated (bleomycin plus mitomycin C, 6 h) and untreated seedlings, 39 genes revealed an increased and 24 genes a decreased expression among the 3200 highly expressed clones (representing approximately 1200 individual genes because of redundancy of the cDNA library). Of the 4900 clones with a low transcriptional level, the expression of 500 clones was found to be altered and 57 genes with increased and 22 genes with decreased expression were identified by sequence analysis of 135 identified clones. The HDCA results were validated by real-time PCR analysis. For about 80% of genes (34 out of 42), alteration in expression was confirmed, indicating the reliability of the HDCA for transcription profiling. DNA damage and stress-responsive genes encoding, for instance transcription factors (myb protein and WRKY1), the ribonucleotide reductase small subunit (RNR2), thymidine kinase (TK), an AAA-type ATPase, the small subunit of a DNA polymerase and a calmodulin-like protein were found to be strongly upregulated. Also, several genes involved in cell cycle regulation revealed significant alteration in transcription, as detected by real-time PCR analysis, suggesting disturbance of cell cycle progression by mutagen treatment.

Keywords: *Arabidopsis*, colony array, genotoxin, real-time PCR, transcriptome.

Introduction

Living beings are constantly exposed to biotic and abiotic challenges from the environment. Responses to these challenges have been developed during evolution. In plants, responses such as the rapid synthesis of stress-responsive proteins are particularly important because of their sessile lifestyle. Expression of stress-responsive genes upon biotic threats (Chen *et al.*, 2002; Glazebrook, 1999; Maleck *et al.*, 2000; Schenk *et al.*, 2000) or abiotic hazards (Chen *et al.*, 2002; Desikan *et al.*, 2001; Seki *et al.*, 2002; Thimm *et al.*, 2001) has been extensively analysed. A large number of studies with one group of the abiotic hazards, the genotoxins, including alkylating agents, UV light, ionising irradiation and DNA-chelating or -modifying chemicals, have been conducted. Genotoxins may destroy or alter the genetic information directly or via mis-repair and are used to study DNA damage and repair in human cells (Meyer *et al.*, 2002; Sesto *et al.*, 2002; Tusher *et al.*, 2001), in yeast (Hanway *et al.*, 2002; Mercier *et al.*, 2001; Paesi-Toresan *et al.*, 1998; Schaus *et al.*, 2001) and in plants (Doucet-Chabeaud *et al.*, 2001; Gallego *et al.*, 2000; Lebel *et al.*, 1993; Li *et al.*, 2002; Liu *et al.*, 2000; Puchta *et al.*, 1995; Vonarx *et al.*, 1998; West *et al.*, 2000).

In the past, expression analysis could only be performed at a single-gene dimension. The recent development of array technologies enables the expression of tens of thousands of genes to be studied simultaneously (Aharoni and Vorst, 2001; Lockhart and Winzeler, 2000; Zhu and Wang, 2000) and even the analysis of the genome-wide transcription profiles. Genome-wide expression profiles in response to DNA-damaging conditions have been successfully studied in yeast (Schaus *et al.*, 2001). New repair genes were identified, and clustering of repair genes according to their

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expression kinetics was performed. Applications of DNA arrays to study transcriptome alterations in *Arabidopsis* have contributed to the elucidation of biotic and abiotic stress responses (Schenk *et al.*, 2000; Seki *et al.*, 2002). However, a global view as to genotoxic effects on the transcriptome of *Arabidopsis* is not yet available. We therefore analysed the genome-wide expression profile for *Arabidopsis* in response to DNA damage with high-density colony array (HDCA). The usually low transcript abundance of most repair genes under non-induced conditions results in the risk of their under-representation within libraries derived from untreated controls. The colony arrays were thus generated with clones of a cDNA library derived from genotoxin-treated cells. To confirm the data obtained by the HDCA, 42 of the up- or downregulated genes were re-evaluated by real-time PCR. Real-time PCR analysis was extended to study effects of DNA damage on cell cycle genes as well as on known repair genes.

Results

Global investigation of transcriptome alterations after genotoxic treatments requires an effective induction of DNA damage and a large-scale detection method to determine the transcription level of individual genes. The flow chart (Figure 1) shows our strategy to study the genotoxic effect on the *Arabidopsis* transcriptome. Suitable genotoxin treatment conditions were determined using the Comet assay for the detection of DNA damage, and dot blot analysis for the detection of transcriptional induction of damage-inducible marker genes. A cDNA library largely representing the transcriptome of genotoxin-treated *Arabidopsis* cells was then established, and the insert size and redundancy of the clones were controlled. An HDCA with 27 000 clones was generated and used for expression analysis. Clones showing altered expression were identified and sequenced. Real-time PCR analysis was used to verify the results from the HDCA.

Application of dot blot analysis to obtain conditions for upregulation of DNA repair genes

Previously we have used the Comet assay to study the integrity of *Arabidopsis* genome after exposure to genotoxins (Menke *et al.*, 2001). The alkylating agents methyl methane sulphonate (MMS) and methyl nitroso urea (MNU), the radiomimetic bleomycin, the cross-linking agent mitomycin C and the herbicide maleic hydrazide, a structural isomer of uracil, were used for the treatment. Over a wide range of concentrations and treatment times, fragmentation (or cross-linking in case of mitomycin C) of nuclear DNA was detected (Menke *et al.*, 2001). Based on these results, we have treated *Arabidopsis* seedlings and suspension cultures to investigate the response to the corresponding genotoxins by using a set of selected repair-related genes. For this purpose, a preliminary dot blot analysis with PCR fragments of 35 genes involved in DNA repair, recombination and replication such as the AtRecQl gene family

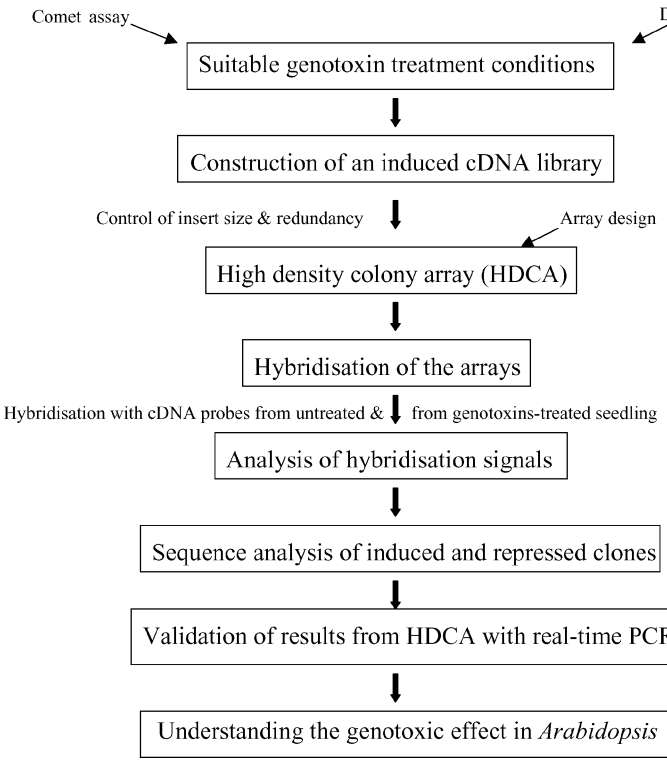

Figure 1. Flow chart describing the strategy for the expression profiling upon genotoxic stress in *A. thaliana.*

(Hartung *et al.*, 2000), the AtSpo11-like genes (Hartung and Puchta, 2001), the mismatch repair genes MutS homologue (MSH)2 and MSH6 (Ade *et al.*, 1999), the toposimerases Topo3α and Topo3β (At5g63920, At2g32000), and the excision repair genes xeroderma pigmentosum B (XPB) and xeroderma pigmentosum D (XPD) (Ribeiro *et al.*, 1998, At1g03190) was performed. The inducibility of Rad51 (Doutriaux *et al.*, 1998; Ries *et al.*, 2000), poly (ADP-ribose) polymerase (PARP) (Doucet-Chabeaud *et al.*, 2001) and gamma-irradiation responsive protein (gr1; Deveaux *et al.*, 2000) by DNA-damaging agents has already been reported for *Arabidopsis*. Although the genotoxins caused DNA damage according to the Comet assay, only the use of a combination of bleomycin (1–1.5 μg ml^{-1}) and mitomycin C (50–75 μM) yielded a strong effect. A twofold increase at the transcription level was detected for the repair genes Rad51, Rad17, PARP-2 and gr1 after 2 h of treatment (not shown). The alteration rate could be further increased up to fourfold after 6–8 h of treatment. No further increase was found after 24 h of treatment. Thus, we decided to construct a cDNA library after 6-h treatment of an *Arabidopsis* suspension culture with bleomycin (1.5 μg ml^{-1}) plus mitomycin C (66.7 μM).

