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Detection of Specific DNA Lesions by a Combination of Comet Assay and FISH in Plants

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We have studied comet formation on Vicia faba nuclei embedded in agarose and treated with the endonucleases DNase I (to produce SSBs or DSBs at random sites), *Fok*I (to produce DSBs preferentially within *Fok*I repeats), or *Eco*RI (to produce DSBs at random sites but not within *Fok*I elements). DNase I–induced SSBs were detected when enzyme treatment was followed by alkaline denaturation. DSBs efficiently mediated comet formation using neutral conditions. FISH with DNA probes, detecting specific chromosomal domains such as *Fok*I element-containing heterochromatin, NORs, or telomeres, was done on comets. 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 for the applied restriction endonucleases within these domains. The data confirmed the expectation that the observed comet formation was based on enzyme-specific DNA breakage. Environ. Mol. Mutagen. 35:132–138, 2000. © 2000 Wiley-Liss, Inc.

Key words: comet assay; *Vicia faba*; endonucleases; single-/double-strand breaks; fluorescence in situ hybridization; chromosomal domains

INTRODUCTION

Single-cell gel electrophoresis (SCGE or comet assay) was first described by Östling and Johanson [1984] under neutral conditions, and by Singh et al. [1988] and Olive [1989] under alkaline conditions, as a simple protocol for determining genotoxic events in single cells. It is based on the observation that DNA affected by genotoxic agents migrates in electric fields from nuclei embedded in an agarose layer toward the anode, giving the appearance of a comet with head and tail. The proportion of DNA transferred to the tail can be detected after DNA staining with fluorochromes and quantified by measuring fluorescence intensity using a CCD-camera. The average proportion of DNA in the tail of numerous individual comets is taken as a measure for genotoxic damage (for review, see Fairbairn et al., 1995). For most genotoxins, comet formation is detectable immediately after exposure. Even detection of crosslinks is possible by decreased DNA migration when nuclei are irradiated additionally with γ-rays [Merk and Speit, 1999] or by increased DNA migration after some time when DNA breaks are formed by endogenous repair enzymes [Miyamae et al., 1997].

Protocols for comet assay with three plants (*Vicia faba*, *Allium cepa*, *Nicotiana tabacum*) have been introduced [Koppen and Verschaeve, 1996; Navarrete et al., 1997; Gichner and Plewa, 1998; Jiang et al., 1998; Koppen and Angelis, 1998; Stavreva et al., 1998; Angelis et al., 1999]. Because of the solid cell wall of plants, the comet assay is done by embedding isolated nuclei instead of whole cells into agarose. The comet assay on plants may provide a valuable tool for the assessment of environmental and experimental genotoxic impacts. Because of its easy use, it may complement other test systems measuring different endpoints of genotoxicity. This requires a higher degree of standardization and a better understanding of the molecular basis of comet formation. Further studies as to the type(s) of lesion and the DNA fractions involved in comet formation under neutral and alkaline conditions, as well as on the correlations of comet data with other phenomena of genotoxicity, are necessary.

Santos et al. [1997], McKelvey-Martin et al. [1998], and Bock et al. [1999] were the first to report on the application

Abbreviations: AA, alkaline denaturation and alkaline gel electrophoresis; AN, alkaline denaturation and neutral gel electrophoresis; DSB, double-strand break; EtBr, ethidium bromide; FISH, fluorescence in situ hybridization; FITC, fluorescein isothiocyanate; NN, neutral gel electrophoresis without prior alkaline denaturation; NOR, nucleolus organizing region; PI, propidium iodide; SSB, single-strand break.

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This paper is dedicated to Professor Rigomar Rieger at the occasion of his 70th birthday.

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of fluorescence in situ hybridization (FISH) to comet preparations from human cells and described the distribution of DNA from distinct chromosomal domains between the head and tail of comets.

