

The Alkaline Single-Cell Gel Electrophoresis/Comet Assay: a Way to Study DNA Repair in Radicle Cells of Germinating *Vicia faba*

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Abstract. Dry seeds are known to accumulate DNA damage with time of storage. Repair of DNA lesions during germination of *Vicia faba* seeds was followed in the radicles using the alkaline single-cell gel electrophoresis/comet assay. In this assay nuclei were liberated, mixed with agarose and spread out over a microscope slide. After lysis of the nuclear membrane and unwinding of the DNA duplex, DNA was stretched during electrophoresis, giving a comet-like migration pattern. The more DNA was damaged, the higher its mobility. DNA repair took place rapidly the first hours of imbibition and more slowly until ca 33 h after onset of germination. A small amount of heavily damaged cells remained present. Labelling with BrdU provided the possibility to localize repair patches and replicated sites in the comet migration pattern. At 15 h of germination, incorporation of BrdU in radicle DNA was situated at random over the entire comet. At 33 h, DNA repair was more or less accomplished and BrdU was mainly localized in the 'heads' of most comets.

At low moisture content seeds of most crop plants remain viable for many years. Even in this dry state, however, degradative cellular changes take place. Lesions in macromolecules accumulate with time, not least in the structure of nuclear DNA (Cheah and Osborne, 1978). Only when the seed is placed in water and metabolic activity is reactivated, replacement synthesis of proteins, lipids, RNA and repair of lesions in DNA can take place (Rhoderick and Osborne, 1993).

The single-cell gel electrophoresis (SCGE)/comet assay offers the possibility to study DNA integrity. In this assay cells and/or nuclei embedded in agarose are lysed to remove the nuclear membrane and proteins. DNA is then unwound (optional) and electrophoresed

for a short time (few minutes). DNA-repair activities, the way of DNA packaging, and DNA integrity in general can influence its migration capacity in the electric field. The migration patterns resemble a 'comet' with a head of intact DNA and a tail containing fragments and DNA loops stretched out of the head (Östling and Johanson, 1984; McKelvey-Martin et al., 1993; Fairbairn et al., 1995). The quantity of DNA migrated in the tail of the comet pattern is an indication of DNA integrity. Some variants of the technique exist, mainly differing in pH of the electrophoresis buffer: the neutral assay where DNA is left double stranded, and the alkaline assay with unwound DNA (Tice, 1995). The assay was often used for animal cells to study DNA damage and repair (Olive et al., 1990; Collins et al., 1996; Buschfort et al., 1997), nuclear architecture (Klaude et al., 1996; Santos et al., 1997; Singh and Stephens, 1997), and apoptosis (Olive and Banáth, 1995; Fairbairn and O'Neill, 1996). Recently, it has been used for the study of different plant tissues (Koppen and Verschaeve, 1996; Navarette et al., 1997; Gichner and Plewa, 1998; Koppen and Angelis, 1998) and seeds (Cerdeira et al., 1997; Koppen and Cerdeira, 1997).

In the experiments described here, DNA integrity and repair in radicle cells of germinating *Vicia faba* (field bean) seeds was studied by use of the alkaline SCGE assay. 5-bromo-2'-deoxyuridine (BrdU) labelling was applied to localize repair regions in the comet pattern.

Material and Methods

Plant material

Two-year-stored dry *Vicia faba* L. (field bean) seeds, which still had a germination capacity of 95%, were imbibed in water for different times at 22°C in the dark. The water was replaced every 24 h. Six hours before collecting the seeds, they were put in Hoagland medium (Sigma, Bornem, Belgium) containing 50 M 5-bromo-2'-deoxyuridine (BrdU) (Sigma, Bornem, Belgium) and 1 M 5-fluorodeoxyuridine (FdU) (Sigma, Bornem, Belgium). After the imbibition time the ca 5 mm-long radicles were cut off and stored in liquid nitrogen.

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Abbreviations: AP – apurinic/aprimidinic, BrdU – 5-bromo-2'-deoxyuridine, BSA – bovine serum albumin, DABCO – 1, 4-diazabicyclo[2.2.2]octane, FdU – 5-fluorodeoxyuridine, FITC – fluorescein isothiocyanate, LMP – low melting point, PBS – phosphate-buffered saline, PI – propidium iodide, SCGE – single-cell gel electrophoresis.

Alkaline single-cell gel electrophoresis (SCGE)/ comet assay

The alkaline comet assay was carried out on the isolated nuclei of the radicles. Therefore, each radicle was chopped with a razor blade in 60 μ l ice-cold MBS (MES – morpholinoethanesulphonic acid, Sigma, Bornem, Belgium)-buffered saline: 80 g NaCl, 2 g KCl and 0.5 g MES for 1 litre, pH 5.8) with 10 mM EDTA. After filtration through a nylon cloth with a pore size of 50 μ m, 10 μ l of the rough nuclei suspension were mixed with 300 μ l 0.8% low-melting-point (LMP) agarose (Gibco BRL, Merlebeke, Belgium) in phosphate-buffered saline (PBS). The mixture was layered on a microscope slide – pre-coated with 100 μ l 0.5% normal-melting-point agarose (NMP, Gibco BRL, Merlebeke, Belgium) – by use of a cover slip and allowed to solidify on ice. Afterwards the slides were put in a solution of 2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Na-lauroyl-sarcosine, pH 10, for at least 1 h at 4 °C to lyse nuclei and to permit deproteination of DNA (Singh et al., 1988). Then, the slides were placed in a horizontal electrophoresis chamber with high-alkali electrophoresis buffer containing 0.3 M NaOH, 1 mM EDTA (pH~13.5). They were left for 20 min in this buffer to allow DNA unwinding and expression of alkali-labile sites followed by electrophoresis for 10 min at 1 V/cm and 300 mA. After electrophoresis, the slides were neutralized in ice-cold 0.4 M Tris (pH 7.5), washed in ice-cold distilled water and allowed to air dry. All steps were performed under yellow light. Dried slides were stained with 5 μ g/ml propidium iodide (PI, Sigma, Bornem, Belgium) for 5 min (Koppen and Angelis, 1998).