Construction of an Arabidopsis *cDNA library from treated suspension culture*

The cDNAs derived from genotoxin-treated cells were cloned into the vector pBK-CMV and propagated in *Escherichia coli* (see Experimental procedures). In total, 27 000 clones were randomly selected and transferred into 384-well microtitre plates for the arraying. In order to estimate the coverage of the library, 312 randomly selected clones were sequenced. Of these, 244 represented different genes, indicating only 22% redundancy of the clones. Assuming that the distribution among the population of *Arabidopsis* transcripts follows the Poisson distribution, the selected 27 000 clones should cover at least 10 000–14 000 independent genes (>40% of the estimated number of *Arabidopsis* genes; The Arabidopsis Genome Initiative, 2000).

HDCA analysis

Selected clones from the cDNA library were arrayed onto nylon membranes. On each membrane, 9000 clones were arrayed in a double-spotting manner (each clone spotted in duplicate), giving a total of 18 000 spots per membrane. The randomly selected 27 000 clones were spotted onto three membranes. Each membrane was hybridised to the reference probe (cDNAs from untreated seedlings). After stripping, the same membranes were hybridised to cDNAs from treated seedlings (bleomycin (1.5 μg ml^{-1}) plus mitomycin C (66.7 μM), 6 h). After a renewed stripping, the procedure was repeated with new cDNA probes of control and genotoxin-treated seedlings, respectively, which were obtained from an independent experiment. Images of signals were recorded for each hybridisation experiment. After subtraction of the background value and normalisation, the change in the signal intensities obtained after hybridisation with probes from control versus those from genotoxin-treated seedlings was evaluated for the individual spots. Figure 2 shows images of a part of one membrane comprising 3000 spots hybridised with cDNAs from (a) control and (b) treated seedlings. The clearly separated individual spots demonstrate the feasibility of the HDCA for transcriptome analysis. In total, 16 200 (60%) out of the 27 000 clones showed a signal higher than the background level. Among these, 8100 clones showed a very low signal (<1.6 times the background level) and therefore were excluded from further analysis. Of the remaining 8100 clones, about 3200 clones showed a strong signal (>five times the background level under either treated or untreated conditions) and 4900 showed a weaker but detectable signal (≥1.6- and <5 times the background, Table 1). For most of the clones, the DNA content was similar (data not shown), and thus we interpreted strong signals as indicating a high expression level and weaker signals as indicating a low expression level.

As a result of the colony spotting (entire bacteria instead of isolated DNA), a higher background and a lower signal intensity than those of arrayed oligonucleotides or PCR fragments was observed. However, for about 8100 clones (with high or at least detectable expression) corresponding to 3000–4200 genes (because of the redundancy), the analysis could be performed. Results of scatter plots (Figure 3) show that genotoxic effects on transcription profiles can be analysed for *Arabidopsis* using the HDCA. As a control, signals from two hybridisations with two control probes (untreated) obtained from independent experiments were compared (Figure 3a). As expected, the variability of the intensity for most spots corresponding to highly expressed genes (≥five times the background (300 arbitrary units (AU)) under either treated or untreated conditions) did not exceed the twofold boundaries that define an alteration. In contrast, after hybridisation with a probe from genotoxin-treated seedlings to the same array membrane, the scatter plot (Figure 3b) revealed for 30 spots a signal shift beyond the twofold boundaries when compared to that obtained with the probe derived from control seedlings, indicating up- or downregulation of the corresponding genes. Altogether, 300 clones of the high and about 500 clones of the lower expression level were found to represent genes of altered expression after genotoxin treatment (Table 1).

Genes up- or downregulated by the genotoxic treatment

For all 300 clones with a high expression level and a ≥twofold alteration, the sequences were determined (Table 2). In total, 39 genes could be identified among

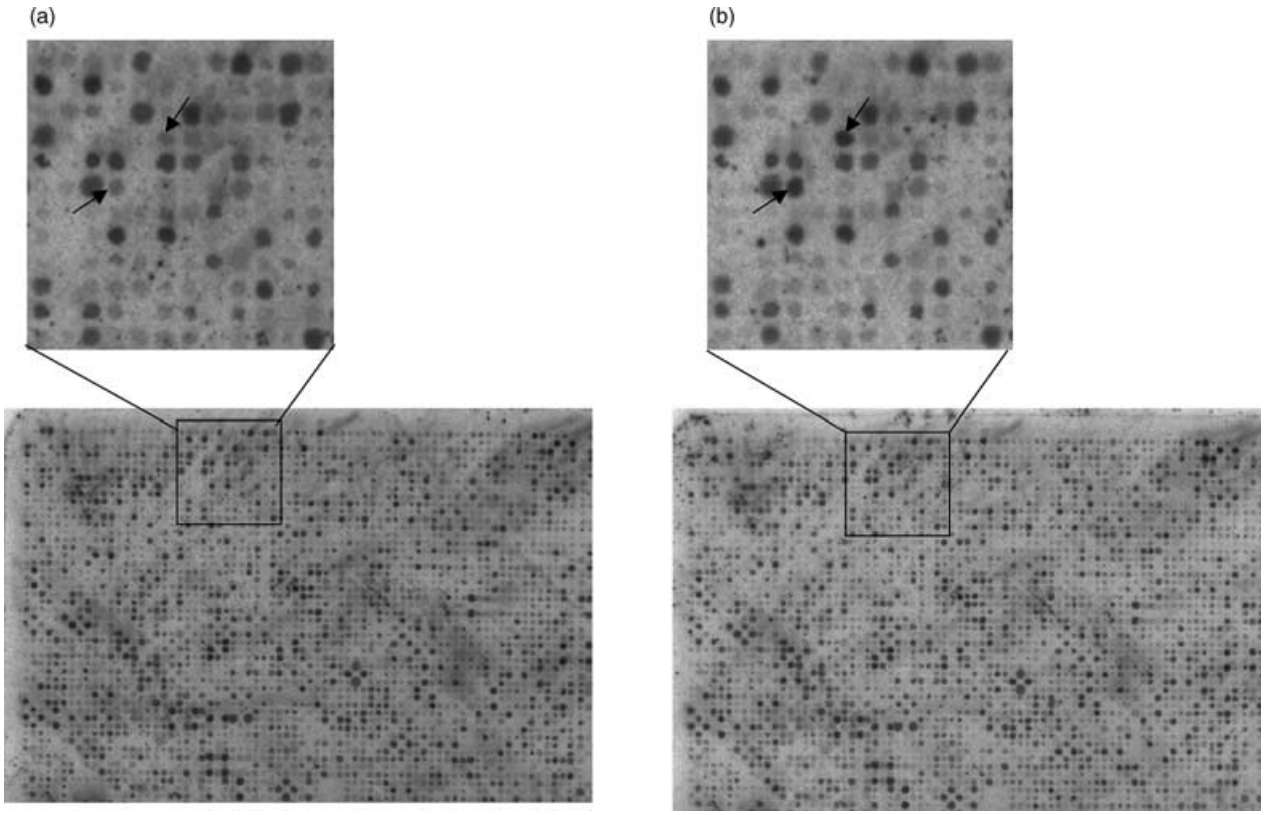

Figure 2. Images of the HDCA with cDNA clones derived from genotoxin-treated *Arabidopsis* cells after hybridisations.
Images of one-sixth (12 cm × 8 cm) of the membrane scanned with an image analyser BAS-3000 (Fujifilm) are shown. The same membrane was hybridised with cDNA probes from untreated (a) and after stripping, with cDNA probes from genotoxin-treated seedlings (bleomycin (1.5 μg ml^{-1}) plus mitomycin C (66.7 μM) for 6 h) (b). Duplicated spots for the gene RNR2 show a 10-fold increase at the transcription level (arrows).

