Review

PLANT GENOTOXICITY: A MOLECULAR CYTOGENETIC APPROACH IN PLANT BIOASSAYS*

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It is important for the prevention of DNA changes caused by environment to understand the biological consequences of DNA damages and their molecular modes of action that lead to repair or alterations of the genetic material. Numerous genotoxicity assay systems have been developed to identify DNA reactive compounds. The available data show that plant bioassays are important tests in the detection of genotoxic contamination in the environment and the establishment of controlling systems. Plant system can detect a wide range of genetic damage, including gene mutations and chromosome aberrations. Recently introduced molecular cytogenetic methods allow analysis of genotoxicity, both at the chromosomal and DNA level. FISH gives a new possibility of the detection and analysis of chromosomal rearrangements in a great detail. DNA fragmentation can be estimated using the TUNEL test and the single cell gel electrophoresis (Comet assay).

KEY WORDS: *DNA damage, environment, fluorescent* in situ *hybrydization,* in vitro *culture, plant genome, transgenic plants*

From the beginning, humans have been polluting the environment. However, the intensity and the severity of different kinds of pollution has drastically increased over the last few decades. The increase of pollution by the release of genotoxic chemicals and the increase of radiation levels has affected the ecosystem and the health of organisms, including humans (1). There is a need for quick and precise methods for the detection and evaluation of air, water and soil contamination and their effects on organisms (2).

Plants comprise a large portion of our biosphere and constitute a vital link in the food chain. Due to the highly conserved structure of the genetic material, it is possible to use a broad variety of species in genotoxicity tests. The most widespread methods are based on the use of bacterial indicator species, yeasts, fungi, insects and mammalian cells or laboratory rodents (3, 4). Several higher plant bioassays for screening and monitoring environmental mutagens have been established (5, 6).

It is very important to select a proper plant to assess the quality of air, water and soil. The influence of environmental mutagens on a plant depends not only on the type of mutagen, exposure time, dose and interaction with other factors, but also on the plant species, genotype, and stage of development (7, 8).

Plant response to mutagenic treatment can be considered on different levels of organization: from DNA, chromosome, and genome to the whole organism. This review will concentrate only on the chromosome and genome level.

Plant genome

Genome – a complete haploid set of chromosomes is characteristic for every species. Plant genome, especially angiosperms, is characterised by the variability in the genome size from 1C=0.05 pg (*Cardamine amora*) to 1C=127.4 pg (*Fritillaria assyriaca*) and by the chromosome number from

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2n=4 (*Haplopappus gracilis*) to 2n=640 (*Sedum suaveolens*), morphology and size (9, 10). Among plants, polyploids are also very common. Genome size and chromosome number are characteristic for each plant, but they can change during the cell cycle due to DNA replication and cell division. During development, they progress through endoreduplication cycles. Environmental conditions can also affect the DNA amount in the nuclei or modify the endoreduplication pattern of plants growing in polluted areas (11-13).

Plant bioassays

Plant mutagenicity bioassays have been in existence for many years. *Stadler* reported the effect of chemical and physical agents on chromosomes in 1928. *Levan* proposed the first test on *Allium* (14). Now, plant bioassays are well-established systems and are used for screening and monitoring environmental chemicals with mutagenic and carcinogenic potential (15, 16). The International Program on Chemical Safety (IPCS) collaborative study on higher plant genetic systems for screening and monitoring environmental pollutants was initiated in 1984. It is a cooperative venture of the United Nations Environment Program, the International Labour Organization and the World Health Organization. Its goal was to develop methodologies for improving the assessment of risks from chemical exposure (17, 18). Under the sponsorship of the IPCS, 17 laboratories from diverse regions of the world participated in evaluating the utility of four plant bioassays for detecting genetic hazards of environmental chemicals (2).

Using plant bioassays for testing and monitoring environmental chemicals or pollutions has many advantages. They are easy to handle, inexpensive and in many cases more sensitive than other available systems (6). There are some limitations as well, such as the longer life cycle of most plants than bacteria, yeast or *Drosophila* and some biochemical differences between plants and mammals. The differences between plant and animal cells have led to the lack of general recognition of plant genotoxicity assays. Limited data from plant bioassays are applicable only when we wish to extrapolate them directly to human. There are many reports on the excellent correlation of the plant system with the mammalian system (17).

