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A New Use for Transgenic Plants – Environmental Biomonitors

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Introduction

Environmental factors such as temperature changes, light intensity, and water availability keep all organisms in a constant stage of genomic flux (Harwell *et al.*, 1994). In addition to natural influences, there is a growing contribution of anthropogenic pollution. The rapid economic progress of human beings is associated with stressors such as xenobiotic chemicals or nutrients. Most of the recent environmental pollution is due to careless industrial development. Mistakes and malfunctions of man-made equipment have led to the release to the environment of an enormous amount of heavy metal salts, as well as radioactive material. By the early nineties, the worldwide release of cadmium had reached an alarming 22 000 tons per annum. For copper, this had become 954 000 tons p.a., for lead 796 000 tons p.a., and zinc as much as 1 372 000 tons p.a. (Alloway and Ayres, 1993). The explosion of reactor 4 of the Chernobyl NPP led to extremely complex patterns of radioactive contamination in Ukraine and throughout Europe. The total activity of the radioactive material released during this accident has been estimated to have reached 3.2×10^8 Ci (IEAE International Conference, 1996). About 6% of continental Europe is still contaminated to more than 0.5 Ci/km^2. These, and other pollutants, have had a great impact on the well-being of living organisms. It is therefore of considerable importance to have a 'biological alarm system' that would alert us to the potential threat of environmental toxicants.

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Abbreviations: DSBs, double-strand breaks; GFP, green fluorescent protein; uidA (GUS), β-glucuronidase marker gene; HR, homologous recombination; *lacI*, *lacZ*, β-galacatosidase operon; MMS, methyl methanesulphonate; MNU, *N*-methyl-*N*-nitrosourea; NPP, nuclear power plant; PAHs, polycyclic aromatic hydrocarbons; PCBs, polychlorinated biphenyls; ROS, reactive oxygen species; TrMCN, *Tradescantia* micronuclei; TrSHM, *Tradescantia* stamen hair-mutation; UVB, ultraviolet-B; UVC, ultraviolet-C; 2,4-D, 2,4-dichlorophenoxyacetic acid.

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In this review, we consider the new application of transgenic plants as sensitive biomonitors of environmental pollution. We will also try to summarize the major environmental pollutants and their influence on plant life. Since it is impossible to cover the huge range of natural and man-made contaminants in detail, we will highlight the influence of radiation (ultraviolet and ionizing), heavy metal salts, and some organic chemicals, including various pesticides, herbicides, and insecticides, on plants.

ULTRAVIOLET RADIATION

The protective shield, the stratospheric ozone layer, which attenuates almost all solar ultraviolet-B (UV-B) and all ultraviolet-C (UV-C) radiation is being depleted by contaminants such as chlorofluorocarbons (Frederick, 1990; Kerr and McElroy, 1993). It is, and will be, accompanied by an increase in terrestrial UV-B irradiance, especially at wavelengths below 300 nm. Besides the influence on human health, UV-B radiation has a great impact on plant growth and development, as well as on plant genome stability (Tevini, 1993; Ries *et al.*, 2000). Terrestrial plants are especially vulnerable to UV-B due to their requirement for sunlight for photosynthesis. It has been predicted that a 1% decline in ozone could lead to a 1% reduction in crop yields (Coohill, 1991). UV-B damages organic molecules such as DNA, proteins, membranes, and phytohormones (Kramer *et al.*, 1991; Pang and Hays, 1991; Tevini, 1993). In addition, the toxicity of many environmental organic contaminants is activated and enhanced by light, as many of these toxic chemicals have strong absorbance bands in the UV-B range (Huang *et al.*, 1993; Ren *et al.*, 1994). Importantly, depending on the UV-B level, plants can either be damaged by higher doses or adapt to lower doses. Unfortunately, the definition of what constitutes a 'low' and a 'high' dose is not precise, and depends on the plant species.

IONIZING RADIATION

Radiation with the potential to significantly affect plants can come from various sources, either natural, i.e. cosmic or terrestrial, or anthropogenic. Natural radiation represents the background level to which all organisms are constantly exposed, whereas anthropogenic sources directly altered by human activity represent a major environmental threat. The majority of research regarding the influence of radiation on plants has been performed using individual plant species. Although there is a great variability between plants in their tolerance to radiation exposure, it is generally considered that a 10% affect on lethality represents about a 50% reduction in crop yield (Dugle and Mayoh, 1984). The ability of a plant to resist exposure to radiation is dependent upon the growth stage in which exposure occurs, as well as the particular plant organs (parts) that receive the irradiation. Cells that undergo intensive mitotic or meiotic division, e.g. meristems and reproductive cells, are more at risk than the other cells (Holst and Nagel, 1997). Depending upon the cell and the absorbed dose or exposure, various physiological processes can be disrupted, leading to changes in either single processes or whole metabolic pathways. These processes include alteration of the breakdown of the complex carbohydrates to hexoses and trioses, protein fragmentation, production of enhanced quantities of anthocyanins, and small stress-related, heat shock proteins (Holst and Nagel, 1997).

Ionizing radiation has been shown to both directly and indirectly (via production of free radicals such as hydrogen peroxide) influence the integrity of organic molecules in the cell, including DNA. Whereas high doses of radiation would cause extremely high levels of DNA strand breaks incompatible with cell survival, significantly lower doses of radiation would mostly contribute to a heavy mutation load. The mutations would result in changes to the morphology and anatomy, as well as productivity, of subsequent generations of the exposed plants.

HEAVY METALS

Metals are highly genotoxic environmental contaminants, although they are essential nutrients at low concentrations. With respect to their genotoxic potential, metals can be grouped to: essential and relatively non-toxic (e.g. zinc); essential, but extremely toxic at elevated concentrations (e.g. copper); non-essential but highly cytotoxic (e.g. cadmium) (Horn, 1984; Thiele, 1992). Toxic heavy metals cause DNA damage, and their carcinogenic effects in animals and humans are most probably related to their mutagenic potential (Hartwig, 1995; Knasmüller *et al.*, 1998). Although no common mode of action has been proposed which could explain the mutagenic and carcinogenic potential of different metals (Hartwig, 1995), two models currently predominate: 1) the formation of ROS leads to DNA damage; 2) heavy metals interfere with DNA repair and/or replication processes (Hartwig, 1995).

Metal ions such as Cu^{2+}, Ni^{2+}, and Cd^{2+}, can cause the formation of hydroxyl radicals and reactive oxygen species through Fenton-type reactions (Imlay and Linn, 1988). The resulting free radicals damage DNA. Point mutations can appear as a direct result of nucleotide damage by ROS, or as a result of nucleotide misincorporation due to malfunction of DNA repair under the influence of free radicals. Strand breaks can arise as damage to sugar–phosphate backbones, or transiently during the repair processes. Some metals and metalloid elements, such as Ni^{2+}, As^{3+}, and As^{5+}, are known to alter cytosine methylation patterns, causing either hypo- or hypermethylation of DNA (Mass and Wang, 1997; Lee *et al.*, 1998). Hypomethylation may increase susceptibility of the chromosomes to breakage. Hypermethylation may also cause chromosomal instability by inhibiting cell cycle-dependent checkpoint controls, with deleterious effects on the cell (Mass and Wang, 1997; Lee *et al.*, 1998).

ORGANIC CHEMICALS

Industrial development has increasingly exposed the biosphere to an expanding variety of chemicals of an anthropogenic nature. According to Gunther and Pestemer (1990), these 'environmental chemicals' represent a broad class of man-made substances that are hazardous not only to humans, but also to all biological organisms. Ten years ago, chemical invasion already included more than 20 000 man-made compounds (O'Connor *et al.*, 1991). These contaminants not only sediment in the soil or water but are also taken up by organisms in the process of bioaccumulation. Being sedentary organisms, plants often accumulate significantly higher levels of toxic compounds than other organisms. Being at the beginning of the food chain, plants are also the first step in the process of bioaccumulation. The process of bioaccumulation in plants may be confined to a single process of xenobiotic transfer from the

surrounding environment to the roots and shoots, with very little metabolic transformation (McCrady *et al.*, 1990). Organic chemicals like polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), and other compounds containing aromatic rings, are extremely genotoxic and carcinogenic. Many of these substances are volatile, and toxicity studies which can mimic the conditions of water and air pollution can be conducted by growing plants in sealed Petri dishes in medium supplemented with toxicants.

The steady increase in the use of pesticides in agriculture, as well as in domestic households, in the past two decades has resulted in a substantial increase in soil and water pollution. In the United States, Perelra and Hostettler (1993) have demonstrated that agricultural pesticides have degraded water quality along the Mississippi River and its tributaries. Inputs of the five major herbicides, atrazine, metolachlor, alachlor, and simazine, along the Mississippi River valley have contaminated nearby ground water and surface water (Perelra and Hostettler, 1993). Similarly, high concentrations of the organic pesticides, tetrachloroethane, atrazine, and nitrilotriacetate, have been found in a lake in Switzerland (Ulrich *et al.*, 1994). As organic chemicals, most pesticides probably cause DNA damage in exposed organisms.

