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Review

Single Cell Gel Electrophoresis (Comet) assay with plants: Research on DNA repair and ecogenotoxicity testing

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HIGHLIGHTS

- SCGE technique is now expanding in plant biology.
- SCGE is a versatile tool to evaluate a number of DNA processes.
- Combination of plants and SCGE as biosensor asset to monitor environment.
- Recent reports describe SCGE analysis for improvement of Mutation Breeding protocol.

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ABSTRACT

Single Cell Gel Electrophoresis is currently used to investigate the cell response to genotoxic agents as well as to several biotic and abiotic stresses that lead to oxidative DNA damage. Different versions of Single Cell Gel Electrophoresis have been developed in order to expand the range of DNA lesions that can be detected and guidelines for their use in genetic toxicology have been provided. Applications of Single Cell Gel Electrophoresis in plants are still limited, compared to animal systems. This technique is now emerging as a useful tool in assessing the potential of higher plants as stable sensors in ecosystems and source of information on the genotoxic impact of dangerous pollutants. Another interesting application of Single Cell Gel Electrophoresis deals with Mutation Breeding or the combined use of irradiation and *in vitro* culture technique to enhance genetic variability in elite plant genotypes. SCGE, in combination with *in situ* detection of Reactive Oxygen Species (ROS) induced by γ-rays and expression analysis of both DNA repair and antioxidant genes, can be used to gather information on the radiosensitivity level of the target plant genotypes.

Contents

Abbreviations: ALS, alkaline labile site; AP, apurinic/apyrimidinic; BER, Base Excision Repair; CEA, Cytosine Extension Assay; CPD, cyclobutane pyrimidine dimer; DSB, double strand break; endoIII, endonuclease III; FISH, Fluorescence *In Situ* Hybridization; FPG, formamidopyrimidine N-glycosylase; GG-R, Global Genome-Repair; HDR, high dose rate; HR, Homologous Recombination; IR, ionizing radiation; LDR, low dose rate; MNU, *N*-methyl-*N*-nitrosourea; NER, nucleotide excision repair; NHEJ, Non Homologous End Joining; OGG1, 8-hydroxyguanine DNA glycosylase; 8-oxo-dG, 7,8-Dihydro-8-oxoguanine; PAHs, polycyclic aromatic hydrocarbons; ROS, Reactive Oxygen Species; SCGE, Single Cell Gel Electrophoresis; SSB, single strand break; TC-R, Transcription-Coupled Repair; UDG, uracil-DNA-N-glycosylase.

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1. Introduction

The use of Single Cell Gel Electrophoresis (SCGE) or Comet assay, first reported by Ostling and Johansons (1984), and subsequently modified and validated (Collins, 2004), allows the quantitative and qualitative study of DNA damage in nuclei isolated from single cells that are embedded in agarose and transferred on microscope slides. The SCGE approach is currently used to investigate the cell response to genotoxic agents as well as to several biotic and abiotic stresses that inevitably lead to oxidative DNA damage. This technique is also utilized to characterize animal and plant mutants lacking specific DNA repair functions or genes involved in DNA damage sensing/signaling and chromatin remodeling (Collins, 2004; Kozak et al., 2009; McArt et al., 2010; Wentzel et al., 2010; Bohmdorfer et al., 2011; Kamisugi et al., 2012). Advantages and limitations of SCGE in ecogenotoxicological and biomonitoring studies have been largely discussed in animal systems (Kumaravel et al., 2009).

Plants are exposed to a wide range of environmental pollutants and for this reason they can be used for monitoring the presence of chemical and physical mutagens in polluted habitats. Moreover, there is interest in replacing the animal models currently used in pharmacological and toxicological research with plants. Although this seems a difficult goal, in some cases plants might enable researchers avoiding or limiting tests on animals. Bartha et al. (2010) investigated the effects of the common antipyretic agent acetaminophen (paracetamol) on the Indian mustard (*Brassica juncea* L. Czern.). Acetaminophen is metabolized in the liver through different pathways, among which is hydroxylation catalyzed by the cytochrome P450 enzyme system which produces the cytotoxic compound *N*-acetyl-*p*-benzo-quinone and a similar detoxification pathway has been recently proposed in plants (Huber et al., 2009). According to the 'green-liver' concept (Schroder and Collins, 2002), detoxification of acetaminophen in the Indian mustard resembles the mammalian metabolism and high drug concentrations were found to cause oxidative stress and irreversible cellular damage *in planta* (Bartha et al., 2010). Within this context, SCGE application for toxicological research using plant cells as substitute for animals will necessarily require a deeper investigation to unravel the plant detoxification pathways.

2. SCGE assay: a versatile technique

Different versions of SCGE have been developed in order to expand the range of DNA lesions that can be detected and guidelines for their use in genetic toxicology have been provided (Angelis et al., 1999; Tice et al., 2000). It should be underlined that SCGE measures only the occurrence of DNA breaks and all the versions of this technique currently available lead to conversion of the target lesions into DNA breaks. Preparation of samples for SCGE analysis is a crucial step performed rapidly and accurately, otherwise the profile of damaged DNA will be lost. In plants, due to the presence of cell wall, SCGE is carried out by embedding isolated nuclei into agarose. In most cases, nuclei are released by chopping the plant tissue with a sharp blade and there is no need for further sample processing after treatments (Koppen and Verschaeve, 1996). The main differences in the starting step for nuclear DNA extraction required to carry out SCGE analysis in animal and plant cells are depicted in Fig. 1. Agarose-embedded animal cells, e.g. lymphocytes, are treated with hypertonic lysis buffer to disrupt the plasma membrane while incubation with a non-ionic detergent allows removal of cell debris, such as membranes, dissolving nucleosomes. Plant materials, tissues or suspension cultures,