the upregulated clones and 24 genes among the down-regulated clones (the remaining 237 clones are redundant for these 63 genes). The most pronounced increase (10-fold) was found for putative genes encoding ribonucleotide reductase small subunit (RNR2), ATPase-associated with diverse cellular activity (AAA)-type ATPase and thymidine kinase (TK). The putative defence-related genes encoding a calmodulin-like protein, glutathione S-transferase (GST) and singlet oxygen resistant (SOR1) are increased two- to fivefold. As the HDCA was based on a cDNA library derived from genotoxin-treated cells, genotoxin-responsive genes were found to be strongly enriched. Especially, the RNR2 gene, which is associated with DNA-damage response, was detected 36 times (Table 2). Also, putative genes for TK

Table 1 Compilation of results of genotoxic effect on *Arabidopsis* transcriptome for the HDCA analysis

Total	
Number of clones arrayed	27000 (100%)
Estimated number of independent genes on HDCA	10000–14000
Hybridisation signal/expression level	
Number of clones without detectable expression level	10800 (40%)
Number of clones with very low expression (signals smaller than 1.6 times the background)	8100 (30%)
Number of clones with low expression (signals bigger than 1.6 times and smaller than 5 times the background)	4900 (18%)
Number of clones with high expression (signals bigger than five times the background, either treated or untreated)	3200 (12%)
Genes with altered expression	
Number of clones with increased (*x*)/decreased (*y*) expression of low expression level	500 (of which 135 sequenced); $x = 400$; $y = 100$
Number of clones with increased (*x*)/decreased (*y*) expression of high expression level	300 (all sequenced); $x = 201$; $y = 99$

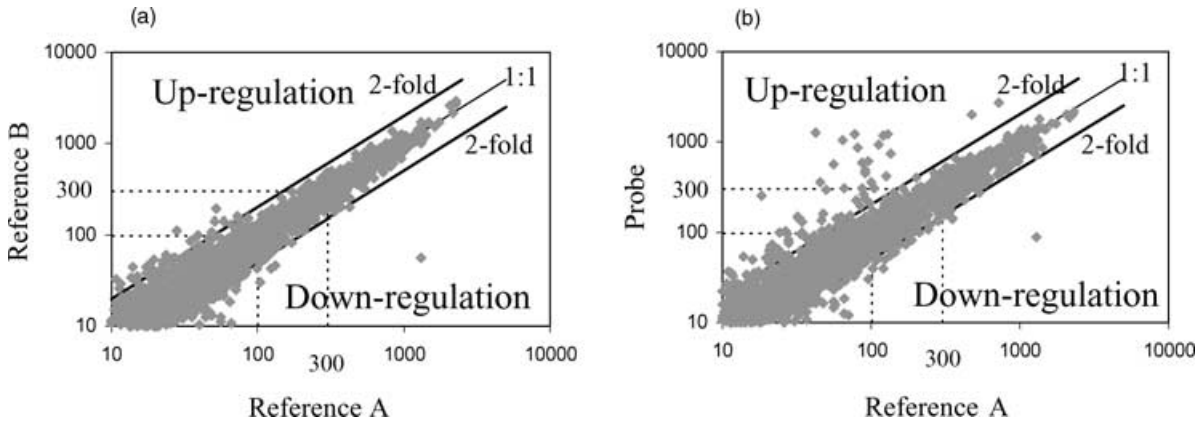

Figure 3. Scatter plot of the hybridisation signals from one-sixth part of a HDCA membrane with genotoxin-treated and untreated probes.
The hybridisation signals per spot, analysed with the program ARRAY VISION (version 5.1), were plotted. As a control, signals obtained with cDNA probes from untreated seedlings (sample reference B) were plotted against those obtained with cDNA from untreated seedlings of an independent experiment (sample reference A) (a). The effect of the genotoxin treatment is shown in (b) where signals of hybridisation with cDNA probes from genotoxin-treated seedlings (bleomycin (1.5 μg ml^{-1}) plus mitomycin C (66.7 μM) for 6 h) were plotted against those obtained from cDNA of control seedlings (sample reference A). Signals with intensity below 100 AU (1.6 times the background level) are considered as too weak and are not subjected to the transcriptional analysis. Signals with intensity above 300 AU (five times the background level) under either treated or untreated conditions are regarded as an indication of high expression. Solid lines indicate a twofold alteration. A spot with the signal values *x* of approximately 1500 and *y* of approximately 100 on the lower right side on both plots was detected to be a hybridisation artefact.

Table 2 Effect of genotoxin treatment on expression of genes with high expression level identified by the HDCA analysis

Locus entry	Repetition	Description of the gene	Functional classification	Transcriptional change (in fold)
Increase				
At2g18190	6	Putative AAA-type ATPase	Cell cycle control	10
At3g27060	36	Ribonucleotide reductase small subunit, putative RNR2	Deoxyribonucleotide metabolism/ DNA repair	10
At3g07800	11	Putative thymidine kinase	Pyrimidine nucleotide metabolism	10
At1g76180	4	Hypothetical protein/dehydrins signatures	Unknown	5
At5g49480	19	NaCl-inducible Ca^{2+}-binding protein-like; calmodulin-like	Unspecified signal transduction	5
At1g29400	1	RNA-binding protein MEI2, putative	Meiosis	5
ATCHRIV83	4	Unknown, base 156021–156371 in ATCHRIV83	Unknown	5
At2g34520	1	Mitochondrial ribosomal protein S14	Protein synthesis	4
At1g17180	9	Putative glutathione transferase	Metabolism/defence	4
At1g09815	4	Unknown	Not found in MATDB	4
At3g52140	1	Putative protein, 150-kDa protein cluA	Translation	3
At3g13520	5	Arabinogalactan-protein AGP12	Unknown	3
At2g22430	9	Homeodomain transcription factor (ATHB-6)	Transcriptional control	3
At3g48140	8	B12D-like protein	Unknown	3
At5g53560	1	Cytochrome b_5	Lipid biosynthesis	3
At4g02520	6	Atpm24.1 glutathione S transferase, GST	Metabolism/defence	3
At1g17170	3	Putative glutathione transferase	Metabolism/defence	3
At5g10980	11	Histone H3.2 protein	Biogenesis of chromosome structure	3
At4g34180	1	Putative protein, slr2121	Unknown	3
At3g52590	2	Ubiquitin/ribosomal protein CEP52	Protein synthesis	3
At4g24690	6	Putative protein	Unknown	3
At4g33865	8	Ribosomal S29 subunit	Unknown	3
At5g27760	3	Putative protein	Unknown	3
At2g41410	5	Calmodulin-like protein	Unspecified signal transduction	2
At3g60360	1	Putative protein/CGI-94 protein	Unknown	2
At1g30230	6	Elongation factor 1-beta, putative	Translation	2
At4g31300	2	Multicatalytic endopeptidase complex, beta subunit	Storage proteins	2
At5g65360	3	Histone H3	Organisation of chromosome structure	2
At5g26210	1	Nucleic-acid-binding protein-like	Transcriptional control	2
At5g64260	2	phi-1-like protein	Unknown	2

Table 2 continued

Locus entry	Repetition	Description of the gene	Functional classification	Transcriptional change (in fold)
At3g46820	1	Phosphoprotein phosphatase	Cellular communication/signal transduction	2
At2g38230	2	Similar to SOR1 from the fungus *Cercospora nicotianae*	Biosynthesis of vitamins/stress response	2
At5g42980	1	Thioredoxin (clone GIF1)	Electron/hydrogen carrier	2
At5g54940	1	Translation initiation factor-like protein	Translation	2
At3g54640	2	Tryptophan synthase alpha chain	Amino acid metabolism	2
At2g23090	2	Unknown protein	Unknown	2
At4g05320	3	Polyubiquitin (ubq10)	Protein modification, degradation	2
At4g09320	5	Nucleoside-diphosphate kinase	Nucleotide metabolism	2
At2g25210	3	60S ribosomal protein L39	Protein synthesis	2
Decrease				
At4g13940	5	Adenosylhomocysteinase	Amino acid metabolism	−3
At3g53460	1	RNA-binding protein cp29 protein	Cellular organisation	−3
At3g23830	3	Glycine-rich RNA binding protein, putative	Unknown	−2
At3g16420	4	Putative lectin, jacalin-like	Defence-related proteins	−2
At1g74560	1	Putative SET protein, phospatase 2A inhibitor	Cell cycle control	−2
At5g56030	2	Heat shock protein 81-2	Stress response	−2
At5g47210	18	Putative protein, RNA binding protein	Nucleus	−2
At3g58610	2	Ketol-acid reductoisomerase	Amino acid biosynthesis	−2
At3g57150	3	Putative protein/DYSKERIN (NUCLEOLAR PROTEIN NAP57)	Nuclear organisation	−2
At1g07930	2	Elongation factor 1-alpha	Translation	−2
At1g07940	3	Elongation factor 1-alpha	Translation	−2
At5g60390	10	Translation elongation factor eEF-1 alpha chain (gene A4)	Translation	−2
At2g21660	3	Glycine-rich RNA binding protein	Unknown	−2
At4g39260	2	Glycine-rich protein (clone AtGRP8)	Unknown	−2
At5g59690	5	Histone H4-like protein	Nuclear organisation	−2
At4g24280	1	hsp 70-like protein	Stress response	−2
At4g17520	1	Nuclear RNA binding protein A-like protein	Nuclear organisation	−2
At1g43170	9	Hypothetical protein/Ribosomal protein L3 signature	Protein synthesis	−2
At2g31610	9	40S ribosomal protein	Protein synthesis	−2
At2g41840	11	40S ribosomal protein S2	Protein synthesis	−2
At4g17390	1	60S ribosomal protein L15 homologue	Protein synthesis	−2
At4g18730	1	Ribosomal protein L11, cytosolic	Protein synthesis	−2
At3g53870	3	Ribosomal protein S3a homologue	Protein synthesis	−2
At3g60750	1	Transketolase-like protein	Chloroplast organisation, biosynthesis	−2