In this investigation, we describe comet formation in *Vicia faba* nuclei after induction of single- or double-strand breaks with specific endonucleases under neutral conditions (NN) and with an alkaline denaturation step (AN), respectively. The comet assay after alkaline denaturation and alkaline electrophoresis (AA) was not performed. This most sensitive variant of the comet assay is usually less suitable to establish time/dose response relationships because saturation (~100% of DNA in tail) is reached very fast. Furthermore, it is not clear whether the same lesions are detected more efficiently under AN conditions or whether other types of damage are responsible for the higher sensitivity [Angelis et al., 1999].

The proportions of DNA in comet tails were compared with those of FISH signals obtained with DNA sequences located at specific chromosome domains and differing in recognition/cutting sites for the enzymes applied. This could be done using computer software that allows one-step measurement of the fluorescence intensity of FISH signals and of DNA stain for each comet in different filter sets. Using this approach, we could check whether the DNA fractions in tails reflect the expected distribution of the endonuclease-induced breaks. Since this approach proved to be successful, it will be possible in the future to compare the chromatin domains involved in comet formation with the chromosomal distribution of primary DNA lesions by immunolocalization [Sauvaigo et al., 1998] as well as with the localization of other endpoints of genotoxicity (chromosomal aberration, sister chromatid exchanges), which proved to be highly specific after treatment of *V. faba* root-tip meristems with distinct types of mutagens [Schubert et al., 1986; Baranczewski et al., 1997].

MATERIALS AND METHODS

Preparation and Treatment of Nuclei for the Comet Assay

The procedure was basically as described by Angelis et al. [1999]. Nuclei of the field bean *Vicia faba* were obtained from 5 to 10 untreated root tips chopped on ice with a razor blade in 500 μl PBS (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4, pH 7.5) and 5 mM EDTA. The resulting suspension (~80 nuclei/μl) was filtered through a 30-μm mesh and a 30-μl aliquot was mixed with 90 μl 0.5% normal melting point agarose (Biozyme, Oldendorf, Germany) at 42°C. Two drops of 60 μl were pipetted separately onto a poly-lysine-coated slide, which was additionally precoated with a dried layer of 0.5% agarose. Each drop was covered with a 22 × 22-mm coverslip and solidified on ice. After removal of coverslips, the nuclei were lysed by incubation in high-salt buffer (2.5 M NaCl, 10 mM Tris-HCl, pH 7.5, 1% *N*-laurylsarcosinate) for 20 min at room temperature (~20–24°C). The gels were then equilibrated three times each for 5 min in enzyme buffer on ice and endonuclease treatment (*Fok*I: 120 U in 10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl; *Eco*RI: 80 U in 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl; DNase I: 3 ng in 50 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$—for preferential induction of single-strand breaks—or 10 mM $MnCl_2$ for increased induction of double-strand breaks) for 5 to 30 min was performed in a coplin jar containing 75 ml of buffer at room temperature. The restriction endonucleases were purchased from Roche (formerly Boehringer, Mannheim, Germany). The reaction was stopped by 100 mM EDTA in 50 mM Tris–HCl, pH 7.5, on ice. After the enzyme treatment, the slides were equilibrated three times each for 5 min in 1× TBE (90 mM Tris–borat, 2 mM EDTA, pH 8.4) on ice and then either exposed to alkaline denaturation (two times for 15 min 0.3 M NaOH, 5 mM EDTA, pH 13.5, at room temperature in the dark) followed by electrophoresis in 1× TBE at 1 V/cm, 15 mA for 6 min (AN protocol) or electrophoresed for 8 min in 1× TBE at 1 V/cm, 15 mA, without prior denaturation (NN protocol). The longer electrophoresis time was chosen for better separation of head and tail, since after the NN protocol a slower DNA migration was observed. This extended time of electrophoresis had no effect on the percentage of DNA in tail. After electrophoresis the slides were dehydrated in ethanol and air-dried.