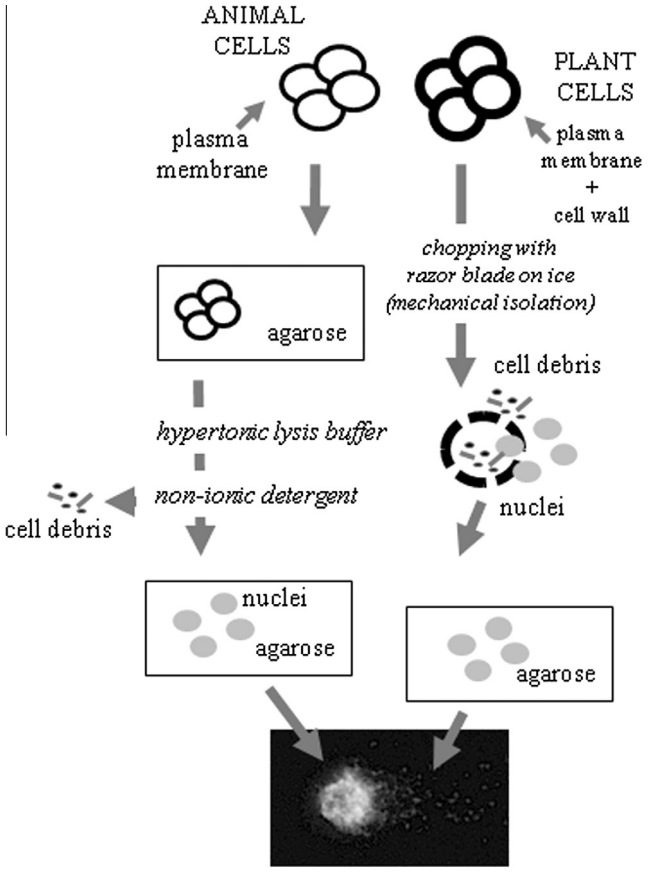

Fig. 1. Schematic representation of the different procedures required to produce intact DNA for SCGE assay in animal and plant cells.

contain thick cell walls and chopping with a ice-chilled razor blade can efficiently ensure the mechanical extraction of nuclei.

2.1. The comet tail

As a first step in SCGE analysis, nuclear membrane and histones are removed by incubation with an high salt solution and nucleoids are released. When nuclear DNA is subjected to electrophoresis under neutral or alkaline conditions, DNA regions close to damaged DNA form a comet tail in the presence of electric field. The latter is visualized using a fluorescent dye and DNA damage is scored by visual or computerized image analysis. The coiled/relaxed nuclear DNA represents the comet head.

In the case of neutral SCGE, lysis and electrophoresis are carried out at pH 9.5, a condition that prevents DNA unwinding, and consequently only double strand breaks (DSBs) can be detected (Olive and Banath, 2006). Using pH $\geqslant$ 10.0, DNA unwinding takes place and single strand breaks (SSBs) are visualized (Collins, 2004). Under strong alkaline conditions (pH > 13.0) SSBs are formed from alkali-labile sites (ALS). If enhanced DNA migration is observed at pH > 13.0 compared to the pattern observed at lower alkaline pH, this response is due to induction of ALS. By contrast, the accumulation of either DNA–DNA or DNA–protein cross-links reduces the DNA ability to migrate in the agarose gel, independent on pH. These two types of cross-linking lesions can be however distinguished by incubating DNA with Proteinase K prior to electrophoresis, since the enzyme is active only on DNA–protein cross-links (Collins, 2004).

There is still no agreement on how the comet tail is formed. The dynamics of comet formation are directly related to DNA organization within chromatin in the form of 'matrix attachment sites' and 'loops'. In the undamaged cells, the loops are tightly supercoiled but the treatment with mild detergents releases histones and other DNA binding proteins and the DNA remains supercoiled inside the nuclear skeleton. In the presence of SSBs, the superhelix tension within loops is released and they form a 'halo' visible around the nucleus. According to the current hypothesis, relaxation of DNA loops may represent the primary event for comet formation under neutral and alkaline conditions. However, if neutral two-dimensional electrophoresis is carried out, the comet tails can migrate only during the first electrophoretic step but they cannot move in the second direction, possibly due to the fact that the DNA loops are attached to nuclear structures (Hall et al., 1991). Conversely, under alkaline electrophoresis conditions comet tails are able to move in both electrophoresis directions, suggesting that the alkaline comet tails consist of free DNA fragments (Klaude et al., 1996).

Besides strand breaks, other factors determine the ability of specific DNA sequences to migrate into the tail. Regions associated with the nuclear matrix, such as replicating DNA and actively transcribed domains, do not migrate into the tail under standard alkaline conditions, suggesting that chromosomes with high gene density are more resistant to DNA damaging agents (Kwasniewska et al., 2012).

2.2. Use of lesion-specific enzymes

The sensitivity of SCGE and the range of applications are increased by digesting DNA with lesion-specific endonucleases, DNA repair enzymes. The comparison between results produced in presence and in absence of the lesion-specific enzyme allows estimating the number of specific lesions, since additional strand breaks are formed at the enzyme-sensitive sites (Collins, 2011). The different SCGE versions currently available, relying on the use of lesion-specific enzymes, are summarized in Fig. 2.