BIOLOGICAL ASSAYS FOR BIOMONITORING

With the appearance of new toxicants, new studies for evaluating the potential hazardous effects on living organisms were required. Among the first laboratory tests used in such studies was the Ames test (Levin and Ames, 1986), and a variety of gain and loss of function tests of *E. coli*-based systems (Miller, 1985; Schaaper and Dunn, 1991). Based on prokaryotic organisms, these tests were often insensitive to the influence of pollutants. Thus, these methods were not useful for evaluating the influence of environmental factors on higher eukaryotes, since the exposure of these organisms to environmental pollutants is not regulated solely by the amount of contamination present but, more importantly, by the bioavailability of the compound and the duration of contact with the organism. Several other methods have been used to assess the genotoxicity of water and soils: the alkaline single cell–gel electrophoresis assay (a 'Comet assay') (Hartmann and Speit, 1994; Gichner *et al.*, 2000; Gichner *et al.*, 2001), sister chromatid exchange assay (Šiviková and Dianovský, 1995), the somatic eye mutation test in *Drosophila* (Rasmuson, 1985), and transgenic mouse-based systems (Sacco *et al.*, 1997; Mayer *et al.*, 1998).

Very few organisms are actually effective as biomonitors of environmental conditions (Sheehan and Loucks, 1994). Ideally, for field biomonitors test-organisms should absorb and integrate doses of toxicants from polluted water and soil. The use of animal-based models is problematic, due to their mobility: however, animals could be used for evaluating the influence of particular mutagens, such as defined concentrations of chemicals, or doses of radiation (ionizing and UV). For example, the recently developed transgenic zebrafish assay, which uses the non-active *lacI* transgene as a target gene, could potentially be applied for the evaluation of water quality (Amanuma *et al.*, 2000). Some tests using model organisms have been successfully used for genotoxicity studies. The transgenic mouse recombination-based assay (Murti *et al.*, 1994) uses the restoration of the *lacZ* gene in germ cells, and the *Drosophila melanogaster* system is based on the reversion of a recessive eye

colour mutation (Green *et al.*, 1986). However, it is difficult to estimate their use in environmental studies, especially for the analysis of effects of chronic exposure, as these models do not permit the study of the influence of complex patterns of soil pollution.

The other organisms with the potential to be used as biosensors, namely plants, are the major source of energy for nearly all higher eukaryotes, and play an active role in transferring contaminants to higher trophic levels (McVey and Macler, 1993). Plants are an essential part of a healthy environment, since they produce oxygen and organic carbon, essential factors for animal survival. Plants are also the basic components of agriculture and forestry, and thus, their well-being is an indicator for that of humans and the environment.

Conversely, unsatisfactory environmental conditions lead to reduced plant growth; therefore, many plant species are used as indicators for adverse environmental factors. Not surprisingly, phytotoxicity testing has long been used as part of ecological risk assessment of industrial and agricultural chemicals, food additives, solid wastes, and chemically and radioactively polluted soil and water. In addition, many reports have shown that plants have important roles in improving wastewater treatment and in cleaning contaminated water and soil, the so-called phytoremediation. Importantly, transgenic plants are not only exploited for improving the quantity and quality of food but, recently, systems have been developed for environmental biomonitoring (Kovalchuk *et al.*, 1998b, 1999, 2001; Ries *et al.*, 2000). The focus of this review is to discuss the use of different non-transgenic plant systems, which have been analysed for sensitivity to mutagenic treatment and as biosensors for measuring chemical and radioactive environmental pollution. We summarize the establishment, testing, and examination in the field of transgenic plants that were used for biomonitoring. We also propose new systems that can be used efficiently as biosensors of environmental pollution.

Classical plant biosensors

Several studies report the use of plants as genetic toxicity biosensors for environmental pollutants (Ichikawa, 1992; Grant, 1994; Grant and Salamone, 1994). Most systems used to study mutations in plants are based on the detection of chromosomal aberrations in *Allium cepa* (Fiskesjo, 1988), *Tradescantia* (Ichikawa, 1992), or *Vicia faba* plants (Kanaya *et al.*, 1994). The *Allium cepa* chromosome aberration test provides a rapid screen for the toxic effects of chemicals (Grant, 1994; Nielsen and Rank, 1994), and was the first of nine plant assay systems evaluated in the Gene-Tox Program of the US Environmental Protection Agency (Constantin and Owens, 1982; Grant, 1994). Because of their big nuclei, root-tip cells of *Allium cepa* are used for the assessment of aberrant anaphase and telophase during mitosis. The assay is based on the evaluation of the percentage of cells undergoing mitosis, the estimation of the number of aberrant versus normal mitotic events, and different fractions of chromosomal aberrations, such as bridges, fragments, vagrant and sticky chromosomes, c-mitotic events, and multipolar anaphases (Fiskesjo, 1988). This test has been used extensively for wastewater monitoring (Smaka-Kincl *et al.*, 1996). Scoring the incidence of chromosomal aberrations and micronuclei in root-tip cells is a simple method for studying the effects of different mutagenic agents such as mercury,

selenium, zinc, and cadmium compounds, as well as pesticides (Gulati *et al.*, 1994; Borboa and de la Torre, 1996).

Tradescantia is another important plant used for environmental mutagenesis studies. This plant is utilized in three different bioassays: i) chromosome aberration assay based on observation of visible chromosome damage during mitosis in root-tips, pollen-tubes, and microspores; ii) stamen-hair mutation assay – a point mutation assay based on the expression of a recessive gene for flower colour in heterozygous plants (TrSHM); iii) cytogenetic test based on the formation of micronuclei that result from chromosome breakage in the meiotic pollen mother cells (TrMCN) (Rodrigues *et al.*, 1997). *Tradescantia* has been used in various studies for evaluating air quality, water and soil pollution. *In situ* assays conducted in public parking garages in Illinois (USA) revealed a correlation between the rate of production of micronuclei and the intensity of traffic, with a positive dose–response relationship (Ma and Harris, 1985). The TrMCN assay revealed positive results when estimating the mutagenic effects caused by gaseous emissions from a municipal waste storage facility (Ma *et al.*, 1996) and a landfill vent pipe (Ma, 1994; Ma *et al.*, 1996). In a study of industrial wastewater in Mexico, a higher incidence of micronuclei was found relative to tap water controls (Ruiz *et al.*, 1992). The clastogenicity of several chemicals commonly found in hazardous waste sites was evaluated in a series of experiments with the use of TrMCN (Sandhu *et al.*, 1989). The assay proved to be extraordinarily sensitive in the assessment of bioremediation measures at the sites, permitting the detection of differences in mutagenicity of the soil polluted with creosote (polyaromatic hydrocarbons) before and after remediation (Baud-Grasset *et al.*, 1993).

Some other plant systems have been applied to environmental studies. Tobacco plants heterozygous for the *Sulphur* (*Su*) nuclear gene, which affects the chlorophyll content in leaves, have been used for the study of mutagenicity of different chemicals (Friedlender *et al.*, 1996). Mutations in this gene determine a co-dominant phenotype, exhibiting the genotype of *Su/Su* (dark green), *Su/su* (light green), or *su/su* (albino) plants (Burk and Menser, 1964). Irradiation of *Su/su* seeds with 20 krad gamma radiation and treatment with different concentrations of methyl methanesulphonate (MMS) or *N*-methyl-*N*-nitrosourea (MNU) caused a strong increase in the number of dark green spots on light green leaves in comparison to non-treated plants (Friedlender *et al.*, 1996). These plants could be used in environmental mutagenesis studies, although the types of molecular changes that occur to create this phenotype are unknown.

Biomonitoring of radioactive pollution with classical tests

One of the first studies monitoring the influence of chronic radioactive pollution was conducted in Japan, where the TrSHM assay was used *in situ* to monitor for ionizing radiation in the vicinity of nuclear power plants (Ichikawa, 1981). Significantly increased mutation frequencies were correlated with wind direction and operation periods of the nuclear facilities. Cebulska-Wasilewska (1992) observed that an increase in the spontaneous mutation frequencies of *Tradescantia* correlated with the contamination in Cracow caused by the blow-out of the Chernobyl nuclear reactor 700 km away.

The *Allium cepa* test was also used to estimate the mutagenicity of ionizing radiation. Inhibition of root development in onion bulbs, and increased incidence of chromosomal aberrations in root cells after exposure to radiation (Cortes *et al.*, 1990; Mateos *et al.*, 1992; Paradiz *et al.*, 1992, 1995) were documented. These effects were also observed in space-exposed onion seeds (Wang, 1994). We have reported the application of the *Allium cepa* test for evaluating the chronic influence of ionizing radiation stemming from nuclear polluted soils (Kovalchuk *et al.*, 1998a). We observed a dose-dependent increase in the level of chromosome aberrations in the root-tip cells of onion seeds germinated on radioactively polluted soils.

Other studies estimating the genotoxic effects of ionizing radiation showed: 1) an enhanced incidence of DNA strand breaks in chronically exposed plant populations (Syomov *et al.*, 1992); 2) chromatin structure changes of seedlings and callus tissues derived from exposed wheat and barley (Reshetnikov *et al.*, 1996); 3) an elevated frequency of chromosomal aberrations in rye and wheat grown within the exclusion zone (Shkvarnikov, 1990); 4) morphogenetic changes in populations of *Plantago lanceolata* L. grown from seeds of first and second generation post-accident plants (Frolova and Popova, 1990); and 5) an increase in the number of embryo-lethal mutations in *Arabidopsis thaliana* populations grown in the 30 km zone around the Chernobyl NPP (Abramov *et al.*, 1992).

Recently, we have developed a new microsatellite-based assay for monitoring radiation-induced germ-line mutation in plants. We profiled two, initially genetically identical wheat (*Triticum aestivum* L.) populations. Plants were grown in the experimental plot 'Chistogalovka' in the Chernobyl exclusion zone, on peat-bog soil with a mean surface density of radioactive contamination of 900 Ci/km^2, and an external gamma dose rate of 980 mR/h. A genetically identical population of wheat plants was grown in uncontaminated soil, and served as control. A nearly four-fold increase in heterozygosity at 13 microsatellite loci was found among the offspring of exposed plants. As calculated in our previous study (Kovalchuk *et al.*, 2000c), such a difference in microsatellite stability could be due to a 6.5-fold increase in germ-line mutation rate in the parental generation. We also analysed the changes observed at molecular level (Kovalchuk *et al.*, 2003a).