Fluorescence in Situ Hybridization (FISH) on Comets

The probes were derived from *V. faba* genomic DNA and labeled with digoxygenin-11-dUTP (Roche) by PCR using primers specific for the 59-bp *Fok*I repeat [Kato et al., 1984], the 25S-rRNA gene, the intergenic spacer (IGS), and the $(TTTAGGG)_n$ telomeric repeat. The hybridization mixture contained 2–4 μl of PCR sample per comet-gel, 30% deionized formamide, 2× SSC (0.3 M NaCl, 30 mM sodium citrate), and 10% dextran-sulfate. Probes were denatured at 80°C for 10 min and immediately cooled down on ice. The target DNA was denatured by 0.3 M NaOH at room temperature for 15 min, then gels were neutralized in 0.4 M Tris–HCl, pH 7.5, on ice for 3 min, dehydrated in ethanol, and air-dried. The hybridization mixture (15 μl) was dropped on each of the prewarmed gels, covered with a coverslip, and incubated overnight at 37°C in a wet-chamber.

Before detection of probes, the slides were washed three times each for 5 min in 25% formamide in 2× SSC at 30°C and three times each for 5 min in 2× SSC at 37°C. Then, a 40-μl aliquot of blocking solution (3% bovine serum albumin, 4× SSC) was dropped on each gel and covered with parafilm for 30 min at 37°C in a wet-chamber. The FISH signals were visualized with sheep anti-digoxigenin-FITC conjugate followed by two rounds of amplification employing rabbit anti-sheep-FITC and anti-rabbit-FITC antibodies (Roche). After short dehydration in ice-cold ethanol, the slides were air-dried and stored at 4°C in the dark. If not indicated otherwise, chemicals were purchased from Sigma (Germany).

Evaluation of Comets

Comets were evaluated using a fluorescence microscope (Zeiss Axioplan) equipped with filter-sets for propidium iodide/ethidium bromide (PI/EtBr) and fluorescein isothiocyanate (FITC), and inspected with an objective of 40 × and a projective of 0.5 × magnification by a CCD-video camera connected to a two-processor Intel Pentium computer-unit run under the Windows NT operating system (Microsoft Corp.). The comet-assay-module of the image analysis system Lucia (LIM, Prague) provides a double-staining mode, which facilitates measurement of DNA content and FISH signals in head and tail of comets, simultaneously within two channels for comparison. The first channel was used for the measurement of DNA stained by EtBr (5 μg/ml in water) and for definition of comet-head and comet-tail. The second channel was used to evaluate the distribution of the FISH signals between the predefined areas of comet head and tail (Fig. 1). Usually, 100 individual comets for each experimental point were measured (50 from each of the two independent comet gels). Both data sets (DNA content and FISH signals) were processed with the software program Sigma Plot (Jandel Corp.). For group comparison, the data

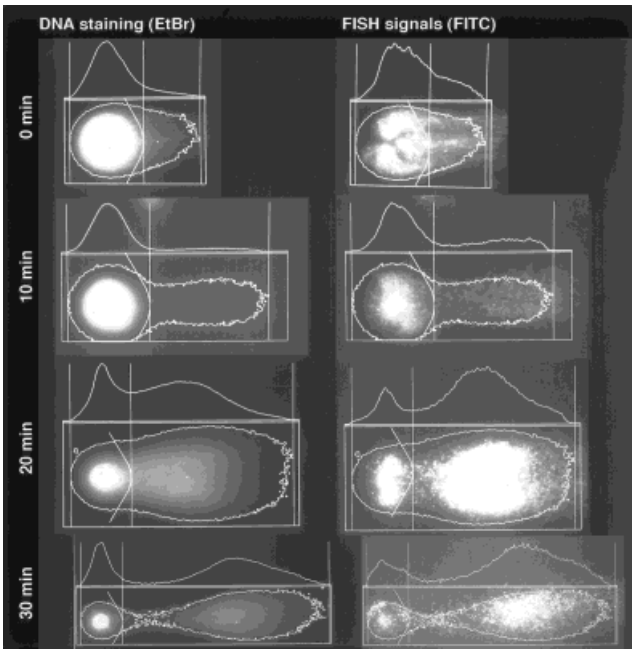

Fig. 1. Typical comets (NN-variant) after 0, 10, 20, and 30 min *Fok*I treatment (ethidium bromide–stained, left) and additional FISH with *Fok*I elements (FITC fluorescence, right). Demarcation of comet heads and tails and comparative fluorescence intensity profiles as provided by the software system Lucia (LIM, Prague). Although hybridization signals were more effective in detection of DNA in tail (right panel) than EtBr, for comparison of the relative proportions of DNA and FISH signals, the tail areas defined by EtBr were used.