The modified base 7,8-dihydro-8-oxoguanine (8-oxo-dG) represents the most common lesion produced under oxidative stress conditions, routinely used as a biomarker of oxidative DNA damage

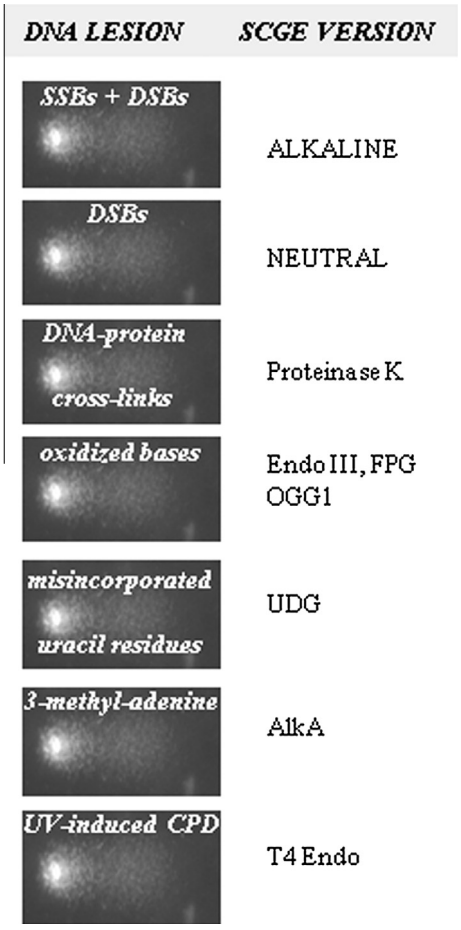

Fig. 2. The versatility of SCGE is highlighted in this schematic representation that covers the multiple modified versions of the assay, currently available for basic/applied research purposes and ecotoxicology analyses. The DNA lesions evidenced by the different SCGE versions are shown.

(Delaney et al., 2012). 8-oxo-dG and other damaged bases can be measured using lesion-specific enzymes. Endonuclease-III (Endo III or Nth) and formamidopyrimidine N-glycosylase (FPG) detect oxidized purines and pyrimidines (Collins et al., 1996; Collins, 2004). The use of the human enzyme 8-hydroxyguanine DNA glycosylase (hOGG1) in SCGE was investigated by Smith et al. (2006).

The enzyme 3-methyladenine DNA glycosylase II (AlkA) preferentially acts on 3-methyladenine while uracil-DNA-N-glycosylase (UDG) reveals misincorporated uracil residues (Shina and Hader, 2002; Collins, 2004). In addition, T4 endonuclease V, which recognizes UV-induced cyclobutane pyrimidine dimers (CPDs), can be used in SCGE assays (Collins et al., 2001).

2.3. SCGE-FISH

The combination of SCGE assay and Fluorescence *In Situ* Hybridization (SCGE-FISH) allows to localize chromosomes or genes within the comet head or in the tail (Santos et al., 1997; Schlormann and Glei, 2012) and to monitor the damage repair rates within gene-specific sequences (Horvathova et al., 2004). The position of fluorescence signals indicates whether the sequence of interest lies within the undamaged (head) or within a damaged (tail) DNA region. If gene-specific signals from tail are repositioned to head during the recovery period, thus there is evidence for repair activity within and around the locus of interest.

Comet formation in *Vicia faba* nuclei treated with specific restriction endonucleases was first investigated by standard SCGE and SCGE-FISH (Menke et al., 2000a). The amount of DNA migrating in comet tails was compared with FISH signals obtained using probes derived from specific chromosome domains. The distribution of FISH signals between the head and tail of comets indicated to which degree these domains were damaged and reflected the distribution of cleavage sites within these domains for the applied restriction endonucleases. *Eco*RI digests uniformly the transcriptionally active euchromatin and cleaves less efficiently in heterochromatin that contains a 59-bp tandem repeat, the *Fok*I element, characterized by a recognition site specific for the *Fok*I enzyme. Differently from *Eco*RI, *Fok*I digests moderately euchromatin and almost completely heterochromatin. The distribution of FISH signals obtained with a probe specific for the *Fok*I repeat revealed the predominance of *Fok*I-element-mediated FISH signals in tails. These experiments confirmed that, also in plants, SCGE-FISH is a reliable tool (Menke et al., 2000a).

As for the advantages of SCGE-FISH applications in plants, it has been proposed that this innovative approach might be particularly useful for evidencing the genotoxic effects environmental mutagens in plants. SCGE-FISH provides valuable information concerning the DNA damage localized within specific genomic regions and this might help a better understanding of the biological impact of hazardous compounds. Several chemicals are routinely used in agriculture, to delay bud development in commercially relevant plants, such as vegetables, which can be stored for prolonged periods before they reach consumers. There is the need to assess the proper dose of active ingredient for commercial formulations, in order to avoid undesired consequences on cultivated plants and derived products. Negative effects on the efficiency of seed germination have been also demonstrated (Marcano et al., 2004). One of the most investigated chemicals in this context is maleic hydrazide (MH, 1,2-dihydro-3,6-pyridazinedione), a structural isomer of uracil which inhibits lipid, protein and nucleic acid synthesis with clastogenic effects (Gichner et al., 2000a). In a recent work, Kwasniewska et al. (2012) reported on the use of alkaline SCGE-FISH for the detection of DNA damage induced in plants by MH. The distribution of 5S and 25S rDNA sequences within the comets in seedlings of *Crepis capillaris*, exposed to this genotoxic compound, was analyzed. These authors showed that SCGE-FISH can be utilized to understand the relationship between chromatin structure, DNA damage and repair distribution in the plant genome with respect to rDNA. Results suggest that chromatin fibers including rDNA regions were rarely formed and that these regions condensed in dots. Rapp et al. (2000) found that chromosomes with higher density of active genes were most often localized in the head of the comet and therefore they hypothesized that DNA regions with high gene density are subjected to very effective DNA repair, faster than that occurring in heterochromatin.