(detected 11 times), a calmodulin-like protein (detected 19 times) and an AAA-type ATPase (detected six times) showed a high redundancy. A twofold decrease was observed for genes presumably encoding RNA-binding proteins and heat shock proteins. Differential effects (up- and downregulation) of the mutagen treatment on transcriptional level were observed for histones and ribosomal proteins (Table 2).

Of approximately 500 clones which were either upregulated (approximately 400 clones) or downregulated (approximately 100 clones) with a lower but still detectable expression level ($>$1.6 times the background level $=$ 100 AU), 135 clones were sequenced (Table 3), 57 showing increase and 22 showing decrease in transcripts (the remaining 56 clones were redundant clones of these 79 genes). Genes associated with DNA repair, pathogen defence and cell cycle control, e.g. E2 ubiquitin-conjugating-like enzyme (Ahus5), mismatch repair protein T (MutT) and DNA polymerase epsilon, were upregulated two- to threefold. An increase in the levels of transcripts for myb and WRKY transcription factors was also detected. However, because of the lower signal intensity, the results have to be considered with care and the expression data for such genes should be confirmed via alternative approaches (see later).

The upregulated genes are functionally classified in Table 2. Among these, defence/stress-responsive genes and genes involved in cell cycle control are more often found than expected on the basis of their proportion within the *Arabidopsis* genome. From the 39 upregulated genes with high expression level, 17 genes (60% of 27 upregulated genes with functional classification; the other 12 unknown genes were not taken into consideration) are stress-responsive, defence-related, cell-cycle-regulating or DNA-repair genes,

Table 3 Effect of genotoxin treatment on expression of genes with low expression level identified by the HDCA analysis

Locus entry	Repetition	Description of the gene	Functional classification	Transcriptional change (in fold)
Increase				
At3g04120	2	Glyceraldehyde-3-phosphate dehydrogenase C subunit (GapC)	Metabolism	4
At5g03780	1	Myb-like protein	Unknown	4
At1g08260	1	Hypothetical protein, DNA-directed DNA polymerase epsilon	DNA synthesis, cell cycle checkpoints	3
At1g43900	1	Unknown protein, Protein phosphatase 2C signature	Unspecified signal transduction	3
At3g60420	1	Putative protein, prib5	Metabolism	3
At5g57560	1	TCH4 protein	Metabolism	3
At2g04050	5	Hypothetical protein	Detoxification	3
At2g42680	1	Unknown protein	Transcriptional control	3
At3g13510	1	Unknown protein	Unknown	3
At3g27630	1	Hypothetical protein	Unknown	3
At5g48020	1	Unknown protein	Unknown	3
At3g07230	2	Putative wound-induced basic protein	Unknown	3
At1g21720	1	Putative 20S proteasome beta subunit PBC2	Cytoplasmic and nuclear degradation	3
At1g02930	2	Putative glutathione S-transferase	Biosynthesis, detoxification	3
At5g58070	4	Outer membrane lipoprotein-like	Biogenesis of plasma membrane	3
At1g43890	2	GTP-binding protein RAB1Y, putative	Unspecified signal transduction	3
At2g41630	3	Transcription factor IIB	General transcription activities	3
At3g45730	3	Putative protein	Unknown	3
At4g33630	5	Hypothetical protein	Unknown	3
At1g29150	3	19S proteosome subunit 9, putative	Cytoplasmic and nuclear degradation	2
At5g59890	1	Actin depolymerising factor 4-like protein	Biogenesis of cell wall (cell envelope)	2
At2g44620	1	Acyl carrier protein	Lipid biosynthesis	2
At1g17290	1	Alanine aminotransferase, putative	Amino acid biosynthesis	2
At1g29850	1	Similar to TF-1 apoptosis related protein 19	Unknown	2
At5g19550	9	Aspartate aminotransferase Asp2	Amino acid degradation	2
At1g01470	1	Similarity to 1-phosphatidylinositol-4-phosphate 5-kinase, AtPIP5K	Unknown	2
At5g19440	1	Cinnamyl-alcohol dehydrogenase-like protein	Metabolism	2
At3g18600	1	DEAD box helicase protein, putative	Chromatin modification	2
At3g57870	1	E2 ubiquitin-conjugating-like enzyme Ahus5	Cytoplasmic and nuclear degradation	2
At3g53990	6	Hypothetical protein, ER6	Unspecified signal transduction	2
At2g29460	1	Putative glutathione S-transferase	Biosynthesis, detoxification	2
At5g51440	1	Mitochondrial heat shock 22 kDa protein-like	Mitochondrion, stress response	2
At2g43760	1	Putative molybdopterin synthase large subunit	Metabolism of vitamins	2
At1g68760	1	Putative mutT protein	Unknown	2
At5g59420	2	Oxysterol-binding protein-like	Metabolism, cell death	2
At5g44070	1	Phytochelatin synthase	Unknown	2
At1g64520	1	Proteasome regulatory subunit, putative	Cell cycle control	2
At2g19750	3	40S ribosomal protein S30	Ribosome biogenesis	2
At1g52300	2	60S ribosomal protein L37, putative	Ribosome biogenesis	2
At4g29390	2	Ribosomal protein S30 homologue	Ribosomal proteins	2
At2g16590	9	Unknown	Unknown	2
At4g24920	1	Protein transport protein SEC61 gamma subunit-like	Protein targeting, sorting	2
At3g46210	1	Putative protein, tRNA nucleotidyltransferase	RNA degradation	2
At5g53300	3	Ubiquitin-conjugating enzyme E2 (ubiquitin-protein ligase 10)	Protein modification	2
At4g05050	1	Strong similarity to polyubiquitins, UBQ14	Cytoplasmic and nuclear degradation	2
At1g15270	1	Unknown protein	Unknown	2
At1g42960	1	Hypothetical protein	Unknown	2
At1g51160	1	Hypothetical protein	Vesicular transport	2