Most higher plant bioassays are based on the detection of chromosomal aberrations, sister chromatid exchanges, and recently, on the analysis of DNA strand breaks. In some systems, point mutations are analyzed, e.g. chlorophyll mutations in leaves, waxy mutations or embryo mutations of *Arabidopsis* (19).

Cytogenetic tests

Cytogenetic tests analyse the frequency and type of chromosome aberrations in mitotic cells and the frequency of micronuclei in interphase cells. Genotoxic agents cause DNA damage, which is either repaired or otherwise leads to alterations of the DNA. Chromosome aberrations are the consequence of DNA double strand break which was unrepaired or repaired improperly. Broken chromosome ends without telomeres become "sticky" and may fuse with other broken chromosome ends. The result of these chromosomal rearrangements are acentric fragments, dicentric bridges observed in mitotic cells of the first cell cycle after mutagenic treatment (Figure 1a-c) or micronuclei in the interphase cell in the next cell cycle (Figure 1d).

The classical test for studying the effects of chemicals on plant chromosomes is the *Allium* test, which was developed by *Levan* in 1938 (14). It uses the root tips from bulbs. *Allium* has eight pairs of relatively large chromosomes; this allows for the easy detection of chromosome aberrations. The plant material is available all year round. The micronucleus test was developed parallel to chromosome aberration assays (20). Micronuclei are extranuclear bodies of chromatin material formed as a consequence of chromosome breakage or aneuploidy. The frequency of cells with micronuclei is a good indicator of the cytogenetic effects of tested chemicals. Similarly, chromosome

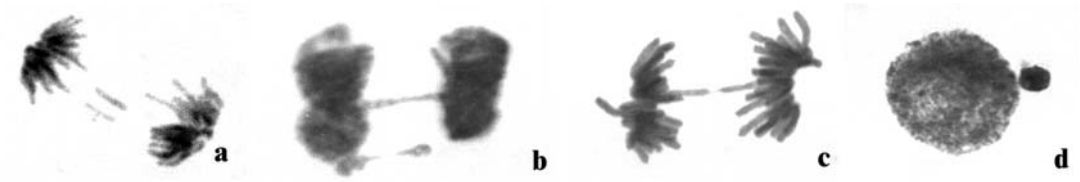

Figure 1 *Chromosome aberrations in root tips cells of Hordeum vulgare after MH treatment: a – two fragments in anaphase; b – anaphase bridge and acentric fragment; c – anaphase bridge; d – interphase nuclei with micronucleus.*

aberration and micronuclei tests are conducted with other plant species such as *Vicia faba* (21), *Crepis capillaris* (22), *Hordeum vulgare* (23).

The *Allium* chromosome aberration test was applied to estimate the genotoxicity of soils in Ukraine in the area contaminated by the Chernobyl accident. It revealed a dose-dependent increase in the frequency of aberrant mitoses of 1.6-23.8 % from control values. The results showed high genotoxicity of radioactively polluted soil and the efficiency of the *Allium* test as a quick biological test for environmental and genetic risk assessment (24).

One of the most suitable plants for detecting different types of xenobiotics is *Tradescantia*. This plant is especially useful for evaluating a hazardous condition in the environment (25). There are two main tests: the stamen hair mutation (Trad-SH) test and the micronucleus assay (Trad-MCN). The first is based on the heterozygosity for flower colour in *Tradescantia* clones. Clone 4430 is a hybrid of *T. hirsutiflora* and *T. subacaulis* reproduced only asexually, through cloning. The visual marker for mutation induction is a phenotypic change in the pigmentation of the stamen cells from the dominant blue colour to recessive pink (26). The Trad-MCN test is based on the frequency of micronuclei in tetrad cells induced in male meiotic cells by the tested mutagen (27). These tests may be used under laboratory, or *in situ* exposure conditions, for monitoring air or water, or for testing radioactive or chemical agents (28-30).