The genotoxic effect of the monofunctional alkylating agent *N*-methyl-*N*-nitrosourea (MNU) on root tip nuclei of *V. faba* was tested by SCGE-FISH (Menke et al., 2000b). Detection of the *Fok*1 elements within comets demonstrated the involvement of these heterochromatin-specific sequences in MNU-mediated damage. Spivak et al. (2009) used SCGE-FISH to analyze Transcription-Coupled Repair (TC-R) mechanisms in cells exposed to low doses of genotoxic agents. The localization of damage and the repair efficiency in transcriptionally active DNA sequences may result in different biological endpoints (mutation, transformation, or cell death), compared to damage accumulated throughout the genome (Global Genome-Repair, GG-R). Different DNA probes have been successfully used with SCGE-FISH for the analysis of damage and repair of specific genomic loci (Hovhannisyan, 2010).

Based on these findings, the potential of SCGE-FISH applications in plants deserves more attention, particularly in view of the increasing amount of agrochemicals that are currently released in the market (Jeschke, 2010).

2.4. Detection of DNA methylated sites using modified SCGE assay

DNA methylation plays a crucial role in maintaining genome stability and detection of the global DNA methylation profiles under different environmental conditions is an essential step towards the comprehension of epigenetic mechanisms both in animals and plants (Saze et al., 2012). DNA methylation can be monitored, using the isoschizomeric restriction enzymes *Hpa*II and *Msp*I that recognize the same tetranucleotide sequence (5′-CCGG-3′) but own differential sensitivity to DNA methylation. *Hpa*II is active only when the internal cytosine is unmethylated while *Msp*I digests the CCGG sequence whether or not the internal cytosine is methylated.

Wentzel et al. (2010) have used the different sensitivity to DNA methylation of *Hpa*II and *Msp*I enzymes to demonstrate that SCGE can provide information on the global DNA methylation status of cultured cells. The human hepatoma cell line HepG2 exposed to the demethylating agent azacytidine was used and analyzed by SCGE. Nucleoids prepared from HepG2 cells were treated with *Hpa*II and *Msp*I and then subjected to alkaline SCGE. Higher methylation levels at the CCGG sites resulted in differences in the amount of DNA migrated in the comet tails of *Hpa*II-digested nucleoids versus *Msp*I-digested nucleoids (Wentzel et al., 2010). Results obtained with SCGE were validated by means of Cytosine Extension Assay (CEA) which uses an *Hpa*II-digestion followed by a single nucleotide extension reaction with [^{3}H]-dCTP. Incorporation of [^{3}H]dCTP into DNA is directly proportional to the number of *Hpa*II-cleaved methylated CpG sites. The methylation-sensitive SCGE was also tested with cells exposed to succinylacetone which causes aberrant DNA methylation patterns (Wentzel et al., 2010). Finally, methylation-sensitive SCGE was carried out using the *Not*I enzyme. In this case, the assay can provide information on the presence of DNA methylation in CpG islands located within gene promoters. It is believed that transcription modulation is influenced by methylation, at least in animal cells (Yamamoto et al., 2007).

Although no reports are currently available, describing the application of the methylation-sensitive SCGE assay in plants, it might become an interesting tool to assess epigenetic variation in plant-based commercial systems whenever low-cost and time-saving screening is required. This specific need has been recently envisaged for *Taxus* cell suspension cultures used as sources of antineoplastic agents. Changes in DNA methylation profiles occurring during prolonged culture time may lead to the progressive loss of taxol biosynthesis and long term effects associated with subculture are not fully understood (Fu et al., 2012).

3. Evaluation of DNA damage/repair by SCGE

The response to genotoxic stress induced by environmental pollutants requires the activation of a complex network of DNA repair processes, such as Nucleotide and Base Excision Repair (NER, BER), Homologous Recombination (HR) and Non Homologous End Joining (NHEJ). Genome integrity is thus preserved through the coordinated action of several DNA repair pathways (Gill and Tuteja, 2010; Balestrazzi et al., 2011a, 2012). Nowadays, there is increasing attention towards the molecular response to genotoxic effects caused by environmental stresses. A fast and reliable technique for the quantitative and qualitative evaluation of the DNA damage/repair response such as SCGE has proven useful in the characterization of plant cells defective in DNA repair (Hartung et al., 2002; Heitzberg et al., 2004; Kozak et al., 2009; Waterworth et al., 2009; Bohmdorfer et al., 2011; Kamisugi et al., 2012; Donà

et al., 2013). Similarly, the combination of SCGE assay with *Saccharomyces cerevisiae* mutants defective in DNA repair functions has been indicated as a valuable approach to elucidate the mechanisms of DNA damage sensing/repair in eukaryotic cells (Oliveira and Johansson, 2012).

3.1. Cell cycle

A novel application of SCGE assay deals with analysis of the DNA damage/repair response in relation to cell cycle. The availability of tools able to assess DNA damage accumulated during the different phases of cell cycle and the resulting repair ability is crucial for predicting the level of resistance to radio- and chemotherapy (Goodarzi and Jeggo, 2012). Although the cell-cycle dependent repair of DSBs by means of HR or NHEJ has been characterized (Delacote and Lopez, 2008), recent studies have highlighted alternative repair pathways able to remove oxidative DNA damage, interstrand cross-links and other types of lesions acting during different cell cycle phases (Frankenberg-Schwager et al., 2008; McCabe et al., 2009). The finding that *Arabidopsis* mutants defective in specific NHEJ components, can remove DSBs with kinetics similar to those found in wild type plants, highlighted the presence of a novel repair pathway besides the known NHEJ mechanism (Kozak et al., 2009). In this case, neutral SCGE was successfully used to monitor cell-cycle dependent fluctuations in DSBs by comparing the response of young seedlings and mature leaves (Kozak et al., 2009).