Importantly, we could show a statistically significant difference in the germ-line mutation rate on a relatively small sample size. Detection of the same increase in mutation rate by standard genetic techniques would be possible only with the use of nearly one million plants. This, together with other unique characteristics of plants, such as a high growth rate and short generation time, makes the proposed technique a potentially powerful approach for monitoring radiation-induced germ-line mutations. The current data also raise an important issue of the genetic hazard of chronic internal and external radiation exposure to the germ-line, showing that the apparent rate of induced microsatellite germ-line mutation is far higher than can be predicted from existing estimates of absorbed doses of exposure.

Transgenic plants as biosensors of environmental contamination

The systems described above are sensitive and useful, yet the changes most of them measure are not understood at the molecular level. Additionally, most of them require either specific knowledge or sophisticated equipment. In our previous studies, we

have developed easy, rapid, cheap, and precise assays for the study of genotoxicity of radioactively and chemically polluted soil. Our assays are based on the restoration of the transgene activity in the *Arabidopsis thaliana* or *Nicotiana tabacum* plants, transformed with non-active β-glucuronidase (*uidA*) marker gene.

RECOMBINATION REPAIR ASSAY

To develop a 'plant recombination' system, *Arabidopsis thaliana* and *Nicotiana tabacum* plants have been transformed with two overlapping, non-functional, truncated versions of a chimeric β-glucuronidase (*uidA*) marker gene as a recombination substrate (Swoboda *et al.*, 1994). In cells in which events of HR at this transgenic locus have occurred, the *uidA* gene was restored. Upon histochemical staining, cells expressing β-glucuronidase, and their progeny, could be localized precisely as blue sectors on white plants, representing recombination events that restored the disrupted gene (*Figure 2.1a*). This enabled a quantitative assay. These plants have previously been reported to respond to the influence of different DNA damaging agents, such as UV-C irradiation and MMS treatment, with increased frequency of homologous recombination (Puchta *et al.*, 1995).

Ionizing radiation is a potent mutagene, which causes biochemical modifications of DNA bases and double-strand breaks. The latter are, in part, repaired by homologous recombination (Puchta and Hohn, 1996), leading to the restoration of β-glucuronidase activity in the 'plant recombination' system. These events thus represent a measure for the level of DNA strand breaks in the analysed gene and, by inference, of the plant genome.

The evaluation of the genome stability of plants exposed to ionizing radiation is important, not only because many agricultural areas are polluted with long-life radioisotopes but also because of an increasing biological availability of ^{137}Cs and ^{90}Sr in the contaminated territories (Bondar' *et al.*, 1994). Consequently, we planned and conducted large scale environmental monitoring experiments with the use of transgenic *Arabidopsis thaliana* and *Nicotiana tabacum* plants. In several parallel studies, we sampled soils from different contaminated areas of the Ukraine with various levels of radioactive pollution. We performed field and laboratory experiments using the soil from the Chernobyl exclusion zone – zone 1 (seven experimental plots) (Kovalchuk *et al.*, 1998b) – as well as in different areas of the other three contaminated zones (zones 2–4) (Kovalchuk *et al.*, 1999). The radioactivity of the soil samples from the exclusion zone (for ^{137}Cs) varied from 1472 kBq/kg in the 'Red Forest' plot to 5 kBq/kg in the Lelev plot, and 24 Bq/kg in the control area. The external gamma dose rate ranged from 11 μR/h in the control area to 977 μR/h in Chistogalovka I and up to 8500 μR/h in the 'Red Forest'.

The use of plants as bioindicators has allowed us to calculate the absorbed dose of radiation for *A. thaliana* grown both in the field and in laboratory conditions. This dose was calculated as the sum of the external and the internal dose. The internal dose is the consequence of uptake of radionuclides by plants grown in the laboratory or in the field, whereas the external dose is due to external exposure and believed to be fully absorbed by plants. Although the internal dose was nearly identical for plants grown in the field or laboratory, the external dose differed significantly between these two groups because, at the plant level, approximately 40 m^2 of surrounding land contributed

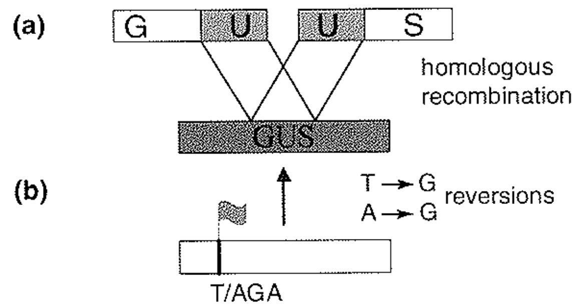

Figure 2.1. Transgenic 'recombination' and 'mutation' systems for the detection of environmental mutagens. Activation of the β-glucuronidase (GUS) gene via either homologous recombination or point mutation was visualized as blue spots after histochemical staining. The two parts of the truncated, overlapping GUS gene can be in either orientation with respect to each other.

to the formation of the external γ dose rate (Moiseev and Ivanov, 1991; Kovalchuk *et al.*, 1998b). In the laboratory, over 50% of the absorbed dose was due to internal exposure, whereas in the field it contributed only 20–25%. Moreover, we noted a growing contribution from external exposure to the total dose absorbed by the plants with an increase in soil surface contamination in open-field conditions. Hence, laboratory tests conducted on the same soil samples provided additional insight into the plant uptake and impact of contaminants on plants, thereby maximizing the value of information obtained from field biomonitoring. Remarkably, it was possible to correlate recombination frequency not only to the level of the soil contamination but also to the absorbed dose. In both cases, we obtained a strong and significant correlation, closely approximated by either an exponential growth regression model, or double exponential regression model, the Bateman-type function (Sokal and Rohlf, 1995).

We have detected a hazardous influence of a chronic ionizing radiation on the stability of the plant genome in both sets of experiments (field and laboratory) conducted in the exclusion zone. We observed a dose-dependent 8.4-fold increase of homologous recombination in plant populations over controls at pollution levels up to 300 Ci/km^2, and an 11.0-fold increase at pollution levels up to 1000 Ci/km^2 in the open-field (*Figure 2.2*, recombination line). Interestingly, with even higher levels of radioactive pollution, we noticed a substantial decrease of homologous recombination, to 2.6-fold above the control level (*Figure 2.2*, recombination line). There are several possible explanations for these findings. The decrease of recombination can be due to either the toxic effects of high levels of radiation killing the cells, or completely disturbing the whole repair mechanism. Alternatively, it may be due to a shift to illegitimate recombination, as a major strand-break repair pathway. In the latter case, the transgene cannot be restored, and the influence of radiation cannot be monitored. On the other hand, the observed increase in recombination-like events in irradiated somatic and germ cells may be far greater than can be predicted from various forms of DNA damage induced by ionizing radiation (Dubrova, 1998). This would mean that the increase in recombination is not caused by direct damage of DNA (targeted events) but, rather, it is the consequence of non-targeted effects caused by

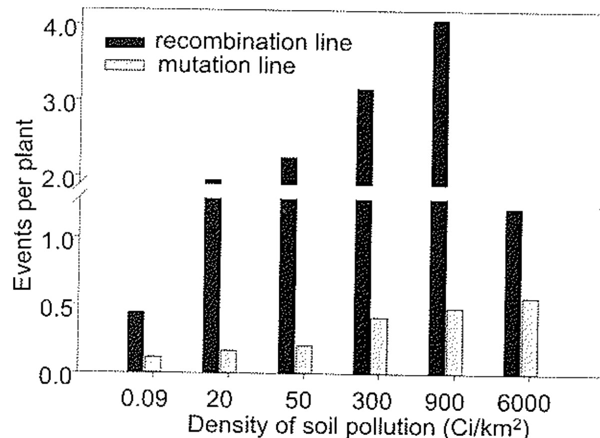

Figure 2.2. The level of homologous recombination and point mutations represents an average number of blue sectors per transgenic *A. thaliana* plant. Plants were grown in Chernobyl in soils with different mean density of pollution. The data are combined from two independent experimental sets (Kovalchuk *et al.*, 1998b, 2001).

radiation elsewhere in the genome. If such a hypothesis is true, then the observed decrease in recombination frequency may be the result of some saturation effect on the system involved in this process.

As the Chernobyl exclusion zone is uninhabitable, it is probably not of primary importance for the evaluation of the long-term effects of ionizing radiation on the plant genome. However, estimation of the genetic biohazard of substantially lower levels of soil contamination is urgently required. We conducted experiments in the following inhabited areas: zone of enhanced radiological control – villages located in the Snyatyn district of the Ivano-Frankivsk region; obligatory resettlement zone – villages and towns located in the Narodichi district of the Zytomir region; and uncontaminated areas as a control. The activity of ^{137}Cs ranged from 22 Bq/kg in control samples to 6549 Bq/kg in inhabited areas. The mean density of soil contamination calculated from the activity of the samples varied between 0.1 and 30 Ci/km^2, respectively. Experiments performed in the zone of obligatory resettlement (zone 2) and enhanced radiological control (zones 3,4) revealed a statistically significant dose-dependent increase of HR (Kovalchuk *et al.*, 1999). Of special importance is the fact that we were able to detect a difference in the frequency of homologous recombination between the plants grown on the clean soil (22 Bq/kg) and plants grown on the soil with a level of contamination as low as 1.5–3.3 Ci/km^2 (188–575 Bq/kg).