The sister chromatid exchange (SCE) test is a well-known, highly sensitive cytogenetic tool for detecting DNA damage. The test is based on DNA segregation, which occurs in chromosomes according to a semiconservative model of DNA replication. SCE involves symmetrical exchange at one locus between sister chromatids that does not alter chromosome length and genetic information. Sister chromatids are visualised through the methods of incorporating bromodeoxyuridine (BrdU) into chromosomal DNA and different staining of chromatids containing DNA with BrdU and chromatids without BrdU (31). The frequency of SCEs per chromosome set increases after treatment with genotoxic agents (Figure 2 a,b). SCE method can be applied in both plant and mammalian cells. Plant species used for SCE test should have a low number of chromosomes, relatively large, such as *Vicia faba* and *Allium cepa* (32, 33). *Crepis capillaris* is especially convenient for analysing the frequency of SCE. This species has only three pairs of morphologically differentiated chromosomes (34, 35). It allows studying SCEs frequency in each chromosome type (Figure 2 c, d).

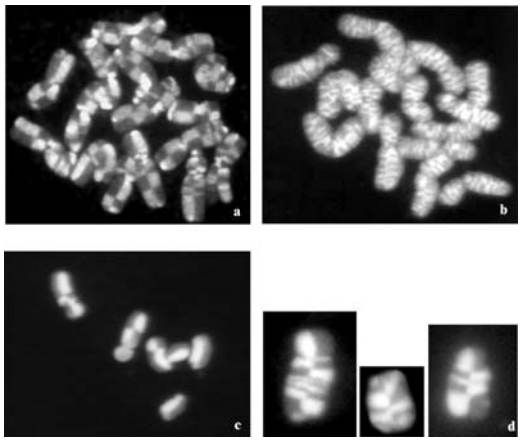

Figure 2 *Sister chromatid exchanges (SCEs) in* Allium cepa *(a, b) and* Crepis capillaris *(c, d) root tips. DAPI 11 staining: a, c – control, full chromosome complement. Chromosomes show low level of spontaneous SCEs; b - full chromosome complement, chromosomes with high level of SCEs after MH treatment; d – SCEs in all chromosomes types after MH treatment.*

Detection of DNA breaks

The development of molecular biology and the application of molecular techniques in cytogenetic studies has made progress in the methods of detection and the estimation of genotoxicity of different agents.

The Comet assay was established for investigating the process of apoptosis in animal cells and then it was adapted to plant cells (36). This test allows not only the detection of single and double stranded DNA breaks in the nucleus, but also the measuring of the level of DNA migration through an agarose gel in an electric field – Figure 3 (37). This is also a useful tool to investigate the capacity of DNA repair of damage induced by different types of mutagens and various damage levels in different cell types (38-40). Computerised image analysis system measures the amount of DNA in the head and in the tail, and the length of the tail. The tail moment (TM) can be calculated to express DNA damage (41). The Comet assay was used to detect DNA damage in nuclei of several plant species isolated from leaves or root tissue after mutagenic treatment (42). The reduction of DNA damage was observed at various recovery times after mutagenic treatment (43).

Another test used to identify apoptosis that has found application in genotoxicity studies is the TUNEL (TdT-mediated dUTP nick end labeling) test (44). The

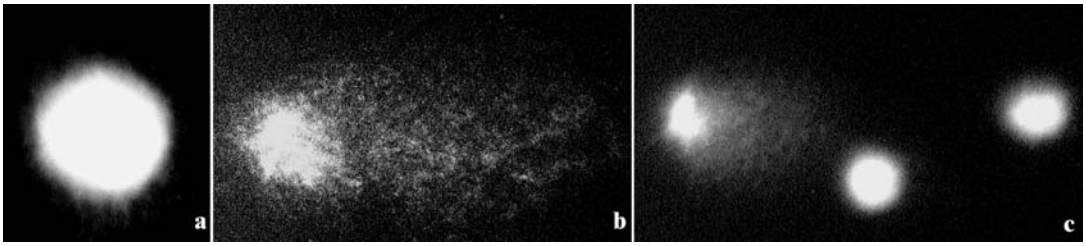

Figure 3 *Comet assay in* Crepis capillaris *cells: a - control nuclei, not damaged; b, c- cells treated by mutagen (MH), nuclei with different level of DNA damage are shown.*

polymerization of labelled nucleotides to DNA strand breaks *in situ* is catalysed by terminal deoxynucleotidyl transferase (Figure 4). The advantages of the TUNEL test include detection of DNA breaks at a single nucleus, short time of assay and easy screening of labelled nuclei. This test is recommended for the preliminary evaluation of genotoxicity of any new tested agent (45).