It has been hypothesized that the total comet fluorescence intensity might correspond to a phase-specific position within the cell cycle and indeed Kruszewski et al. (2012) demonstrated that SCGE allows to obtain a reliable cell cycle distribution comparable with that obtained by flow cytometry. Two different populations of blood mononuclear $CD34^+$ cells, namely quiescent (G_0 phase) cells and cells stimulated to enter the cell cycle (S phase), respectively, were analyzed. No differences were observed in terms of DNA damage accumulation in proliferating and quiescent cells while the lowest amounts of DNA damage were measured in the G_2/M subpopulation. It was also noticed that a large variability in terms of comets was evident both in quiescent and proliferating cells. This finding suggests that other intrinsic factors, such as chromatin remodeling, might be responsible for the large heterogeneity in the observed DNA damage (McArt et al., 2010).

When X-rays treatments were carried out on quiescent and proliferating cells, respectively, S-phase cells accumulated higher levels of DNA damage than cells in G_0/G_1 and G_2/M phases and this might be due to the presence of active replication forks. Under alkaline conditions, the replication forks behave as SSBs and thus S-phase cells own a larger amount of DNA that can migrate during electrophoresis (Olive and Banath, 1993). A correlation between cell cycle phase and DNA damage accumulation was highlighted in *Pisum sativum* cells exposed to chromium (Cr) by comparing SCGE and flow cytometry (Rodriguez et al., 2011). Cell cycle arrest occurred at G_2/M boundary, resulting in decreased proliferation in roots and abnormal mitosis that led to mixoploidization, a mixture of diploid and tetraploid cells. The observed increase in DNA content revealed by SCGE was in agreement with results from flow cytometry (Rodriguez et al., 2011).

Advantages in the use of SCGE assay for the analysis of cell cycle-related DNA damage/repair responses might be envisaged in the study of seed vigor. In seeds, cell cycle progression strictly correlates with the physiological state providing molecular indicators of the different phases of germination as well as priming treatments (Balestrazzi et al., 2011b; Ventura et al., 2012). Alkaline SCGE has been used to assess the level of DNA strand breaks in embryos from cherry (*Prunus* spp.) seeds subjected to gamma ray treatment and prolonged storage (Setsuko and Tooru, 2002). The DNA damage/repair response evidenced by SCGE in seeds under stressful conditions can be correlated with the seed ability to survive adverse environments, as demonstrated in the desert plant *Artemisia sphaerocephala* by Yang et al. (2011).

3.2. SCGE-based DNA repair assay

The individual DNA repair capacity might be used as a biomarker of susceptibility to genotoxic stress. With the 'Challenge assay' or SCGE-based DNA repair assay, cells are treated with a damaging agent and the removal of the damage is then monitored over time in order to study the kinetics of DNA repair. This version of SCGE is commonly used to monitor the rejoining of strand breaks in cells (An et al., 2010). Different parameters have been described in literature, among which is the DNA repair half-time or the time required for cells to remove 50% of the DNA damage (Kozak et al., 2009). Repair of SSBs is generally rapid with a half time of 10 min while repair of oxidized and alkylated bases takes a few hours (Azqueta et al., 2009).

In a different version of the SCGE-based DNA repair assays, cells are pre-treated with a DNA-damaging agent to induce specific lesions and then embedded in agarose on a microscope slide. Subsequently, cells are lysed and the damaged nucleoids are used as a substrate to test the repair ability of cellular extracts prepared from tissues (Langie et al., 2011). The damaged nucleoids are incubated with the cellular extract and the resulting repair activity is monitored at different time points, using the SCGE assay. Several parameters, e.g. the agarose concentration, seem to influence the ability of DNA repair proteins from the cellular extract to penetrate the gel and reach the nucleoids while keeping sample on ice during preparation minimizes the activity of endogenous proteases. The test was also validated using cellular extracts from OGG1−/− mice, lacking the OGG1 repair function, and wild type controls, respectively, and also extracts obtained from young and aged tissues (Langie et al., 2011). A possible advantage deriving from the use of the SCGE-based DNA repair assay in plants might be associated with the screening of cultivars that show natural variation in DNA repair capacity (Teranishi et al., 2004).

4. Plant cells as tools to monitor environmental pollution *in situ*

Environmental pollutants exert genotoxic effects that can be evidenced by different methodologies, including SCGE. Higher plants might be used as stable sensors in ecosystems, as a source of information on the genotoxic impact of dangerous agents, such as the urban airborne particulate mixtures. Plants are highly sensitive to environmental pollutants, including hydrophilic and lipophilic chemicals and heavy metals. They are sessile organisms continuously challenged with mixtures of pollutants at higher concentrations compared to humans and animals and particularly leaves represent an ideal target for biomonitoring purposes since they can accumulate large amounts of pollutants.

SCGE can be used to assess the phytoremediation potential of plant species/genotypes towards specific genotoxic compounds in contaminated soils. The SCGE sensitivity highlights the plant performance in absorbing the pollutant, through accumulation in roots and shoots, providing useful informations for the selection of environmentally compatible species/genotypes. The intrinsic features of this technique allow the fasten, large-scale screening both at the plants and pollutant level. A successful application of SCGE in the field of phytoremediation, described by Chakraborty and Mukherjee (2011), deals with fly ash, a by-product of thermal power plants whose disposal requires extensive amounts of land and water. Vetiver (*Vetiveria zizanioides* L.) plants, cultivated on fly ash, revealed the ability to phytostabilize this pollutant by means of massive root growth. No DNA damage was evidenced

Table 1
SCGE applications in plants. For the different SCGE versions and practical applications, the plant species and related references are indicated.