Table 3 continued

Locus entry	Repetition	Description of the gene	Functional classification	Transcriptional change (in fold)
At1g54520	1	Hypothetical protein	Unknown	2
At1g63000	1	Unknown protein	Metabolism	2
At3g11860	1	Hypothetical protein	Unknown	2
At3g29310	1	Hypothetical protein	Unknown	2
At4g32020	1	Putative protein	Unknown	2
At5g18310	2	Putative protein	Unknown	2
At5g39800	1	Hypothetical protein	Unknown	2
At5g56980	1	Putative protein	Unknown	2
At5g26170	1	Putative protein, DNA-binding protein WRKY1	Transcriptional control	2
Decrease				
At3g08580	1	Adenylate translocator	Nucleotide transporters	−2
At3g09820	1	Putative adenosine kinase	Purine nucleotide metabolism	−2
At5g12940	1	Putative protein, DRT100	Unspecified signal transduction	−2
At3g19820	1	Cell elongation protein, Dwarf1	Unknown	−2
At4g20360	1	Translation elongation factor EF-Tu	Translation	−2
At5g57870	1	Eukaryotic initiation factor 4, eIF4-like protein	Translation	−2
At5g22880	1	Histone H2B like protein	Nucleus	−2
At3g12390	1	Hypothetical protein	Translational control	−2
At2g19480	1	Putative nucleosome assembly protein	Cell cycle control	−2
At1g48920	2	Eukaryotic putative RNA-binding region RNP-1 signature	Chloroplast, rRNA processing	−2
At5g17870	1	Plastid-specific ribosomal protein 6 precursor-like	Ribosomal proteins	−2
At3g02510	1	Regulator of chromosome condensation (RCC1) signatures	Stress response	−2
At4g24770	3	RNA-binding protein RNP-T precursor	Chloroplast organisation	−2
At3g04840	1	Putative 40S ribosomal protein S3A (S phase specific)	Ribosome biogenesis	−2
At1g55490	1	Rubisco subunit binding-protein beta subunit	Protein folding, stress response	−2
At2g28000	1	Putative rubisco subunit binding-protein alpha subunit	Protein folding, stress response	−2
At1g04820	1	Tubulin alpha-2/alpha-4 chain	Cytoskeleton, cell cycle control	−2
At1g07320	2	Unknown protein	Unknown	−2
At3g44750	1	Putative histone deacetylase	Chromatin modification	−2
At5g26742	3	DEAD box RNA helicase, RH3	Not found in MATDB	−2
At5g59870	1	Histone H2A-like protein	Nuclear organisation	−2
At5g59970	1	Histone H4-like protein	Nuclear organisation	−2

which, in *Arabidopsis*, constitute approximately 20% of the genome (The Arabidopsis Genome Initiative, 2000).

Real-time PCR to confirm the results from the HDCA analysis

Our HDCA analysis provided a global view about transcriptome response to genotoxic stress in *Arabidopsis*. However, as a result of the limited sensitivity of this method, a precise determination of the up- or downregulation rate was not possible. It has been suggested that the expression data obtained from array analyses generally require further verification, in particular for genes with a low expression level (Rajeevan *et al.*, 2001). Therefore, we used real-time PCR to confirm the results obtained for 42 genes identified in the HDCA analysis (Table 4). Twenty-one strongly expressed genes, of which 13 are upregulated and 8 downregulated, and 21 weakly expressed genes, of which 12 are upregulated and 9 downregulated, were analysed. Except for three genes (the nucleoside-diphosphate kinase, 40S ribosomal protein S2 and elongation factor 1-alpha), a clear correlation was observed between the results obtained with both approaches for the genes with high expression level. Genes encoding AAA-type ATPase, RNR2 and TK with a 10-fold induction according to the HDCA showed eight-fold (AAA-type ATPase) and approximately 30-fold induction (TK and RNR2) by real-time PCR. For 16 of the 21 genes of low expression level, a correlation was also found. A transcriptional increase in a DEAD box helicase and of the actin-depolymerising factor (both with low expression level) could not be confirmed by real-time PCR as the downregulation of a putative histone deacetylase, and of the DEAD box RNA helicase RH3 and an unknown gene (At1g07320). A 68-fold increase was found for the transcript of a myb protein (Table 4). In addition, a WRKY1 transcription factor and a hypothetical DNA-directed DNA

Table 4 Comparison of gene expression determined by HDCA/dot blot and real-time PCR after genotoxin treatment

		Transcriptional change (in fold)		
		HDCA	Dot blot	Real-time PCR
High expression				
At2g18190	Putative AAA-type ATPase	10		8.0
At3g27060	Ribonucleotide reductase small subunit, putative RNR2	10		28
At3g07800	Putative thymidine kinase	10		30
At1g29400	RNA-binding protein MEI2, putative	5		1.9
At1g17170	Putative glutathione transferase	3		1.9
At5g49480	NaCl-inducible Ca^{2+}-binding protein; calmodulin-like	5		6.3
At1g09815	Unknown	4		4.9
At4g24690	Unknown	3		2.1
At5g27760	Unknown	3		1.9
At2g22430	Homeodomain transcription factor (ATHB-6)	3		1.9
At5g10980	Histone H3.2	3		1.7
At5g65360	Histone H3	2		4.6
At4g09320	Nucleoside-diphosphate kinase	2		−1.4
At3g53460	RNA-binding protein cp29 protein	−3		−1.5
At3g16420	Putative lectin, jacalin-like	−2		−1.6
At3g57150	Putative protein/DYSKERIN (NUCLEOLAR PROTEIN NAP57)	−2		−1.3
At1g74560	Putative SET protein, phospatase 2A inhibitor	−2		−1.3
At4g39260	Glycine-rich protein (clone AtGRP8)	−2		−1.4
At5g59690	Histone H4-like protein	−2		−1.9
At2g41840	40S ribosomal protein S2	−2		1.3
At1g07930	Elongation factor 1-alpha	−2		1.0
Low expression				
At5g03780	myb-like protein	4		68
At1g08260	Hypothetical DNA-directed DNA polymerase epsilon	3		16
At1g21720	Putative 20S proteasome beta subunit PBC2	3		1.8
At5g26170	Putative protein, DNA-binding protein WRKY1	2		21
At1g01470	Similarity to 1-phosphatidylinositol-4-phosphate 5-kinase	2		3.5
At1g29150	19S proteosome subunit 9, putative	2		2.2
At5g53300	Ubiquitin-conjugating enzyme E2	2		1.3
At3g53990	Hypothetical protein, ER6	2		1.9
At3g57870	E2 ubiquitin-conjugating-like enzyme Ahus5	2		2.4
At1g68760	Putative mutT protein	2		2.5
At3g18600	DEAD box helicase protein, putative	2		1.0
At5g59890	Actin depolymerising factor 4-like protein	2		−1.5
At5g59870	Histone H2A-like protein	−2		−3.5
At5g22880	Histone H2B-like protein	−2		−4
At5g59970	Histone H4-like protein	−2		−3.1
At3g02510	Regulator of chromosome condensation (RCC1) signatures	−2		−1.4
At4g24770	RNA-binding protein RNP-T precursor	−2		−1.7
At1g04820	Tubulin alpha-2/alpha-4 chain	−2		−1.4
At1g07320	Unknown	−2		−1.2
At3g44750	Putative histone deacetylase	−2		−1.1
At5g26742	DEAD box RNA helicase, RH3	−2		1.2
Repair genes				
At4g02390	PARP-2		3	50
At5g20850	Rad51		2	81
At5g66130	Rad17		2	7.2
At2g31320	PARP-1		Low expression	5.5
Reference genes				
At1g49240	Actin			1.7
At1g42970	GapB			2.3
At5g42990	UBC18			1.1
At1g70600	60S RP L27A	1		1.0

Downregulation of a gene is indicated by '−'.

Table 5 Effect of genotoxin treatment on expression of cell cycle genes determined by real-time PCR

Locus entry	Description of the gene	Transcriptional change (in fold)
At3g48750	CDC2A, cyclin-dependent kinase (CDK)	1.1
At3g54180	CDC2B, cyclin-dependent kinase	1.2
At1g20930	CDC2-k, Cdc2 kinase	−1.2
At1g77390	CycA1;2, A-type cyclin	2.3
At4g37490	CycB1;1, B-type cyclin	26
At5g06150	CycB1;2, B-type cyclin	−2.1
At4g35620	CycB2;2, B-type cyclin	1.0
At4g34160	CycD3;1, D-type cyclin	1.0
At2g23430	ICK1, CDK inhibitor	1.0
At3g50630	ICK2, CDK inhibitor	−1.2
At3g12280	RB, tumor suppressor retinoblastoma protein	1.3
At2g27960	CKS1, CDK interacting protein	1.0
At3g48160	DEL, DP-E2F-like	−2.5
At2g36010	E2Fa, transcription factor	2.0
At1g02970	WEE1, negative regulator of CDK	7.4

polymerase epsilon subunit were also highly upregulated (about 20-fold). We also analysed by real-time PCR the genotoxic effect on four housekeeping/reference genes encoding Actin-8, glyceraldehyde-3-phosphate dehydrogenase subunit B (GapB), ubiquitin-conjugating enzyme 18 (UBC18) and the 60S ribosomal protein L27A (At1g70600). Actin-8 and GapB are usually considered to be persistently expressed over a wide range of conditions. Our results with real-time PCR indicated an alteration of about twofold for these two genes while the expression of UBC18 and of the 60S ribosomal protein L27A (the later one was present within the HDCA with unchanged signal intensity) remained unaffected by the genotoxin treatment. In addition, we tested four repair genes, of which three (PARP-2, Rad51 and Rad17) were shown to be genotoxin responsive via dot blot hybridisation (see above). All of these were proved to be upregulated when real-time PCR was applied. For Rad51 a >80-fold and for PARP-2 a 50-fold increase in transcription was found, whereas using dot blot analysis only two- to threefold increases could be detected.