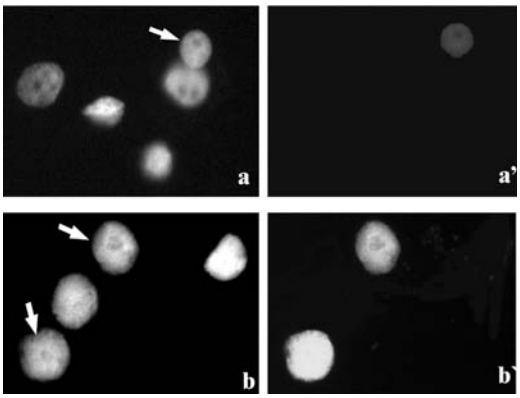

Figure 4 *TUNEL method in* Crepis capillaris *interphase nuclei in control cells (a, a`) and after treatment with X-ray (b, b`): a, b- DAPI staining, all nuclei are seen; arrows indicate damaged nuclei; a`, b` – TUNEL reaction - nuclei with DNA fragmentation are shown.*

FISH – new perspectives for plant bioassays

Changes in chromosomal morphology are usually detected with classical cytogenetic techniques. However, the traditional methods of chromosome staining can fail in the analysis of small changes in chromosome structure. Fluorescent *in situ* hybrydization (FISH) gives new possibilities to study chromosomal aberrations in plant mutagenesis. It allows the detection and a more detailed localization of chromosomal rearrangements, both in mitotic and interphase nuclei (46). Additionally, it helps to understand the mechanisms of the formation of chromosomal aberrations. Until now, DNA probes required for each chromosome have made possible detailed identification of chromosome aberrations using FISH, mainly in human genotoxic studies (47, 48). Even DNA probes for particular plant chromosomes are limited; there are few examples when FISH employing chromosome region-specific DNA probes (e.g. centromere, telomere, rDNA) is helpful in chromosome aberration analysis. It has been possible to detect translocations in chromosomes of tetraploid plants *Arabidopsis thaliana* (49). Chromosome aberration (CA) and micronuclei (MN) tests in combination with FISH using rDNA and telomeric sequences as a probe were conducted to study in great detail the effects of maleic acid hydrazide (MH) on root tip meristem cells of *Crepis capillaris*. Furthermore, FISH revealed spontaneous chromosomal rearrangements in *C. capillaris* hairy root line with 1B chromosome (50). An introduction of FISH to micronucleus test allows analysis of the origin of micronuclei. The aneugenic and clastogenic effects of N-methyl-N-nitrosourea (NMH) treatment in barley plants in MN test combined with FISH using telomere- and centromere-specific probes were reported by *Jovtchev et al.* (51). Furthermore, detailed analysis of chromosomal rearrangements in interphase nuclei using FISH is especially important in tissues in which mutagenic treatment caused a decrease in the frequency of cell divisions.

Transgenic plants as a bioindicators

A new approach to biomonitoring, which involves transgenic plants is based on the integration into the plant genome of a marker gene of known sequences that will serve as target for mutagenic influences. The transgene can be introduced in an active or inactive state and mutation permits the evaluation of the mutagenicity of the tested agents. Two different transgenic systems were designed to study mutagenic influence via point mutations and homologous recombination events (HR). To

analyze point mutations, plants carry one copy of transgene (GUS) per haploid genome inactivated by point mutation. The plants used to screen HR events possess one copy per haploid genome of an overlapping, nonfunctional, truncated version of the GUS marker gene as recombination substrate. GUS is activated via strand-break-induced HR between two repeats. The frequency of point mutation and homologous recombination can be measured by GUS gene-reactivation assay. To date, mainly transgenic *Arabidopsis* and tobacco plants have been used for the biomonitoring of environmental factors (52).