Since similar data were obtained from the experiments using transgenic *Nicotiana tabacum* plants, we could suggest that an increase in radiation-induced homologous recombination is plant-wide rather than *Arabidopsis*-specific. Thus, transgenic plants have been shown to be sensitive for the detection of the genetic hazard, stemming not only from highly contaminated soils located in the exclusion zone but also from the soils with substantially lower levels of contamination.

To evaluate the importance and reliability of our new method, we compared it to the

Allium cepa chromosomal aberration assay. Onion seeds were germinated on the same contaminated soil samples used in the recombination experiments. Three days after germination, mitotic activity of root-tip cells was analysed, and the frequency of aberrant mitoses was evaluated. We could relate the frequencies of HR in the transgenic *Arabidopsis thaliana* plants to the level of chromosomal aberration in mitotic root-tip *Allium cepa* cells, and found a significant correlation ($r > 0.90$, $n = 6$, $P < 0.05$) (Kovalchuk *et al.*, 1998b). The increase of recombination frequency measured in the transgene in *Arabidopsis* was followed by an increase of the aberration frequency observed in onion, particularly in the fraction of bridges and fragments as direct results of single- and double-strand breaks. The decrease of the recombination frequency observed in *Arabidopsis thaliana* plants at the higher pollution levels was related to an increase of the total percentage of aberrant cells, and a decrease of the fraction of bridges and fragments (70.5 ± 7.9 % in the control to 2.7 ± 1.6% in the 'Red Forest', and from 28.8 ± 7.1% in the control to 2.2 ± 1.6% in the 'Red Forest', respectively). However, the increase of aberrant mitosis was mainly due to the contribution of the fraction of cells with c-mitotic effects – sticky and vagrant chromosomes, as well as multipolar anaphases, highly toxic changes usually leading to cell death. These high numbers of lethal aberrations may serve as one of several possible explanations for the reduced frequency of HR in plants grown on highly radioactively contaminated soils.

Acute or chronic contact with radiation may have different effects on the genome integrity. Although in a radioactively contaminated environment plants are mostly exposed to chronic pollution, evaluation of both influences is important. Plants were grown on two chemically different types of soils, each artificially contaminated with equal amounts of ^{137}Cs. A strong and significant correlation between the frequency of HR in plants, the radioactivity of the soil samples, and the doses of radiation absorbed by plants (in all cases $r > 0.9$, $n = 6$, $P < 0.05$) was observed (Kovalchuk *et al.*, 2000a). In addition, we have noted that plants grown in soils with a different chemical composition, but equal radioactivity, exhibited different levels of HR, dependent upon the absorbed dose of radiation. Remarkably, we have observed a much higher frequency of HR in plants exposed to chronic irradiation, when compared to acutely irradiated plants. Although acute application of 0.1–0.5 Gy did not increase the frequency of HR, the chronic exposure of plants to a lower dose (200 mGy) led to a 5–6-fold induction of HR frequency, as compared to the control (Kovalchuk *et al.*, 2000a).

These unexpected results could be explained in several ways. During acute exposure to IR and immediately afterwards, plants probably mobilize protection mechanisms and DNA repair to combat any damage. Nearly two-thirds of double-strand breaks are created by free radicals from ionized water (Ward, 1998). Part of the radicals that are not scavenged via detoxification pathways can be converted into toxic forms such as OH^-, which can directly damage DNA, causing strand breaks. Some of these breaks are apparently repaired by homologous recombination shortly after irradiation. In contrast to acutely irradiated plants, plants grown in the polluted soil have to deal with the constant exposure to IR, and hence, with constant production of free radicals. Although the amount of radicals generated during chronic exposure to low doses of radiation is significantly lower in comparison to acute irradiation, the permanent exposure may induce many more DSBs, which are in part repaired by HR.

Another explanation could be that, after acute exposure, plants utilize the fastest possible mechanism of DNA repair, and a relatively higher percentage of the damage is repaired via illegitimate recombination (end-to-end joining) (Salomon and Puchta, 1998; Britt, 1999). In this case, an increase of the frequency of HR would not be observed, since the repair by illegitimate recombination cannot be detected by the system used.

POINT MUTATION DETECTION ASSAY

The system described above measures the homologous recombination in plants. Although the recombination repair system allowed for estimates of the number of precisely repaired strand breaks created under the influence of a particular mutagene, it could not show the rate of repair mistakes leading to mutations. Somatic mutation events are of particular importance in plants, they do not have a predetermined germ-line, but form their reproductive structures from somatic meristems late in development. Thus, any somatic mutation can potentially be passed on to subsequent generations. In contrast to animals, plants cannot avoid the influence of environmental factors due to their life, and therefore probably require a system dedicated to maintaining genome stability.

In recent studies, we have tried to develop a system that would allow the detection of somatic point mutations. Amber, opal, and ochre stop codons were introduced at several different positions into the β-glucuronidase (*uidA*) gene. These termination codons completely prevented translation of active protein. We generated several transgenic *Arabidopsis thaliana* plant lines carrying these inactivated *uidA* genes in the different chromosomal regions (Kovalchuk *et al.*, 2000b). Spontaneous restorations of *uidA* activity due to the reversion of stop codons to the original codons were observed (*Figure 2.1b*). A large variation in the mutation frequency of individual single copy transformants could be determined. Reversion frequencies were in the range of 10^{-7}–10^{-8} events per base pair, exceeding the previous estimates for other eukaryotes by at least 100 times. The ability of the 'stop-codon' transgenic plants to detect the mutagenic effects of various DNA damaging factors was verified by treatment with UV-C, X-rays, and methyl methanesulphonate (MMS) (Kovalchuk *et al.*, 2000b). Most of the lines reacted strongly to mutagens.

We tested the ability of our new 'mutation' system to detect the mutagenicity of various concentrations of heavy metal ions. In conjunction, we also used a well-defined 'recombination' system. Plants sown on medium contaminated by the heavy metal salts Cd^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, and As_2O_3, exhibited a pronounced uptake-dependent increase in the frequencies of both somatic intrachromosomal homologous recombination and point mutation. Despite some insignificant differences, plants from both systems reacted similarly to the influence of heavy metals. In order to monitor environmental pollution, several soil samples were taken from places contaminated by a nearby oil refinery station. Under laboratory conditions, test plants were sown in soils collected from sites exhibiting different levels of contamination with Pb, Cd, Zn, and other elements. A 4–7-fold increase in the frequency of homologous recombination and a 5–10-fold induction of point mutations in plants grown in the contaminated soils was noted, compared to those grown in the clean control soil (Kovalchuk *et al.*, 2001).

In order to evaluate the relative sensitivity of our transgenic plant systems compared to other genotoxicity tests, we used the data from several studies (Rossman *et al.*, 1991; Bitton and Koopman, 1992; Codina *et al.*, 1993; Bitton *et al.*, 1994; Hartmann and Speit, 1994; Rank and Nielsen, 1994; Kong *et al.*, 1995; Sacco *et al.*, 1997; Steinkellner *et al.*, 1998; Helleday *et al.*, 2000). The parameters tested in the other assays were, of course, different, and are therefore not necessarily comparable. Our transgenic systems appeared to be one of the most sensitive with respect to detecting the mutagenicity of individual heavy metals. There were very few other systems that were able to detect mutagenicity at low concentrations of heavy metals. For example, a concentration of 0.03 mg/L Cd led to a 50% decrease in β-galactosidase activity in the MetPLATE *E. coli* test (Bitton and Koopman, 1992). Similarly, our system also showed more than a two-fold increase in both mutation and recombination levels after exposing plants to as little as 0.01 mg/L of Cd. In the same MetPLATE *E. coli* test, Zn at a concentration of 0.1 mg/L also led to a 50% reduction of β-galactosidase activity (Bitton and Koopman, 1992). Our system detected mutagenicity of Zn at 6.0 mg/L; however, lower concentrations were not tested. Several other systems summarized by Kong *et al.* (1995) were equally sensitive to the transgenic *Arabidopsis* system: EC_{50} (mg/L) upon exposure of several invertebrate species (*Daphnia pulex, Daphnia magna, Ceriodaphnia dubia*) were in the range of 1.8–6.6 for As, 0.05–0.35 for Cd, 0.018–0.23 for Cu, 0.14–7.6 for Ni, 0.53–4.9 for Pb, and 0.05–5.1 for Zn: median lethal concentrations of Cd, Cu, and Zn after 96 h exposure of the fish *Pimephales promelas* were 0.01–2.2 mg/L, 0.02–0.1 mg/L, and 0.33–1.7 mg/L, respectively (Kong *et al.*, 1995). Similarly, our system detected the toxic effect of Cd, Cu, Ni, Pb, and As at concentrations as low as 0.001, 0.05, 0.1, 0.5, and 0.05 mg/L, respectively (Kovalchuk *et al.*, 2001).

Both transgenic *Arabidopsis* systems proved to be more sensitive than the well-established *Tradescantia* MCN assay (Steinkellner *et al.*, 1998). It is quite possible that the difference in sensitivity of these two systems (*Tradescantia* MCN and transgenic *Arabidopsis*) could be explained by the time of exposure to mutagenic compounds. To efficiently mimic the environmental exposure, the mutagene should be applied for a long period of time. While, in our assays, plants were germinated in the presence of a mutagene and were subsequently exposed for 35 days, in the *Tradescantia* MCN assay, cuttings were exposed for 6 h, and intact plants for only 72 h. Therefore, DNA repair mistakes accumulated in our assays for the whole growth period, and allowed us to monitor the outcome at the end of the exposure time. In contrast, in the *Tradescantia* assay, DNA damage occurred only within the short time of the exposure, and possibly for a short time afterward. Additionally, we measured point mutations (many of them would be silent in other assays), as well as homologous recombination (precise repair of DSBs). This subtle kind of DNA damage would not be detected as micronuclei by the *Tradescantia* assay.