Effect of genotoxin treatment on transcription of cell cycle genes in Arabidopsis

Dynamic expression in a cell-cycle-dependent manner was reported for some of the genes described above (Menges *et al.*, 2002). As mutagens usually induce transient cell cycle arrest, the real-time PCR analysis was extended to cell cycle genes. In a genome-wide analysis, Vandepoele *et al.* (2002) identified 61 core cell cycle genes including cyclin-dependent kinases (CDKs), cyclins, CDK/cyclin interacting proteins, and cell cycle regulators in *Arabidopsis*. We determined transcriptional change upon genotoxic challenge (bleomycin (1.5 μg ml^{-1}) plus mitomycin C (66 μM), 6 h) for 15 of these genes including 3 CDKs, 5 cyclins and 4 CDK/cyclin interacting proteins as well as the Rb, E2Fa and DEL proteins (Table 5). Transcription of two genes (CycB1;2 and DEL) was found to be downregulated and of four genes (CycB1;1, CycA1;2, E2Fa and WEE1) to be upregulated (with ≥twofold alteration). The strongest (26-fold) increase in transcript abundance was found for a mitotic cyclin (CycB1;1). Interestingly, a twofold reduction was determined for another B-type cyclin (CycB1;2). A sevenfold increase was found for WEE1, a negative regulator of CDK/cyclin complexes (Table 5).

Discussion

Here we describe the application of the HDCA for a genome-wide transcriptome analysis upon genotoxic stress using a cDNA library derived from genotoxin-treated cells. This ensured that genes expressed mainly or exclusively after DNA damage are enriched within the cDNA population and yielded a high redundancy of genotoxin-responsive genes (Tables 2 and 3) within the HDCA. The contribution of the defence/stress-responsive genes and of genes involved in cell cycle control is greatly enriched compared to their proportion within the *Arabidopsis* genome.

Validation of the HDCA results via real-time PCR

The HDCA approach revealed 300 altered clones representing approximately 1% of the analysed ones and corresponding 63 genes with a high expression level and 500 clones with a lower expression level that showed a deviating transcriptional activity after genotoxin treatment. Of the latter, 135 were sequenced and found to represent 79 genes (Table 3). Forty-two of the 142 identified genes were re-examined with real-time PCR, which is more sensitive and increasingly used for detailed expression analysis and for validation of array data (Meyer *et al.*, 2002). The results of the HDCA and real-time PCR correlated well in the majority (80%) of the genes tested (Table 4). In particular for some genes with a low basic transcription level, real-time PCR analysis (Table 4) showed a distinct difference in alteration of expression, e.g. for myb (68-fold), WRKY (21-fold) and putative DNA polymerase epsilon small subunit (16-fold). In the HDCA analysis, these genes were found to be upregulated two- to fourfold. Four repair genes (Rad17, PARP-1, PARP-2 and Rad51), which were studied first by dot blot analysis, also revealed a strong increase in transcription by real-time PCR (81-fold in case of Rad51). An 82-fold increase in Rad51 transcripts upon DNA damage (by gamma irradiation) was also reported recently (Lafarge and Montane, 2003). The strong increase in transcripts of myb and WRKY1 transcription factors might indicate a DNA-damage-induced regulation of gene expression.

Table 6 Motifs identified in the promoter regions of DNA damage inducible genes

Sequence of motifs	Genes						
	GR1	LigIV	PARP-1	PARP-2	Rad51	RNR2	TK
AACCAT	No	−42 to −37 −436 to −431	No	−63 to −58	No	No	−28 to −23
AAGCAA	No	−92 to −87	−205 to −200 −1052 to −1047	No	No	No	−64 to −59 −703 to −698 −994 to −989
ACAAAT	−587 to −582 −674 to −669	−208 to −203	−810 to −805 −975 to −970	−205 to −200	−615 to −610 −953 to −948	−183 to −178	−161 to −156
ATAAATA	No	−190 to −184	−44 to −38	−300 to −294	−860 to −854	−93 to −87 −830 to −824	No
ATTCAA	−29 to −24 −523 to −518	−502 to −497	−492 to −487 −790 to −785	No	−503 to −498	−126 to −121	−188 to −183 −605 to −600
ATTCTG	−998 to −993	−442 to −437	−475 to −470 −757 to −752 −902 to −897	No	No	−531 to −526	No
CAAAATT	No	No	−563 to −557	−406 to −400 −505 to −499	No	No	−185 to −179
CAGGGCC	No	No	No	No	No	−879 to −873	−646 to −640
CATATA	No	No	−873 to −868	−802 to −797	−932 to −927	No	No
CATGTT	No	No	−930 to −925	−702 to −697 −862 to −857	No	−320 to −315	−383 to −378 −657 to −652
CTTCAAT	−34 to −28	No	No	No	No	−30 to −24 −47 to −41	No
CTTTTGT	−155 to −149 −853 to −847	No	No	−188 to −182	No	−740 to −734	No
GAACCA	No	−437 to −432	No	No	No	−408 to −403	No
GCAATT	−313 to −308	−90 to −85	No	No	−719 to −714 −975 to −970	No	−147 to −142 −1027 to −1022
GGATGT	No	No	−1026 to −1021	No	No	No	−121 to −116 −1019 to −1014
TAAGTT	−891 to −886	−219 to −214	No	−900 to −895	No	−770 to −765 −839 to −834	No
TAATTCT	−1000 to −994	−444 to −438	No	No	−928 to −922	No	−501 to −495
TCTAAA	No	−263 to −258	No	No	−841 to −836	−485 to −480	−239 to −234 −497 to −492
TCTTCAA	−844 to −838	No	No	−98 to −92 −674 to −668	No	−19 to −13 −31 to −25 −48 to −42	−102 to −96
TTGACGAT	No	−273 to −266	No	No	No	−262 to −255	No
TTGGAA	−1028 to −1023	No	No	−579 to −574	−556 to −551	No	−683 to −678
TTTCAAA	No	−118 to −112 −456 to −450	No	−161 to −155 −390 to −384	No	−178 to −172	−180 to −174
TTTGGG	No	−378 to −373 −483 to −478	No	−248 to −243 −822 to −817	−234 to −229	No	−622 to −617

No: motif not present; numbers indicating the distance of the motif from the putative start codon.

Analysis of genes showing genotoxin-mediated alteration in transcription activity

The analysis of the 39 upregulated genes with a high expression level in the HDCA approach revealed that 17 genes (60% of 27 upregulated genes, which could be functionally classified) are involved in stress response, pathogen defence, cell cycle regulation or DNA repair (Table 2). These genes constitute approximately 20% of the *Arabidopsis* genome (The Arabidopsis Genome Initiative, 2000). This contrasts with the results of Birrell *et al.* (2002). These authors could not detect increased transcription of genes that protect against DNA damage by array analysis after genotoxin treatment of yeast.