SCGE-FISH		
Plant species	FISH probe	Reference
Vicia faba	*Fok1* element	Menke et al. (2000a)
Crepis capillaris	5S/25S rDNA	Kwasniewska et al. (2012)
CELL-CYCLE ASSOCIATED SCGE		
	Genotoxic agent	
Pisum sativum	Heavy metal	Rodriguez et al. (2011)
BIOMONITORING		
	Heavy metals	
Bacopa monnieri	Cadmium	Vajpoyee et al. (2006)
Vicia faba	Cadmium	Lin et al. (2007)
Solanum tuberosum	Cadmium	Gichner et al. (2008)
Trifolium repens	Cadmium	Bhat et al. (2012)
Allium cepa	Titanium	Ghosh et al. (2010, 2012)
Nicotiana tabacum	Titanium	Ghosh et al. (2010, 2012)
	Air pollutants	
Nicotiana tabacum	O_3	Restivo et al. (2002)
Ginkgo biloba	CO, SO_2, O_3	Sriussadaporn et al. (2003)
Epipremnum aureum	CO, SO_2, O_3	Sriussadaporn et al. (2003)
Vinca rosea	CO, SO_2, O_3	Sriussadaporn et al. (2003)
Populus tremuloides	O_3	Tai et al. (2010)
	Ionizing radiations	
Vicia faba	X-ray	Koppen and Angelis (1998)
Nicotiana tabacum	Gamma-ray	Gichner et al. (2000b)
Petunia hybrida	Gamma-ray	Donà et al. (2013)
	Chemical mutagens	
Nicotiana tabacum	Ethyl methane-sulfonate	Gichner et al. (2000b)
Nicotiana tabacum	*N*-ethyl-*N*-nitrosourea	Gichner et al. (2000b)
	Organic pollutants	
Sinapsis alba	PHAs	Pakova et al. (2006)
Triticum aestivum	PHAs	Pakova et al. (2006)
Phaseolus vulgaris	PHAs	Pakova et al. (2006)

by SCGE carried out on root nuclei and this finding demonstrated the long-term survival ability of the vetiver plants on the contaminated soil. Interestingly, only limited amounts of the heavy metals found in fly ash were translocated from roots to shoots and the plants were safe for animals. By contrast, other plants such as *Allium cepa* turned out to be highly sensitive to fly ash and high levels of DNA damage were revealed by SCGE (Chakraborty and Mukherjee, 2011).

The advantage of SCGE as a tool to assess the genotoxic effects of a wide range of environmental pollutants have been demonstrated. Roots, stems and leaves are the targets of soil, water and air pollutants which may be available depending on the up-take mechanisms (Poli et al., 1999; Restivo et al., 2002). SCGE-based analysis in different plant tissues is thus a useful source of information. The different SCGE applications reported with plant cells, hereby discussed, are listed in Table 1.

4.1. Heavy metals

Heavy metals emitted from industries, vehicles, and waste incinerators are accumulated in soils, affecting plant growth and crop production. However, the tolerance threshold can vary depending on crop species/genotype (Baath, 1989). Heavy metal accumulation has been investigated in both aquatic and terrestrial plants. Acquatic macrophytes accumulate significant amounts of heavy metals and other genotoxic substances, and are considered useful tools for monitoring pollution. Rooted aquatic plants may be used as alternative to animal models for ecogenotoxicity studies in aquatic ecosystems.

Several reports deals with cadmium (Cd), a non-essential toxic metal that causes genomic instability, producing direct and indirect genotoxic effects, e.g. DNA strand breaks, DNA–protein cross-links, oxidative DNA damage, and causing inhibition of DNA repair enzymes (Whiteside et al., 2010). The sensitivity of the SCGE assay for detecting DNA damage induced by cadmium concentrations higher than 0.01 μM in the wetlands plant, *Bacopa monnieri* L. (Scrophulraceae) was assessed by Vajpoyee et al. (2006). SCGE was used to measure Cd toxicity (60 μM) in rooted potato (*Solanum tuberosum* L.) cuttings to define an improved method for monitoring the effects of agricultural chemicals on crops (Gichner et al., 2008). Alkaline SCGE highlighted the accumulation of DNA damage in *V. faba* leaves exposed to Cd (40 μM), in correlation with oxidative injury to other cell components (Lin et al., 2007). There is limited information on the effects of heavy metals on medicinal plants that have become endangered due to overexploitation, habitat destruction and soil pollution. Bhat et al. (2012) carried out a study on *Trifolium repens*, cultivated as a medicinal herb and fodder for live stock, highlighting the requirement for different bioassays, among which is SCGE, in order to perform the correct evaluation of genotoxic agents. SCGE sensitivity revealed the presence of DNA damage induced by Cd concentrations corresponding to 150–250 mg kg^{-1} of polluted soil.

Nanomaterials own unique properties suitable for a wide range of industrial applications. However, the increasing amounts of nanomaterial in groundwater and soil raise environmental concerns related to alteration of soil and water microflora and consequently the food chain (Oberstorder et al., 2007). Titanium dioxide (TiO_2) nanoparticles are used in several products and in some sectors of agriculture. The genotoxicity of TiO_2 nanoparticles towards plant cells was evaluated using SCGE analysis in *Allium cepa* and *Nicotiana tabacum*. Dose-dependent response of DNA damage accumulation together with enhancement of lipid peroxidation, could be a possible reason for the genotoxic potential of TiO_2 nanoparticles when used at concentrations higher than 1.25 mM (Ghosh et al., 2010). SCGE has been used to assess genotoxicity of silver nanoparticles incorporated into consumer products due to their biocidal effect (Ghosh et al., 2012).