The nuclei on the slides were stained for 1 h at room temperature with 20 μ l anti-BrdU monoclonal antibody (Boehringer Mannheim, Brussels, Belgium) at a concentration of 6 μ g/ml diluted in PBS with 0.1% bovine serum albumin (BSA). After 3–4 min washing in PBS, 20 μ l of a 30x diluted (in PBS + 0.5% BSA) FITC-conjugated rabbit anti-mouse antibody (Boehringer Mannheim, Brussels, Belgium) were added for 30 min at room temperature, in the dark. The slides were washed again 3–4 min in PBS and mounted with a 5 μ g/ml PI counterstain and some drops of antifade containing 0.2 M Tris, 90% glycerine and 2.33% 1,4-diazabicyclo[2.2.2]octane (DABCO) (Fluka, Bornem, Belgium).

Viewing of slides

The comets were viewed with a Zeiss Axioplan fluorescence microscope (200x) using a filter set 15 (Zeiss, 510–560 nm excitation filter, 590 nm barrier filter) for PI and a filter set 10 (Zeiss, 450–490 excitation filter, small band filter) for FITC. Measurements of the comets were done with equipped image analysis system Komet 3.1 (Kinetic Imaging, Liverpool, England). Fifty comets per radicle and time point of imbibition were analysed and this was repeated five times. The percentage of migrated DNA in the tail was used as an indica-

tive parameter for DNA disrapture. Location of BrdU incorporation in the comets was done by eye viewing.

Analysis of data

Calculations and graphs were made using Statistica 5. Significance was calculated by non-parametric Friedman ANOVA. If significant ($P < 0.01$ was taken as a measure), the Wilcoxon matched pairs test was used for pairwise comparison. The variability of the measurements about the median was expressed as 'average deviation'. It was calculated by taking the average of the individual measurements, deviations (in absolute value) from the median.

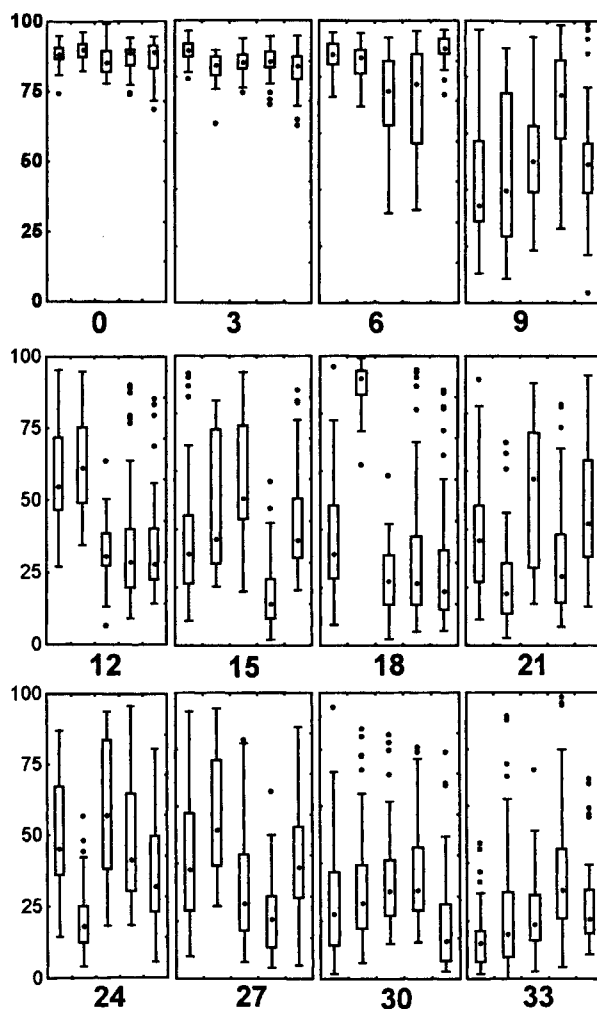


Fig. 1. DNA integrity (% DNA in tail) of *Vicia faba* radicles analysed with the alkaline SCGE assay at different time points (0–33 h) after onset of rehydration. The assay was repeated for five different radicles per time point of imbibition. The box-whisker plots for % DNA migrated in the tail of the comet pattern represent median (●), 25-percentiles (boxes), non-outlier range (whiskers), and outliers (○) = if data point value $> UBV + 1.5*(UBV-LBV)$, or data point value $< LBV - 1.5*(UBV-LBV)$, with UBV and LBV representing the upper and lower value of the box.