For most metals tested, the toxic concentrations detected by cell culture, bacterial and mammalian tests were significantly higher than those 'measured' by our plants. Bacterial systems are less sensitive than ours, but are equivalent to other systems used for genotoxicity studies. For example, the Microtox test is very sensitive to the influence of Cu and Zn, but not Cd and Ni (Kong *et al.*, 1995). Generally, the lower sensitivity of bacterial systems in comparison to our assay may be explained by the shorter exposure time (hours versus days), and possibly better detoxification activities

of prokaryotes. Additionally, different parameters are controlled. In the Microtox test, inhibition of growth and respiration are measured, whereas in our system, DNA damage repair is analysed. Our system is superior in that the long exposure time mimics the extent of environmental exposure, and employs a sensitive target – the DNA.

Another widely used sensitive system, the *Allium cepa* chromosome aberration assay, exhibited higher sensitivity than the Ames and Microscreen tests (Rossman *et al.*, 1991). It is difficult to explain why plant-based assays are more sensitive to heavy metals than other assays, but two factors may contribute: 1) plants probably accumulate a higher concentration of metals than other organisms; 2) since they cannot escape from a toxic environment, plants have evolved to cope with a higher level of repair mistakes in somatic tissues, as indeed was shown in our previous studies (Kovalchuk *et al.*, 2000). Although the hypothesis that 'active' concentrations (concentration in the organism) of heavy metals are higher in plants than in animals is an attractive one, it remains untested. Data comparing the heavy metal uptake in different organisms are lacking.

In order to test the efficiency of the newly generated 'mutation' assay for ionizing radiation biomonitoring, we tested plants on the radioactively polluted soil taken from various contaminated areas around Chernobyl. Seeds were germinated in pots containing contaminated soil, and plants were harvested 35 days after germination. We observed a steady increase in mutation frequency (*Figure 2.2*, mutation line) which was dependent upon the increase in mean density of soil pollution (Kovalchuk *et al.*, 2001). Similar data were obtained for several other transgenic lines that carried different mutation substrates (Kovalchuk *et al.*, unpublished). In contrast to the decrease in the recombination frequency at the higher level of pollution, mutation frequency steadily increased (*Figure 2.2*). This is not surprising, as 'recombination' assay measures precise repair of DSBs, and may malfunction at the higher radiation levels, whereas 'mutation' assay detects various repair mistakes that lead to point mutations.

Transgenic systems may also be utilized to sense other types of environmental mutagens

INCREASED UV-B DETECTION

The transgenic recombination lines have also been used to evaluate potentially mutagenic exposure to various levels of UV-B radiation. Experiments with special sun simulators revealed that elevated UV-B increases the frequency of somatic homologous recombination in a dose-dependent manner (Ries *et al.*, 2000). In addition, the system allowed for the measurement of germ-line recombination either as a result of an inherited late somatic event, or as a meiotic recombination event. Elevated levels of UV-B resulted in an increase in the appearance of plants totally stained blue – plants in which the restoration of the marker gene was inherited by a factor of between two to five (Ries *et al.*, 2000). These data are of great importance, as they indicate the alarming mutagenicity of even marginally higher UV-B levels. Currently, we are working on monitoring the influence of various UV-B levels across Alberta, Canada.

WATER POLLUTION

The transgenic plant biomonitoring assays were also used to assess the genotoxicity of radioactively contaminated water. Water was sampled from the private wells in the villages from the inhabited 'Chernobyl' and uncontaminated control areas. Transgenic *A. thaliana* plants were sown in Petri dishes with medium prepared using control or contaminated water.

Our analyses have revealed that the drinking water from private wells in villages contaminated after the Chernobyl NPP accident was genotoxic. Although radiological analyses of the water samples did not reveal any ^{137}Cs or ^{90}Sr activity, because the concentration of these nuclides fell below the range that can be detected with the standard equipment, we found an increase in homologous recombination frequency in *A. thaliana* cultivated on the water from contaminated regions of the Ukraine. This increase was correlated with the soil surface radioactive contamination. In contrast to common radiation measuring systems, which did not detect traces of radioactivity in the water, our biomonitoring systems proved to be reliable and sensitive, and detected even minute levels of radionuclides (Kovalchuk *et al.*, 2003b).

INFLUENCE OF PESTICIDES

Currently, we are assessing the mutagenicity of different chemicals of organic and non-organic origin by using both transgenic systems. We are analysing the influence of various pesticides that are used either as mixtures or as separate active entities. In particular, transgenic plant assays were successfully applied to evaluate the genotoxicity of Atrazine, simazine, 2,4-D, dicamba, mecoprop, and complex herbicide Killex (Filkowski *et al.*, in press).

Marker genes to be used for the mutagenesis assays

In all our previous studies, we used only one marker gene, β-glucuronidase. Both repair assays were based on the detection of the activity of this marker gene upon the restoration of gene integrity. The minor disadvantage of this visual marker gene is that its detection requires non-vital histochemical staining. It is not feasible to measure the level of point mutations and strand breaks before and after application of the mutagene. To overcome this obstacle, we generated another reporter construct that utilizes the luciferase gene. Detection of luciferase activity is not destructive. A similar recombination substrate was generated, and transgenic *Arabidopsis* and tobacco plants carrying the construct were obtained. Frequent recombination events were observed using an *in vivo* imaging system. A special custom-built LN CCD cooled luciferase camera was used. The major advantage of the luciferase reporter gene is the vital detection of the luciferase activity. The recombination events can be visualized before and after the application of the mutagene (*Figure 2.3a,b*). Another transgenic visual marker that is frequently used in mammalian research, green fluorescent protein (GFP), appeared unsuitable for recombination and mutation assays. Although the whole GFP+ plants are easily detectable, single cells with recombination or mutation events that lead to the restoration of the GFP function were hardly visible, and required laborious microscopic analysis.

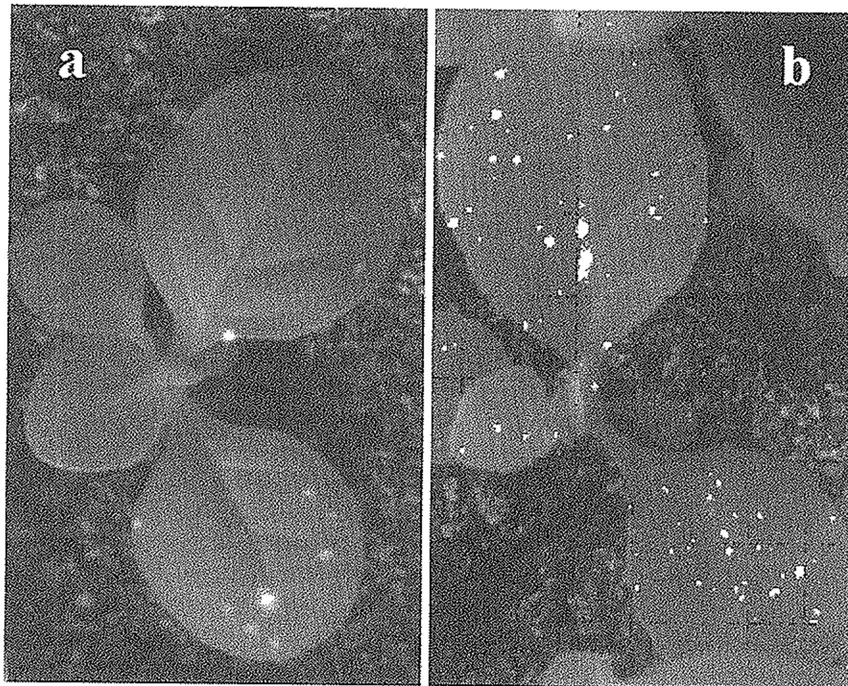

Figure 2.3. Luciferase as a recombination-monitoring transgenic marker. (a) The recombination spots were detected on 5-week-old tobacco plants prior the treatment with 7000 ergs of UV-C. (b) Recombination spots were detected on the very same plants one week after the UV-C treatment.

Other possible transgenic systems for detection of environmental mutagens

The 'recombination' reporter line is ideal for the study of strand break induction; it has an acceptable spontaneous recombination frequency and requires 50–100 plants to be scored for the statistical analysis. The system also provides a large mutation target; recombination repair of the damage to either of two homologous repeats (ca. 500 nt each) would lead to the activation of the transgene. In contrast, 'mutation' lines have much lower frequency of transgene activation, and require 200–1000 plants for the efficient statistical analysis of the data. The low frequency of point mutations which is observed in the plant lines described above is, of course, due to the small target used: a single base pair in the diploid genome. One way to increase the target size would be to design a negatively scorable screen in the form of a repressor–test gene combination: an active repressor binds to the promoter sequence blocking the reporter gene expression (*Figure 2.4*). Tetracycline repressor is a commonly used repressor that efficiently blocks the transcription of the reporter gene, which is driven by a promoter element including a repressor binding site. Inactivation of the function of the tetracycline repressor by any kind of mutation, strand breaks, or point mutations would restore the promoter function and activate the reporter transgene. The number of mutational spots, as a reflection of the transgene activation, is expected to

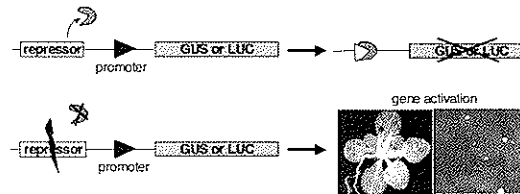

Figure 2.4. Possible tetracycline repressor-based system. Under the normal conditions, the marker gene is not active since the repressor blocks the promoter. Mutations in the repressor region will lead to the release of the activity of the marker gene. This can be effectively visualized as sectors representing the somatic cells with either luciferase or β-glucuronidase activity.

significantly exceed that measured to date. This will reduce the number of plants used, and allow for better statistical analysis of the data. There are, of course, some disadvantages: the system requires repressor binding to be tight, and in addition, the test plants would have to be heterozygous for the gene encoding the dominant repressor to ensure the inactivation of the single copy repressor element by the mutation event.