4.2. Air pollutants

Sriussadaporn et al. (2003) carried out an investigation in the metropolitan area of Tokyo (Japan) with *Ginkgo biloba* L., commonly used as a street tree for its resistance to environmental stresses. In the same study, two ornamental herbaceous species were also tested, pothos (*Epipremnum aureum*) and periwinkle (*Vinca rosea*) both tolerant to environmental stresses. As a result, all the roadside plants exposed to carbon monoxide (CO), sulfur dioxide (SO_2), ozone (O_3) and other pollutants, yielded high levels of DNA damage in leaves, although different responses were observed. *G. biloba* mature leaves turned out to be an adequate system to monitor the chronic effects of environmental pollution. By contrast, an exponential increase in the percentage of DNA damage was observed in periwinkle leaves, indicating that these plants were challenged with high stress, above the resistance threshold. Thus, the adaptive capacity of periwinkle was impaired, resulting in visible injury. Pothos showed a different response, since the cellular functions were destabilized in the early period and then there was adaptation to stress, concomitant with activation of DNA repair. During the final stage of exposure, a stable percentage of DNA damage was observed in photos leaves (Sriussadaporn et al., 2003).

UV radiation can exert harmful or helpful effects, depending on wavelength. The genotoxic effects of UV-B radiation (290–320 nm) derive from the induction of covalently cross-linked photoproducts in DNA, the most prevalent being a *cys–syn* cyclobutane pyrimidine dimer (CPD). T4 endonuclease V binds to CPD in double-stranded DNA, then the enzyme cleaves the N-glycosylic bond of the 5′-pyrimidine of the dimer and breaks the 3′-phosphodiester bond,

releasing an abasic site. T4 endonuclease V has been used in SCGE assays carried out with the acquatic plant *Spirodela polyrhiza*, a model for aquatic toxicity research, exposed to UV radiation (Jiang et al., 2007). The T4 endonuclease V was effective in revealing the dose-dependent DNA damage accumulation only after UV-B treatment. The SCGE assay could also discriminate between the UV-B and UV-A (320–400 nm) mediated injury, since UV-A causes DNA damage as a consequence of ROS accumulation and this damage cannot be photorepaired.

Stratospheric ozone, the major absorber of UV radiation, is also a secondary air pollutant resulting from primary precursor pollutants, such as hydrocarbons that are accumulated in urban locations with heavy traffic. This genotoxic agent is converted to ROS in the leaf intercellular spaces. Restivo et al. (2002) validated the SCGE assay on leaf tissues of *N. tabacum* cultivars characterized by different sensitivity to O_3 ($\geqslant$80 ppb, parts per billion). The Bel W3 cultivar was less efficient in the recovery ability under oxidative stress conditions and for this reason it has been proposed that the use of this cultivar might increase the sensitivity of SCGE for genotoxic risk assessment (Restivo et al., 2002). The effects of increased O_3 concentrations, in terms of DNA damage, were evaluated in trembling aspen (*Populus tremuloides* Michx.) clones and application of SCGE assay to leaves proved an effective approach in detecting DNA damage (Tai et al., 2010) (up to 89 ppb, parts per billion). Differences in the cellular response to DNA damage were observed between aspen clones, revealing different levels of O_3 tolerance/sensitivity (Tai et al., 2010).

4.3. Ionizing radiations and chemical mutagens

The use of SCGE as biomarker of exposure to ionizing radiation (IR) for environmental and occupational purposes is well established in human cells (Collins, 2008). The physical–chemical interaction of IR with the cellular DNA results in SSBs, ALS, DSBs, DNA–DNA and DNA–protein crosslinks, and oxidized bases. In human fibroblast exposed to γ-rays, SCGE showed that DNA damage was efficiently repaired during a recovery period in the range 15 min to 2 h. As for plants, Koppen and Angelis (1998) demonstrated that *V. faba* roots exposed to X-ray (30 Gy) could repair DNA strand breaks, estimating that approximately 50% of DNA damage was repaired in less than 20 min. Similarly, Gichner et al. (2000b) showed that DNA damage induced by γ-rays in the range 20–40 Gy was completely repaired in non-replicating tobacco leaf nuclei after 24 h. According to these results, SCGE analysis of nuclei from plant leaves is not suitable for biomonitoring the late effects of IR, since DNA damage is readily repaired.

In the case of chemical mutagens, much longer time periods were required in animal cells in order to repair DNA damage. As demonstrated by Gichner et al. (2000b), only 37% and 55% of DNA breaks caused by alkylating agents were repaired at 21 and 45 h after treatment. DNA damage in leaf nuclei of ethyl methane-sulfonate (EMS) or *N*-ethyl-*N*-nitrosourea (ENU)-treated tobacco plants persisted over a 72 h recovery period. One possible explanation for the lack of apparent DNA repair in leaf is the long half-life of the alkylating agents used (Gichner et al., 2000a,b). It is possible that a large fraction of alkylated bases is removed by DNA glycosylases, leaving AP sites that may not be readily repaired in quiescent leaf nuclei. Without DNA replication an AP site may represent a silent DNA lesion. By contrast, actively proliferating cells localized in root tips were more sensitive to EMS (Gichner et al., 2000a,b) but DNA repair takes place within a sufficient recovery time and the lethal DNA adducts are removed. This means that leaves represent a suitable material for *in situ* biomonitoring of chemical mutagens since the resulting DNA lesions can be detected for long time after exposure (Gichner et al., 2000a,b).