Plant in vitro cultures in the evaluation of genotoxicity

Adventitious roots (e.g. *Allium cepa*) or primary roots (e.g. *Vicia faba, Crepis capillaris, Pisum sativum*) are the most frequently used for assessing chromosome or DNA damage in higher plant bioassays (14, 21, 53). It follows that to conduct such tests, plant breeding is necessary. Nevertheless, the development of tissue *in vitro* culture and transformation techniques make other tissues attractive as sources of mitotic cells. An example is a culture of transformed roots, so called hairy roots, obtained after the transformation with *Agrobacterium rhizogenes*. Transformed root lines, which are characterised by lateral branching, easily provide many root tip cells. It allows them to be used in cytogenetic analysis in basic plant genome research (54). Additionally, "genetic identity" is a feature of transformed roots which is very important in case the plant is not self-fertile. Unfortunately, a number of altered karyotypes have been found in hairy roots of the majority of species, both during transformation and in long-term *in vitro* culture (55). However, *C. capillaris* hairy roots are a rare example of karyotype and morphology stability after transformation and during long–term culture. Their fast growth, genetic stability and simple conditions of *in vitro* culture, together with simple karyotypes, make them convenient for evaluating chromosome damage. An additional advantage of *C. capillaris* hairy roots is its higher sensitivity to mutagens compared to primary roots. A comparison of the sensitivity of cells of root meristems of seedlings and hairy roots was based on a response to two mutagens: MH (maleic acid hydrazide) and X-ray. Chromosomal aberrations and SCEs tests were used to analyse chromosome changes, whereas TUNEL assay was applied for *in situ* detection of DNA fragmentation. The responses of the transformed roots to analysed mutagens were significantly stronger than the responses of the primary roots, both on the DNA and chromosome level. The cytogenetic effect of MH was similar in seedlings treated with 2 mM MH and in hairy roots treated with a four times lower concentration of mutagen. Furthermore, the same dose of MH caused the death of hairy roots, while it did not affect seedling growth. There were also differences in the frequency of chromosomal aberrations in hairy roots and seedling roots to the same doses of X-rays. Monitoring of DNA breakage in the TUNEL test after MH treatment showed a higher frequency of labelled nuclei in hairy roots than in seedlings, even though the mutagen concentration used to treat hairy roots was four times lower. Irradiation with the same dose caused DNA fragmentation in nuclei with a two times higher frequency in hairy roots than in seedlings (56).

This suggests that all the described features of *C. capillaris* hairy roots, especially their relatively high sensitivity, make them a promising new system for plant bioassaying.

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Sažetak

DETEKCIJA GENOTOKSIČNOSTI S POMOĆU BILJNIH BIOTESTOVA - MOLEKULARNO-CITOGENETIČKI PRISTUP

Za prevenciju oštećivanja molekule DNA zbog onečišćenja okoliša nužno je razumijevanje bioloških posljedica nastalih oštećenjem DNA i molekularnih mehanizama djelovanja genotoksikanata koji vode ili popravku ili promjenama genskog materijala. Do danas je usavršen niz testova za detekciju genotoksičnog djelovanja koji omogućuju identifikaciju supstancija koje reagiraju s molekulom DNA. Raspoloživi podaci pokazuju da su biljni biotestovi vrlo važni u detekciji genotoksičnog onečišćenja okoliša, kao i za uspostavljanje nadzornih sustava u okolišu. Biljni test-sustav može otkriti široki raspon genskog oštećenja uključujući mutacije gena i kromosomske aberacije. Nove molekularno-citogenetičke metode omogućuju analizu genotoksičnog djelovanja na razini kromosoma i molekule DNA. Metoda FISH-a ("*fluorescent in situ hybridization*") pruža nove mogućnosti za detekciju i analizu kromosomskih preraspodjela. Lomovi u molekuli DNA mogu se uspješno detektirati s pomoću metode TUNEL i gel-elektroforeze pojedinačnih stanica (komet test).

KLJUČNE RIJEČI: *fluorescentna* in situ *hibridizacija, genom biljke,* in vitro *kultura, okoliš, oštećenje DNA, transgenske biljke*

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