There are some other possibilities for the efficient detection of transgene activation. It may be possible to introduce simple frameshifts that would abolish the gene activity. Any mutations, deletions, or insertions that restore the frame would potentially activate the transgene. It is also possible to generate plants that carry an inactive version of the antibiotic (or herbicide) resistance gene. The progeny of the exposed plants may be grown in the presence of the selective agent, and resistant plants are scored. This approach will allow for the detection of inherited spontaneous, or induced, mutation events. Although this approach looks appealing, it requires a much longer time for the evaluation of the environmental influence, as well as an extremely large number (2×10^5–10^6 plants, not published) of progeny plants to be scored.

Summary and outlook

Several biological systems for evaluating the influence of environmental pollution have been established previously, yet no simple eukaryotic test system was available. We have introduced a new transgenic approach, which is fast, sensitive, and capable of visualizing mutagene-induced homologous recombination and mutation events. It permitted rapid screening of the genotoxicity of soils in the highly contaminated exclusion zone, as well as in the much lower contaminated inhabited areas polluted as a result of the Chernobyl NPP accident. It also allowed us to monitor the pollution of soil by heavy metals and herbicides, as well as water by radioactivity. As the systems permit data collection after a short period of time (around four weeks), and do not require any sophisticated equipment and specific knowledge for the detection and scoring of recombination events, it can be broadly used for environmental studies. Even large scale experiments for the evaluation of radiation toxicity may be possible. This is of primary importance, considering that areas with a mean density of soil pollution from 1 to 5 Ci/km^2 extend to 12 000 km^2 in Sweden, 11 500 km^2 in Finland, 8600 km^2 in Austria, 5200 km^2 in Norway, and 1300 km^2 in Switzerland (Izrael *et al.*, 1997).

Many hectares of soil and large bodies of water around the globe are polluted with heavy metal salts and organic compounds. Soil and water contaminated with metals and organic toxicants pose a major environmental and human health problem that requires an effective and affordable technological solution. Transgenic biomonitoring systems proffer a solution, as they can be used to detect the potential threat of various pollutants.

Our transgenic biosensors may prove useful in monitoring the efficiency of decontamination of the polluted areas. Various plants have been shown to be useful as phytoremediation devices, removing pollutants from soil and water, accumulating them in their biomass, detoxifying them in some cases, and vaporizing them in others (Rugh *et al.*, 1996; Raskin *et al.*, 1997). By introducing novel genes, particular plants were improved in either their remediation capacity, or in their tolerance to toxic agents (Chaney *et al.*, 1997; Clemens *et al.*, 1999). Our plants could be used as a remediation quality control that allows for the evaluation of the mutagenicity of contaminated soils before and after remediation. Hence, transgenic plants are becoming more and more valuable for biosensoring, efficient clean-up, and post-remediation control of polluted soil and water. Therefore, the utilization of transgenic plants for biomonitoring and remediation represents a significant increase in their importance, adding to their potential as an improved source of food, and possibly medicine.

Importantly, several previous studies, including ours, indicated that plants were often more sensitive to certain types of contaminants than animals (Smith, 1991; Wang and Freemark, 1995). To be an effective 'alarm system', the test organism needs to provide warning of a possible hazard before ecologically significant damage could occur. Plants, being at the base of the food chain, may experience the effects of toxicants sooner than higher trophic levels, thereby reducing the lag period between exposure and significant impact (Lovett Doust *et al.*, 1994).

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References

ABRAMOV, V.I., FEDORENKO, O.M. AND SHEVCHENKO, V.A. (1992). Genetic consequences of radioactive contamination for populations of *Arabidopsis*. *Science of Total Environment* **112**, 19–28.

ALLOWAY, B.J. AND AYRES, D.C. (1993). *Chemical principles of environmental pollution.* London, UK: Chapman and Hall.

AMANUMA, K., TAKEDA, H., AMANUMA, H. AND AOKI, Y. (2000). Transgenic zebrafish for detecting mutations caused by compounds in aquatic environments. *Nature Biotechnology* **18**, 62–65.

BAUD-GRASSET, S., BAUD-GRASSET, F., BIFULCO, J.M., MEIER, J.M. AND MA, T.H. (1993). Reduction of genotoxicity of a creosote-contaminated soil after fungal treatment determined by the *Tradescantia* micronucleus test. *Mutation Research* **303**, 77–82.

BITTON, G. AND KOOPMAN, B. (1992). Bacterial and enzymatic bioassays for toxicity testing in the environment. *Review in Environmental Contamination Toxicology* **125**, 1–22.

BITTON, G., JUNG, K. AND KOOPMAN, B. (1994). Evaluation of a microplate assay specific for heavy metal toxicity. *Archives of Environmental Contamination Toxicology* **27**, 25–28.

BONDAR', P.F., IVANOV, Y.I. AND OZORNOV, A.G. (1994). Estimation of the relative biological availability of Cesium-137 in the fallout and general biological availability in the soils of the radioactively contaminated areas. In: *Problems of agricultural radiology*. Ed. N. Arkhipov, pp 74–88. Kiev, Russia: Naukova Dumka.

BORBOA, L. AND DE LA TORRE, C. (1996). The genotoxicity of Zn(II) and Cd(II) in *Allium cepa* root meristematic cells. *New Phytologist* **134**, 481–486.

BRITT, A.B. (1999). Molecular genetics of DNA repair in higher plants. *Trends in Plant Science* **4**, 20–25.

BURK, L.G. AND MENSER, H.A. (1964). A dominant *aurea* mutation in tobacco. *Tobacco Science* **8**, 101–104.

CEBULSKA-WASILEWSKA, A. (1992). *Tradescantia* stamen-hair mutation bioassay on the mutagenicity of radioisotope-contaminated air following the Chernobyl nuclear accident and 1 year later. *Mutation Research* **270**, 23–29.

CHANEY, R.L., MALIK, M., LI, Y.M. *ET AL.* (1997). Phytoremediation of soil metals. *Current Opinion in Biotechnology* **8**, 279–284.

CLEMENS, S., KIM, E.J., NEUMANN, D. AND SCHROEDER, J.I. (1999). Tolerance to toxic metals by a gene family of phytochelatin synthases from plants and yeast. *EMBO Journal* **12**, 3325–3333.

CODINA, J.C., PEREZ-GARCIA, A., ROMERO, R., AND DE VINCENTE, A. (1993). A comparison of microbial bioassays for the detection of metal toxicity. *Archives of Environmental Contamination Toxicology* **25**, 250–254.

CONSTANTIN, M.J. AND OWENS, E.T. (1982). Introduction and perspectives of plant genetic and cytogenetic assays. A report of the US Environmental Protection Agency Gene-Tox Program. *Mutation Research* **99**, 1–12.

COOHILL, T. (1991). Action spectra again? *Photochemistry and Photobiology* **54**, 859–870.

CORTES, F., DOMINGUEZ, I., MATEOS, S., PINERO, J. AND MATEOS, J.C. (1990). Evidence for an adaptive response to radiation damage in plant cells conditioned with X-rays or incorporated tritium. *International Journal of Radiation Biology* **57**, 537–541.

DUBROVA, Y. (1998). Plant transgenics track Chernobyl's fallout. *Nature Biotechnology* **16**, 1010–1011.

DUGLE, J. AND MAYOH, K. (1984). Responses of 56 naturally-growing shrub taxa to chronic gamma irradiation. *Environmental Experimental Botany* **24**, 267–276.

FISKESJO, G. (1988). The *Allium* test – an alternative in environmental studies: the relative toxicity of metal ions. *Mutation Research* **197**, 243–260.

FILKOWSKI, J., BESPLUG, J., BURKE, P., KOVALCHUK, I. AND KOVALCHUK, O. Recombination and point mutation-monitoring transgenic plants reveal the genotoxicity of commonly used herbicides 2,4-Δ and dicamba. *Mutation Research*, in press.

FREDERICK, J. (1990). Trends in atmospheric ozone and ultraviolet radiation: mechanisms and observations for the northern hemisphere. *Photochemistry and Photobiology* **51**, 757–763.

FRIEDLENDER, M., LEV-YADUN, S., BABUREK, I., ANGELIS, K. AND LEVY, A.A. (1996). Cell divisions in cotyledons after germination: localization, time course and utilization for a mutagenesis assay. *Planta* **199**, 307–313.

FROLOVA, N.P. AND POPOVA, O.N. (1990). Monitoring the seeds of chronically irradiated indigenous populations of *Plantago lanceolata* L. Variability in the progeny. *Radiobiologia* **30**, 446–449.

GICHNER, T., PTACEK, O., STAVREVA, D.A., WAGNER, E.D. AND PLEWA, M.J. (2000). A comparison of DNA repair using the comet assay in tobacco seedlings after exposure to alkylating agents or ionizing radiation. *Mutation Research* **470**, 1–9.