4.4. Organic pollutants

Polycyclic aromatic hydrocarbons (PAHs) represent a class of organic pollutants ubiquitous in the environment, produced by the combustion of fossil fuels and discharge of petroleum-related materials (Skupinska et al., 2004). Due to their hydrophobic properties, PAHs adsorb to suspended particles and are incorporated into sediments, resulting in mutagenic risk for ecosystems. These compounds are converted by the cellular metabolism into electrophilic dihydrodiol epoxides that attack DNA, forming covalently linked bulky adducts on DNA bases. As a result, structural changes at the DNA level occur, that impair transcription and replication (Peltonen and Dipple, 1995). Although NER plays a relevant role in the repair of PAH-induced DNA lesions, other pathways such as BER might be involved (Braithwaite et al., 1998). Although metabolically activated PAHs do not generate strand breaks or ALS, their genotoxicity was demonstrated by SCGE (Vian et al., 2002). When PAHs were tested in V79 CHL (Chinese Hamster Lung) fibroblasts lacking the enzymes required to convert PAHs to genotoxic metabolites, DNA damage was observed using alkaline SCGE in the absence of external metabolic activation. This finding suggested that PAHs act as photosensitizers and produce cytotoxic ROS (Platt et al., 2008).

The SCGE assay was used by Aouadene et al. (2008) with CHO (Chinese Hamster Ovary) cells to detect and characterize the genotoxic profile of river sediments located in industrial and urban areas contaminated with different PAHs, among which phenanthrene and fluoranthene, with an estimated accumulation of 1284.7 $\mu g\ kg^{-1}_{\text{sediment dry weight}}$. Plants might be used as biosensor of PAH pollution, although no reports are currently available. The toxic effects of three homocyclic PAHs, namely phenanthrene, anthracene and fluorene, have been investigated in *Sinapsis alba* L., *T. aestivum* L. and *Phaseolus vulgaris*. This study demonstrated that PHAs trigger the plant antioxidant response, enhancing the activities of antioxidant enzymes (Pakova et al., 2006). Thus, it is reasonable to think that SCGE carried out on plant nucleoids might be used as a toxicity marker of PAHs accumulation. On the other hand, phytoremediation represents an effective approach to remove PAHs from contaminated sites, using plants that metabolize these toxic compounds into less hazardous products. Gao and Zhu (2004) investigated and compared accumulation and translocation of phenanthrene (133 $mg\ kg^{-1}_{soil}$) and pyrene (172 $mg\ kg^{-1}_{soil}$) by 12 plant species. PAHs are transferred from roots into plant tissues and accumulate at levels that correlate with the plant lipid content (Kang et al., 2010). As previously demonstrated for other toxic compounds, SCGE might prove valuable in assessing the phytoremediation potential of plants towards PAHs.

5. SCGE in the context of *in vitro* culture and mutation breeding

Crop improvement relies both on traditional breeding and *in vitro* culture of elite materials and the availability of optimized protocols for clonal propagation or for mutagenesis is essential for operators. In some cases *in vitro* procedures require the use of high concentrations of growth regulators, e.g. auxin, which might result in genotoxic effects (Ateeq et al., 2002). SCGE has been successfully exploited by Costa et al. (2012) in order to select protocols for inducing somatic embryogenesis in Norway spruce (*Picea abies*). Suspension cultures consisting of proliferating proembryogenic masses were incubated with hydrolytic enzymes to remove cell wall and produce protoplasts. The latter were embedded in agarose, subjected to lysis and electrophoresis. Conifer nucleoids turned out to be a suitable material for alkaline SCGE (Costa et al., 2012). Prolonged *in vitro* culture might result in enhanced levels of oxidative stress and thus DNA injury. Activation of DNA

repair mechanisms might then lead to genetic variability. Ventura et al. (manuscript in preparation) have used SCGE to compare DNA damage accumulation and repair response of intact leaves excised from *Petunia x hybrida* plantlets grown *in vitro* and leaf discs cultivated for one week on regeneration medium.

Mutation breeding combines irradiation treatments with *in vitro* culture technique in order to enhance genetic variability in elite plant genotypes. Ornamentals represent the ideal target for mutation breeding since economically relevant traits, e.g. flower color and morphology, plant architecture and variegated leaves, long shelf life, can be easily scored following mutagenic treatment. Mutation breeding is currently performed as a routine technique in several vegetatively propagated ornamental plants, although it is generally acknowledged that protocols need to be optimized on a genotype-base (Shu, 2009). Irradiation treatments can be designed by changing the physical parameters of total dose and dose rate (rate of energy deposition) and there is evidence that exposure to IR, e.g. γ-rays, at low and high dose rate (LDR, HDR) respectively, causes different biological effects. Furthermore, the dynamics of DNA damage accumulation and the molecular mechanisms that regulate recovery from radiation injury as a function of dose rate are poorly explored. Alkaline and neutral SCGE were used by Donà et al. (2013) to investigate the dose rate-dependent response of *Petunia x hybrida* cells belonging to a commercially relevant genotype in order to acquire informations useful for mutation breeding purposes. Exposure to LDR and HDR irradiation resulted in different levels of DNA damage immediately after treatments and a delayed repair response was observed in cells challenged with LDR. The use of SCGE, in combination with the *in situ* detection of ROS induced by γ-rays and the expression profile analysis of both DNA repair and antioxidant genes, allowed to define the level of radiosensitivity of the target *Petunia x hybrida* genotype (Giovannini et al., 2012).

6. Conclusions

The up-date provided in the present review highlights the efforts, made by different research groups in the field of plant biology, in order to translate the recent advances in SCGE application from animal to plant cells. There is strong interest in developing highly reliable and low-cost environmental plant-based sensors able to complement and strengthen the conventional techniques so far routinely used for pollution assessment. In this context, the multiple SCGE versions need to be extensively tested in large-scale representative screenings. On the other hand, the renewed interest in mutation-based techniques for introducing novel genetic variability in elite cultivars might benefit from the useful information that SCGE provides, in terms of radiosensibility/DNA repair response.

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