RNR2, TK and AAA-type ATPase genes were identified as highly upregulated in the HDCA, and their increased

expression was confirmed by real-time PCR (Table 4). In particular, RNR2 appeared repeatedly in the arrays and was highly upregulated (30-fold). In yeast, the inducibility of the RNR2 gene by the DNA-damaging agents 4-nitroquinoline-1-oxide, UVC and H_2O_2 was also reported, and its expression was found to be regulated by the repair genes PSO5/RAD16 (Paesi-Toresan *et al.*, 1998), Rad9, DANN-damage checkpoint (DDC1), DNA-damage uninducible (DUN) and constitutive RNR3 transcription (CRT) (Walsh *et al.*, 2002). In tobacco (Chaboute *et al.*, 2000), yeast and human (Elledge *et al.*, 1992; Greenberg and Hilfinger, 1996), the expression of RNR2 is tightly regulated in a cell-cycle-dependent manner. TK is another strongly upregulated gene (30-fold) according to real-time PCR analysis. The drastic increase in transcription of this enzyme upon genotoxic stress might reflect a high demand for DNA synthesis during repair. The similar requirement of RNR2 and TK for DNA metabolism and their transcriptional response to genotoxins might imply a similar regulation mechanism. Therefore, we have compared the promoter regions of both genes (mapped on chromosome 3) and found motifs like CAGGGCC, TCTAAA, CATGTT, TTTCAAA and TCTTCAA present in both promoter regions (Table 6) at similar positions relative to the putative start codons. Additionally, we compared promoter regions of five other repair genes (GR1, LigIV, PARP-1, PARP-2 and Rad51) of which Rad51, PARP-1 and PARP-2 were found to be upregulated after mutagen treatment, as demonstrated by real-time PCR. Twenty-three candidate motifs (hexamers to octamers; Table 6) were identified by pairwise comparison of sequences. Of these, 18 are present in at least three promoter regions. One motif (ACAAAT) was present in all tested promoter regions. Whereas this motif was present as single copy in LigIV, PARP-2, RNR2 and TK, two copies were found in Rad51, GR1 and PARP-1 promoter regions. Interestingly, in genes with only one copy, the motif was positioned approximately 200 bp upstream of ATG start codon (LigIV, PARP-2, RNR2 and TK; Table 6). In contrast, when two copies of the motif were present, both resided at least 500 bp upstream of start codon (Rad51, GR1 and PARP-1; Table 6). We also detected in some genes unique motives that occurred three times within the putative promoter regions (AAGCAA for TK, ATTCTG for PARP-1 and TCTTCAA for RNR2). Surprisingly, in case of RNR2, the three copies of the motif are located within a fragment of only 36 bp of the promoter region (−13 to −48 bp). The sequences of the 23 motives listed in Table 6 do not resemble any known binding sequences in *Arabidopsis* or yeast. Whether they are involved in regulating gene expression is unknown. Recently, Garcia *et al.* (2003) reported that DNA damage (by ionising irradiation) failed to induce expression of repair genes (GR1, LigIV, PARP-1 and Rad51) in two *AtATM* mutant lines and therefore suggested a common transcriptional regulation of these genes upon DNA damage/ionising irradiation. One could speculate that the motif ACAAAT present in the seven promoter regions tested might be involved in such a regulation. The high similarity of motif sequences and positions within the promoter regions suggests that at least some of the motifs identified in this study might play a role in regulating expression of these genes under DNA damage/stress conditions. Combination of different motifs, their copy numbers as well as their relative positions might help to fine-tune transcriptional response to different types of DNA damage and stress in *Arabidopsis*. However, further *in vivo* and *in vitro* promoter studies are needed to sustain this hypothesis.

A mammalian E2F transcription-factor-binding site (TTTg/cg/cCGC) is present in the promoter region of RNR2 of tobacco (Chaboute *et al.*, 2000) and also in the RNR2 promoter region of *Arabidopsis* (−473 to −466 from ATG of RNR2, complementary strand). The strict cell-cycle-dependent regulation of expression of RNR2 in tobacco was suggested to be associated with the E2F motif (Chaboute *et al.*, 2000). This motif is absent from the promoter regions of the other six repair genes analysed here. We also screened the seven promoters for the presence of myb- and WRKY-binding sites and identified binding sites for the two putative transcription factors that belong to the highly upregulated genes (myb (68-fold upregulated) and WRKY1 (21-fold upregulated); Table 4). The myb core binding sequence (AACGG) was detected only in the promoter region of PARP-1 at two positions (−252 to −248 and −153 to −149). The W-box (TTGACc/t) of WRKY could be detected as single copy in GR1 (−460 to −455) and three copies in Rad51 (−946 to −941, −824 to −819 and −715 to −710). The presence of E2Fa, myb or WRKY binding motifs in some of the seven promoter regions analysed here suggests that expression of the respective genes might be regulated differently under different circumstances.

The highly increased transcription (eightfold) of the AAA-type ATPase might be associated with the rapid protein turnover under stress conditions, as some kinds of AAA-type ATPase are subunits of 26S proteasome (Fu *et al.*, 1999). The transcription of a large number of ribosomal proteins was also altered by the genotoxin treatment with the majority becoming downregulated (three up- and six downregulated genes; Table 2), likely indicating disturbance of protein synthesis by genotoxins.

One of our aims was to identify unknown genes that might be involved in DNA repair. Fifteen highly expressed genes (Table 2) and 24 of low expression (Table 3), which are not yet functionally classified, were identified by the HDCA. We focused on the first analysis (sequence comparison using BLAST program, protein motif search) on three genes (Accession numbers At1g09815, At4g24690 and At5g27760) showing an upregulation confirmed by real-time PCR (Table 4). At5g27760 encodes for a protein of 96 amino acids with high sequence similarity to a putative

protein in rice. Functional motifs could not be detected in both. At1g09815 encodes a small putative protein with sequence similarity to the smallest subunit of DNA polymerase delta. Interestingly, upregulation of a gene coding for the putative small subunit of DNA polymerase epsilon was also detected in our analysis (Table 3 and confirmation with real-time PCR in Table 4). Association of both types of DNA polymerases with DNA-damage-induced replication has been reported for yeast and mammals (Hubscher *et al.*, 2002). Our data indicate that synthesis of DNA as well as production of DNA precursors by TK and RNR2 is upregulated as a response to DNA damage.

The third open-reading frame (ORF) (At4g24690) encodes a large putative membrane protein of approximately 700 amino acids with a zinc finger signature and similarity to one part of the octicosapeptide repeat motif (LKYKDEEGDLVTLAEDSD), which is supposed to bind Ca^{2+} ions. Further sequence homology was found between this unknown gene and NBR1 (next to BRCA1), which also contains the zinc finger signature and the octicosapeptide repeat motif. The exact function of NBR1 is still unclear; however, because of its interaction with FEZ1 (fasciculation and elongation protein zeta-1) and CIB (calcium- and integrin-binding protein), a role in cell-signalling pathways was suggested (Whitehouse *et al.*, 2002). Indeed, several indications exists that signal transduction cascades are involved in the regulation of DNA repair (Ulm *et al.*, 2001, 2002). In future, we will characterise insertion mutants for the respective genes to study their putative involvement in DNA repair.

The cell cycle genes

As mutagens are very potent inducer of transient cell cycle arrest, the real-time PCR analysis was extended to 15 cell cycle genes (Table 5). Altered transcription was found for several genes (three cyclins, one CDK/cyclin interacting protein (WEE1) and two cell cycle regulators (DEL and E2Fa); Table 5). Interference with cell cycle regulation by salt stress in *Arabidopsis* roots was reported by Burssens *et al.* (2000). Initial repression followed by an increased expression was found for CDC2A, CycA2;1 and CycB1;1. Different transcriptional responses were obtained in our study for different types of cyclins and even for members of the same-type cyclins (between 26-fold upregulation of CycB1;1 and a reduction of CycB1;2 transcripts). Upregulation was also detected for CycA1;2 with a function in G2/M and G1/S transition. The strong upregulation of AtCycB1;1 suggests a specific role in response to mutagen stress. Modulation of CycB1;1 expression by binding of myb protein to the *cis*-acting element of *Arabidopsis* CycB1;1 was reported by Planchais *et al.* (2002). The high upregulation of the myb protein found here (68-fold increase; Table 4) further supports their finding. Three of the seven CDK/cyclin interacting proteins and cell cycle regulators tested also showed transcriptional response to genotoxins; transcription of the WEE1 protein, a negative regulator of mitosis which when overexpressed in yeast inhibited cell division (Sorrell *et al.*, 2002), was increased sevenfold, DEL (DP-E2F-like) was downregulated (twofold) and E2Fa upregulated (twofold; Table 5). The function of E2Fa in controlling the start of DNA replication, e.g. by regulating the expression of RNR2, has been studied by Chaboute *et al.* (2000). A cell-cycle-regulating function of the DEL protein is still hypothetical. Altogether, the altered expression of several cell cycle genes indicates that mutagen treatment interferes with regular cell cycle progression. Effects of mutagen-induced DNA damage on transcription of cell cycle genes are apparently complex, e.g. having upregulation of genes acting actually antagonistically (CycB1;1 and WEE1). Using synchronised suspension culture (Menges and Murray, 2002), transcriptional change in cell cycle genes upon DNA damage could be studied more precisely. The finding of an increase in transcription of the DNA damage checkpoint control protein Rad17 (sevenfold; Table 4) further supports apparent influences of mutagens on cell cycle progression in *Arabidopsis*.