GICHNER, T., STAVREVA, D.A. AND VAN BREUSEGEM, F. (2001). o-Phenylenediamine-induced DNA damage and mutagenicity in tobacco seedlings is light-dependent. *Mutation Research* **495**, 117–125.

GRANT, W.F. (1994). The present status of higher plant bioassays for detection of environmental mutagens. *Mutation Research* **310**, 175–185.

GRANT, W.F. AND SALAMONE, M.F. (1994). Comparative mutagenicity of chemicals selected for test in the International Program on Chemical Safety's collaborative study on plant systems for the detection of environmental mutagens. *Mutation Research* **310**, 187–209.

GREEN, M., TODO, T., RYO, H. AND FUJIKAWA, K. (1986). Genetic-molecular basis for a simple *Drosophila melanogaster* somatic system that detects environmental mutagens. *Proceedings of the National Academy of Sciences of the United States of America* **83**, 6667–6671.

GULATI, A., PRAKASH, S. AND GUPTA, S.P. (1994). Genotoxicity testing for relative efficiency of selected pesticides on *Allium cepa*. *Journal of Environmental Biology* **15**, 89–95.

GUNTHER, P. AND PESTEMER, W. (1990). Risk assessment for selected xenobiotics by bioassay methods with higher plants. *Environmental Management* **14**, 381–388.

HARTMANN, A. AND SPEIT, G. (1994). Comparative investigations of the genotoxic effects of metals in the Single Cell Gel (SCG) Assay and the Sister Chromatid Exchange (SCE) test. *Environmental and Molecular Mutagenesis* **23**, 299–305.

HARTWIG, A. (1995). Current aspects in metal genotoxicity. *BioMetals* **8**, 3–11.

HARWELL, M., GENTILE, J., NORTON, B. AND COOPER, W. (1994). Issue paper on ecological significance. In: *Ecological risk assessment issue papers*, EPA/630/R-94/009. Washington, DC, USA: US Environmental Protection Agency.

HELLEDAY, T., NILSSON, R. AND JENSSEN, D. (2000). Arsenic [III] and heavy metal ions induce intrachromosomal homologous recombination in the *hprt* gene of V79 Chinese Hamster Cells. *Environmental and Molecular Mutagenesis* **35**, 114–122.

HOLST, R. AND NAGEL, D. (1997). Radiation effects on plants. In: *Plants for environmental studies*. Eds. W. Wang, J.W. Gorsuch and J.S. Hughes, pp 38–79. Boca Raton, USA: Lewis Publishers.

HORN, N. (1984). In: *Metabolism of trace metals in man*, Vol. II. Eds. O.M. Rennert and W.-Y. Chan, pp 26–52. Boca Raton, Florida, USA: CRC Press.

HUANG, X.-D., DIXON, D. AND GREENBERG, B. (1993). Impacts of UV radiation and photomodification on the toxicity of PAHs to the higher plant *Lemna gibba* (Duckweed). *Environmental Toxicology and Chemistry* **12**, 1067–1077.

ICHIKAWA, S. (1981). *In situ* monitoring with *Tradescantia* around nuclear power plants. *Environmental Health Perspectives* **37**, 145–164.

ICHIKAWA, S. (1992). *Tradescantia* stamen-hair system as an excellent botanical tester of mutagenicity: its responses to ionizing radiations and chemical mutagens, and some synergistic effects found. *Mutation Research* **270**, 3–22.

IEAE. (1996). International Conference: One decade after Chernobyl: Summing up the consequences of the accident. Summary of the Conferences Results.

IMLAY, J.A. AND LINN, S. (1988). DNA damage and oxygen radical toxicity. *Science* **240**, 1302–1309.

IZRAEL, Y.A., DE CORT, M., JONES, A.R. *ET AL.* (1997). In: *Radioecological consequences of the Chernobyl accident*. Eds. A. Karaoglu, G. Desmet, G.N. Kelly and H.G. Menzel, pp 1–10. Luxemburg: European Commission, ECSC-EC-EAEC.

KANAYA, N., GILL, B., GROVER, I. *ET AL.* (1994). *Vicia faba* chromosomal aberration assay. *Mutation Research* **310**, 231–247.

KERR, J. AND MCELROY, C. (1993). Evidence for large upward trends of ultraviolet-B radiation linked to ozone depletion. *Science* **262**, 1032–1034.

KNASMULLER, S., PARZEFALL, W., SANYAL, R. *ET AL.* (1998). Use of metabolically competent human hepatoma cells for the detection of mutagens and antimutagens. *Mutation Research* **402**, 185–202.

KONG, I.-C., BITTON, G., KOOPMAN, B. AND JUNG, K.-H. (1995). Heavy metal toxicity testing in environmental samples. *Reviews in Environmental Contamination Toxicology* **125**, 1–22.

KOVALCHUK, O., KOVALCHUK, I., ARKHIPOV, A., TELYUK, P., HOHN, B. AND KOVALCHUK, L. (1998a). The *Allium cepa* chromosome aberration test reliably measures genotoxicity of soils of inhabited areas in the Ukraine contaminated by the Chernobyl accident. *Mutation Research* **415**, 47–57.

KOVALCHUK, I., KOVALCHUK, O., ARKHIPOV, A. AND HOHN, B. (1998b). Transgenic plants are sensitive bioindicators of nuclear pollution caused by the Chernobyl accident. *Nature Biotechnology* **16**, 1054–1057.

KOVALCHUK, O., KOVALCHUK, I., TITOV, V., ARKHIPOV, A. AND HOHN, B. (1999). Radiation hazard caused by the Chernobyl accident in inhabited areas of Ukraine can be monitored by transgenic plants. *Mutation Research* **446**, 49–55.

KOVALCHUK, O., ARKHIPOV, A., BARYLYAK, I. *ET AL.* (2000a). Plants experiencing chronic internal exposure to ionizing radiation exhibit higher frequency of homologous recombination than acutely irradiated plants. *Mutation Research* **449**, 47–56.

KOVALCHUK, I., KOVALCHUK, O. AND HOHN, B. (2000b). Genome-wide variation of the somatic mutation frequency in transgenic plants. *EMBO Journal* **19**, 4431–4438.

KOVALCHUK, O., DUBROVA, Y., ARKHIPOV, A., HOHN, B. AND KOVALCHUK, I. (2000c). Wheat DNA mutation rate after Chernobyl. *Nature* **407**, 583–584.

KOVALCHUK, O., TITOV, V., HOHN, B. AND KOVALCHUK, I. (2001). A sensitive transgenic plant system to detect toxic inorganic compounds in the environment. *Nature Biotechnology* **19**, 568–572.

KOVALCHUK, O., KOVALCHUK, I. ARKHIPOV, A., HOHN, B. AND DUBROVA, YU. (2003a) Extremely complex pattern of microsatellite mutation in the germline of wheat exposed to the post-Chernobyl radioactive contamination. *Mutation Research*, **525**, 93–101.

KOVALCHUK, O., TELYUK, P., KOVALCHUK, L., KOVALCHUK, I. AND TITOV, V. (2003b) Novel plant bioassays for monitoring the genotoxicity of drinking water from the inhabited areas of the Ukraine affected by the Chernobyl accident. *Bulletin of Environmental Contamination and Toxicology* **70**, 847–853.

KRAMER, G., NORMAN, H., KRIZEK, D. AND MIRECKI, R. (1991). Influence of UV-B radiation on polyamines, lipid peroxidation and membrane lipids in cucumber. *Journal of Phytochemistry* **30**, 2101–2108.

LEE, Y.-W., BRODAY, L. AND COSTA, M. (1998). Effects of nickel on DNA methyltransferase activity levels and genomic DNA methylation. *Mutation Research* **415**, 213–218.

LEVIN, D.E. AND AMES, B.N. (1986). Classifying mutagens as to their possible specificity in causing the six possible transitions and transversions: a simple analysis using the *Salmonella* mutagenicity assay. *Environmental Mutagenesis* **8**, 9–28.

LOVETT DOUST, J., SCHMIDT, M. AND LOVETT DOUST, L. (1994). Biological assessment of aquatic pollution: a review, with emphasis on plants as biomonitors. *Biology Reviews* **69**, 147–186.

MA, T.H. (1994). Landfill or incineration – which is the better way to treat our solid wastes? *Environmental Molecular Mutagenesis* **23**, 40.

MA, T.H. AND HARRIS, M.M. (1985). *In situ* monitoring of environmental mutagens. In: *Hazard assessment of chemicals, current developments, Volume 4*. Ed. J. Saxena, pp 77–106. New York, USA: Academic Press.

MA, T.H., XU, C., POWERS, L. AND LIAO, S. (1996). *In situ* monitoring with the *Tradescantia* bioassays on the genotoxicity of gaseous emissions from a closed landfill site and an incinerator. *Mutation Research* **359**, 39–52.

MASS, M.J. AND WANG, L. (1997). Arsenic alters cytosine methylation patterns of the promoter of the tumor suppressor gene *p53* in human lung cells: a model for a mechanism of carcinogenesis. *Mutation Research* **386**, 263–277.

MATEOS, S., PANNEERSELVAM, N., MATEOS, J.C. AND CORTES, F. (1992). Comparative study of the potentiating effect of caffeine and poly-D -lysine on chromosomal damage induced by X-rays in plant cells. *Mutation Research* **266**, 215–219.

MAYER, C., KLEIN, R.G., WESCH, H. AND SCHMEZER, P. (1998). Nickel subsulfide is genotoxic *in vitro* but shows no mutagenic potential in respiratory tract tissues of Big Blue rats and Muta Mouse mice *in vivo* after inhalation. *Mutation Research* **420**, 85–98.