Perspectives

Our study on the impact of genotoxic stress on gene expression in *Arabidopsis* provided a global view on the transcriptional response of potentially genotoxin-responsive genes. Future studies on those genes that appeared to be up- or downregulated, for which a function in DNA-damage response is not yet known, might provide more details about DNA repair and stress response in *Arabidopsis*. The availability of a complete genome chip (25 K-chip, Affymetrix), which contains perfect and mismatched oligonucleotides for the most putative *Arabidopsis* ORFs, might render the HDCA for *Arabidopsis* dispensable because of the capacity and sensitivity of Affymetrix chip; however, the advantage of easy handling and lower cost of the HDCA is still encouraging the use of the HDCA. As a result of lower stringency required for hybridisation in the HDCA compared to that in the Affymetrix chip, the former is more suitable for studies with different *Arabidopsis* ecotypes and close relatives. In particular, in organisms for which complete genome sequences are not available, the HDCA approach might be the matter of choice for transcription profiling. Real-time PCR is useful to verify and complement the data obtained from the HDCA or other arrays such as Affymetrix chips.

Experimental procedures

Growth conditions and genotoxin treatment of seedlings and suspension cultures from Arabidopsis thaliana

Seeds of *Arabidopsis* (Col-0) were sterilised with 4% sodium hypochloride. About 5 mg seeds were grown in a Magenta-box

in GM medium (with glass beads as solid support) with cycles of 16-h light at 22°C/8-h dark at 20°C for about 2 weeks (Menke *et al.*, 2001). *Arabidopsis* suspension culture (L. Heynh.) was grown under shaking (120 r.p.m.) in Medium 4X (modified after GAMBORG, DSMZ, Braunschweig, Germany) at 24°C in the dark for about 1 week. For the genotoxin treatment, the seedlings were collected and incubated in a 50-ml tube filled with GM medium with or without mutagens for 30 min to 24 h. The concentrations of genotoxins were: MMS and MNU, 1–10 mM; bleomycin, 0.25–1.5 μg ml^{-1}; mitomycin C, 10–500 μM and maleic hydrazide, 0.5–8 mM. After harvesting, the seedlings were either used immediately for RNA isolation or frozen in liquid nitrogen and stored at −80°C.

Isolation of the mRNA

Total RNA was isolated using peqGoldTrifast solution according to the manufacturer's instruction (peQLab, Germany), and mixed with oligo-dT cellulose (peQLab). The bound mRNA was eluted and used as a template for cDNA synthesis. For real-time PCR analysis, the mRNA was treated with DNase-I.

Construction of a cDNA library from mRNA of genotoxin-treated Arabidopsis *suspension culture*

For construction of the cDNA library, 5 μg of mRNA extracted from genotoxin-treated *Arabidopsis* suspension culture (bleomycin (1.5 μg ml^{-1}) plus mitomycin C (66.7 μM) for 6 h) was used. Procedures for the construction of the cDNA library, synthesis of the first- and the second-strand cDNA, cloning of the cDNA into vector (ZAP expression vector) and *in vivo* excision of the phagemid vector pBK-CMW were carried out according to the manufacturer's instructions (ZAP Express cDNA Gigapack III Gold cloning kit, Stratagene, La Jolla, CA, USA). The phage titre was about 10^6, and 27 000 *E. coli* clones were picked into 384-well microtitre plates using a QPix robot (Genetix, UK). The size of the cDNA inserts was estimated by restriction analysis and the coverage of the cDNA library by sequencing of 312 randomly selected clones.

Dot blotting

A dot blot analysis with PCR fragments of 35 genes involved in DNA repair was performed to find out efficient conditions for genotoxin treatment, which allow detection of altered transcriptional activity. PCR fragments of 0.5–1 kb were amplified and blotted onto a nylon membrane (Biodyne B, Pall, Portsmouth, UK). Each spot contained approximately 0.1 μg DNA. Denaturation, neutralisation and fixation processes were carried out according to the manufacturer's instruction (Pall).

HDCA analysis

About 27 000 *E. coli* clones from the cDNA library were arrayed (QPix robot, Genetix, UK) onto three nylon membranes in a double-spotting manner (each clone spotted in duplicate). One membrane (22.2 cm × 22.2 cm, Genetix, UK) contained 9000 clones (18 000 spots). After spotting, the bacterial colonies were grown overnight and then lysed. The DNA was immobilised and fixed onto the membrane using standard protocols.

cDNA probes for hybridisation were synthesised on Dynabeads oligo $(dT)_{25}$ (Dynal, Norway) according to the manufacturer's instruction. The frozen seedlings were ground and lysed, and the lysate was mixed with Dynabeads oligo $(dT)_{25}$. The bound mRNA (from 0.5 g of seedlings per experiment) served as a template for synthesis of a complementary cDNA strand (reverse transcription with Stratascript, Stratagene). After elution of mRNA, the covalently bound cDNA strand served as a template for synthesis of ^{33}P-labelled probes (Megaprime DNA-labelling system, Amersham, England). The probes were eluted from the beads and purified through a 0.2-μm spin column (VectaSpin Micro, Whatman, UK).

Pre-hybridisation (1 h) and hybridisation (overnight, with labelled probes) was carried out at 65°C in the church buffer (1 mM EDTA, 1% BSA + 7% SDS in 0.5 M phosphate buffer (pH 7.5)). Membranes were washed three times (15 min each) with 0.1% SDS + 0.2× SSC at 65°C and then exposed to imaging screens. Images were read using an image analyser FLA-3000 (Fujifilm, Japan). The intensity of the spots was analysed with ARRAY VISION software (version 5.1, Imaging Research, Canada). After background subtraction, the total signal intensities of images of different hybridisations for the same array were used for normalisation. Clones were considered for further evaluation if the signal intensity was 1.6 times the background value (100 AU; Figure 3), and both of the duplicated spots showed the same tendency for at least a twofold expression difference (either up- or downregulation). Alteration rates of identified genes are mean values obtained from two hybridisation experiments. Sequences of all 300 clones with deviating expression at a high expression level and 135 out of the 500 clones showing deviations at a lower expression level were analysed.

The spotted membranes could be re-used up to 10 times after stripping them twice with boiling solution of 0.1% SDS + 0.1× SSC, once with 0.4 M NaOH at 65°C and once again with boiling solution of 0.1% SDS + 0.1× SSC.

Sequence analysis

Sequences of identified clones were compared with the TAIR database using the program BLAST 2.0 (http://www.arabidopsis.org/Blast/). The genes were classified according to MIPS *A. thaliana* database (http://mips.gsf.de/proj/thal/db/tables/tables_func_frame.html).

Real-time PCR

For verification of the data obtained by the HDCA, 42 genes showing altered expression (see Table 4) were selected for analysis by real-time PCR using an iCycler iQ (Bio-Rad, Hercules, CA, USA) and SYBR I as a fluorescence dye. The cDNA strand synthesised with DNase-I-treated mRNA was used as a template. Primer pairs for each gene were designed to amplify fragments of approximately 180 bp. The expression levels were related to that of the 60S ribosomal protein L27A gene (At1g70600) as a standard. A PCR reaction volume of 50 μl contained the first-strand cDNA, DNA polymerase (Hotgoldstar, Eurogentec, Belgium, 1.25 U; in 1× buffer, Eurogentec, Belgium), the corresponding primer pair (each primer 1 μM), dNTP mix (each 0.2 mM), $MgCl_2$ (3 mM) SYBR I and fluorescein dye. After heat activation of the polymerase at 95°C, 45 cycles of denaturation (95°C, 30 sec), annealing (56°C, 30 sec) and amplification (72°C, 30 sec) were performed. During each amplification step, the PCR products were quantified. Melting curves for each PCR reaction were determined by measuring the decrease of the fluorescence with increasing temperature (temperature from 55 to 95°C). The specificity of PCR reactions was confirmed by melting curve analysis on the iCycler iQ as well as by agarose gel electrophoresis of the products. Threshold cycles at which the fluorescence of the PCR product SYBR I complex first exceeded the

background level were determined by the integrated analysis software for each gene. The relative template concentrations were evaluated based on the standard curve for the 60S ribosomal protein L27A gene. Each PCR run was carried out in triplicate. The results are mean values of at least two PCR runs.

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