MCCRADY, J.K., MCFARLANE, J.C. AND GANDER, L.K. (1990). The transport and fate of 2,3,7,8-TCDD in soybean and corn. *Chemosphere* **21**, 359–376.

MCVEY, M. AND MACLER, B.A. (1993). Ecological risk assessment case study: selenium effects at Keterson Reservoir. In: *A review of ecological assessment case studies from a risk assessment perspective.* EPA/630/R-92/005. Washington, DC, USA: US Environmental Protection Agency.

MILLER, J.H. (1985). Mutagenic specificity of ultraviolet light. *Journal of Molecular Biology* **182**, 45–68.

MOISEEV, A. AND IVANOV, V. (1991). *Directory for dosimetry and radiation hygiene*, p 220. Moscow, Russia: Atomizdat.

MURTI, R., SCHIMENTI, K. AND SCHIMENTI, J. (1994). A recombination-based transgenic mouse system for genotoxicity testing. *Mutation Research* **307**, 583–595.

NIELSEN, M.N. AND RANK, J. (1994). Screening of toxicity and genotoxicity in wastewater by the use of *Allium* test. *Hereditas* **121**, 249–254.

O'CONNOR, G.A., CHANEY, R.L. AND RYAN, J.A. (1991). Bioavailability to plants of sludge-borne toxic organics. *Reviews in Environmental Contamination and Toxicology* **121**, 129–155.

PANG, Q. AND HAYS, J. (1991). UV-B inducible and temperature-sensitive photoreactivation of cyclobutane pyrimidine dimers in *Arabidopsis thaliana*. *Plant Physiology* **95**, 536–543.

PARADIZ, J., ŠKRK, J. AND DRUŠKOVIC, B. (1992). Cytogenetic effects of ionizing radiation on meristem. *Acta Pharmacologica* **42**, 397–401.

PARADIZ, J., GABERŠZIK, A. AND ŠKRK, J. (1995). The influence of gamma radiation on growth and development of the onion (*Allium cepa* L.). *Acta Pharmacologica* **45**, 351–354.

PERELRA, W.E. AND HOSTETTLER, F.D. (1993). Nonpoint source contamination of the Mississippi River and its tributaries by herbicides. *Environmental Science Technology* **27**, 1542–1552.

PUCHTA, H. AND HOHN, B. (1996). From centiMorgans to basepairs: homologous recombination in plants. *Trends in Plant Science* **1**, 340–348.

PUCHTA, H., SWOBODA, P. AND HOHN, B. (1995). Induction of homologous DNA recombination in whole plants. *Plant Journal* **7**, 203–210.

RANK, J. AND NIELSEN, M.N. (1994). Evaluation of the *Allium cepa* anaphase–telophase test in relation to genotoxicity screening of industrial wastewater. *Mutation Research* **312**, 17–24.

RASKIN, I., SMITH, R.D. AND SALT, D.E. (1997). Phytoremediation of metals: using plants to remove pollutants from the environment. *Current Opinion in Biotechnology* **8**, 221–226.

RASMUSON, A. (1985). Mutagenic effects of some water-soluble metal compounds in a somatic eye-color test system in *Drosophila melanogaster*. *Mutation Research* **157**, 157–162.

REN, L., HUANG, X.-D., MCCONKEY, B., DIXON, D. AND GREENBERG, B. (1994). Photo-induced toxicity of three polycyclic aromatic hydrocarbons (fluoranthene, pyrene and naphthalene) to the duckweed *Lemna gibba*. *Ecotoxicology and Environmental Safety* **28**, 160–171.

RESHETNIKOV, V.N., LAPTEVA, O.K., SOSNOVSKAIA, T.F. AND ROSHCHENKO, M.V. (1996). Radiation-induced changes in the cellular chromatin of cereal plants cultivated in the area of the Chernobyl Atomic Electric Power Station. *Radiatsionnaja Biologicheskaja Radioecologija* **36**, 567–572.

RIES, G., HELLER, W., PUCHTA, H., SANDERMANN, H., SEIDLITZ, H.K. AND HOHN, B. (2000). Elevated UV-B radiation reduces genome stability in plants. *Nature* **406**, 98–101.

RODRIGUES, G.S., MA, T.H., PIMENTEL, D. AND WEINSTEIN, L. (1997). *Tradescantia* bioassays as monitoring systems for environmental mutagenesis: a review. *Critical Reviews in Plant Sciences* **16**, 325–359.

ROSSMAN, T.G., MOLINA, M., MEYER, L. *ET AL.* (1991). Performance of 133 compounds in the lambda prophage induction endpoint of the Microscreen assay and a comparison with *S. typhimurium* mutagenicity and rodent carcinogenicity assays. *Mutation Research* **260**, 349–367.

RUGH, C.L., WILDE, H.D., STACK, N.M., THOMPSON, D.M., SUMMERS, A.O. AND MEAGHER, R.B. (1996). Mercuric ion reduction and resistance in transgenic *Arabidopsis thaliana* plants expressing a modified bacterial merA gene. *Proceedings of the National Academy of Sciences of the United States of America* **93**, 6667–6671.

RUIZ, E.F., RABAGO, V.M.E., LECONA, S.U., PEREZ, A.B. AND MA, T.H. (1992). *Tradescantia* micronucleus (Trad-MCN) bioassay on clastogenicity of wastewater and *in situ* monitoring. *Mutation Research* **270**, 45–51.

SACCO, M.G., ZECCA, L., BAGNASCO, L. *ET AL.* (1997). A transgenic mouse model for the detection of cellular stress induced by toxic inorganic compounds. *Nature Biotechnology* **15**, 1392–1397.

SALOMON, S. AND PUCHTA, H. (1998). Capture of genomic and T-DNA sequences during double-strand break repair in somatic plant cells. *EMBO Journal* **17**, 6086–6095.

SANDHU, S.S., MA, T.H., PENG, Y. AND ZHOU, X. (1989). Clastogenicity evaluation of seven chemicals commonly found at hazardous industrial waste sites. *Mutation Research* **224**, 437–446.

SCHAAPER, R.M. AND DUNN, R.L. (1991). Spontaneous mutation in the *Escherichia coli*. *Carcinogenesis* **11**, 1087–1095.

SHEEHAN, P.J. AND LOUCKS, O.L. (1994). Issue paper on effects characterization. In: *Ecological risk assessment issue papers*, EPA/630/R-94/009. Washington, DC, USA: US Environmental Protection Agency.

SHKVARNIKOV, P.K. (1990). A cytological study of plants growing under exposure to different radiation levels. *Tsitologija Genetica* **24**, 33–37.

ŠIVIKOVÁ, K. AND DIANOVSKÝ, J. (1995). Sister chromatid exchanges after exposure to metal-containing emissions. *Mutation Research* **327**, 17–22.

SMAKA-KINCL, V., STEGNAR, P., LOVKA, M. AND TOMAN, M.J. (1996). The evaluation of waste, surface and ground water quality using the *Allium* test procedure. *Mutation Research* **368**, 171–179.

SMITH, B.M. (1991). An inter- and intra-agency survey of the use of plants for toxicity assessment. In: *Plants for toxicity assessment: second volume*. Eds. J.W. Gorsuch, W.R. Lower, W. Wang and M.A. Lewis, pp 41–59, ASTM STP 1115. Philadelphia, PA, USA: American Society of Testing and Materials.

SOKAL, R. AND ROHLF, F. (1995). *Biometry*, p 850. New York, USA: W.H. Freeman and Company.

STEINKELLNER, H., MUN-SIK, K., HELMA, C. *ET AL.* (1998). Genotoxic effects of heavy metals: comparative investigation with plant bioassays. *Environmental and Molecular Mutagenesis* **31**, 183–191.

SWOBODA, P., GAL, S., HOHN, B. AND PUCHTA, H. (1994). Intrachromosomal homologous recombination in whole plants. *EMBO Journal* **13**, 484–489.

SYOMOV, A.B., PTITSYNA, S.N. AND SERGEEVA, S.A. (1992). Analysis of DNA strand break induction and repair in plants from the vicinity of Chernobyl. *Science of the Total Environment* **112**, 1–8.

TEVINI, M. (1993). Effects of enhanced UV-B radiation on terrestrial plants. In: *UV-B radiation and ozone depletion: effects on humans, animals, plants, microorganisms, and materials*. Ed. M. Tevini, pp 125–153. Boca Raton, FL, USA: Lewis Publishers.

THIELE, D. (1992). Metal-regulated transcription in eukaryotes. *Nucleic Acids Research* **20**, 1183–1191.

ULRICH, M.M., MULLER, S.R., SINGER, H.P., IMBODEN, D.M. AND SCHWARZENBACH, R.P. (1994). Input and dynamic behavior of the organic pollutants tetrachloroethane, atrazine and NTA in a lake, a study combining mathematical and field measurements. *Environmental Science Technology* **28**, 1674–1685.

WANG, S. (1994). Chromosome aberrations in space-exposed seeds of *Allium cepa* L. *Journal of Genetics and Breeding* **48**, 113–116.

WANG, W. AND FREEMARK, K. (1995). The use of plants for environmental monitoring and assessment. *Ecotoxicology and Environmental Safety* **30**, 289–301.

WARD, J.F. (1998). Nature of lesions formed by ionizing radiation. In: *DNA damage and repair in higher eukaryotes*. Eds. J.A. Nickoloff and M.F. Hoekstra, pp 65–84. Vol II. Totowa, New Jersey, USA: Humana Press.