of two individual comet gels were pooled and tested for an overlap of the 95% confidence interval of the means of both data sets (Table I). Results were additionally assessed by two-dimensional scatter plots, showing the percentage of DNA content, and FISH signals in tails and by box plots (Figs. 2 and 3), giving the mean values and the standard error for both.

TABLE I. Correlation of DNA and FISH Signals in Various Treatments[a]

		Treatment time (min)			
Probe	Protocol	0	10	20	30
		*Fok*I Treatment			
*Fok*I elements	AN	−[b]	○[b]	+[b]	+
		○	○	+	
		−	−	−	○
		○	○	+	+
	NN	+	+	+	+
		○	+	+	+
		○	+	+	+
rDNA	AN	−	−	−	○
		○	○	○	○
	NN	○	○	−	−
		○	○	○	
		*Eco*RI treatment			
*Fok*I elements	AN	−		−	−
	NN	+	+	−	−
rDNA	AN	+	○	−	−
	NN	+	−	−	
TTAGGG	AN	−	−	−	−
	NN	−	−	○	−
		DNase I treatment ($+MgCl_2$)			
rDNA	AN	○	○	○	○
	NN	○	○	○	○
		DNase I treatment ($+MnCl_2$)			
rDNA	AN	+	○	○	[c]
	NN	+	○	○	[c]

[a] Probes: *Fok*I elements, rDNA, telomeric repeats in comet tails after treatment of *Vicia faba* nuclei with the enzymes *Fok*I, *Eco*RI, and DNase I, respectively, applying the AN or the NN protocol of comet assay.
[b] ○, no significant differences (p = 95%) between the proportions of DNA and of FISH signals in tail; +, significantly more FISH signals than DNA in tail; −, significantly less FISH signals than DNA in tail.
[c] See Table II.

RESULTS AND DISCUSSION

Controls

Nuclei electrophoresed after high-salt lysis and incubation in the respective treatment buffer for 30 min at room temperature served as control for comet formation. Comparing different experiments as well as repetitions of specific experiments, we found that the percentage of DNA in tails of control nuclei may vary considerably. The differences were most obvious when controls of the neutral assay (NN) and those with an alkaline step (AN) were compared. After alkaline denaturation, between 1.7 and 27.1% of the DNA were found in the comet tails with a mean value of 13.3% (calculated from 16 experiments with 100 comets each). In experiments without alkaline denaturation, these values ranged from 16.1 to 31.6% with an overall mean of 24.6% (Table II). Higher control values under NN as compared to alkaline conditions were observed regularly and also reported by Joyce et al. [1999] and Angelis et al. [1999]. The reason for this effect is not yet clear.

Exceptionally high control values (not included in the preceding figures) were observed after incubation of nuclei in a buffer containing 10 mM $MnCl_2$. $MnCl_2$ was necessary to increase DSB induction by DNase I. In this case (AN protocol), 54.2 to 79.4% of the DNA was found in tail as opposed to 29.3 to 36.7% when NN conditions were applied (Table II). This may be related to the strong DNA unwinding effect of the transitional metal cation Mn^{2+} at low ionic strength, based on the specific type of DNA binding by transition metal ions. Whereas Mg^{2+} and other alkaline earth metals bind to a DNA helix by coordinating two phosphate groups and thus stabilizing the molecular structure, transition metals can additionally chelate the N_7 of a guanine group and an adjacent phosphate [Luck and Zimmer, 1972]. As a consequence, bases are presumably tilted, mediating a reduction of the melting temperature and an unwinding of the double helix, which in turn might increase DNA migration.

Additional control experiments under AN conditions

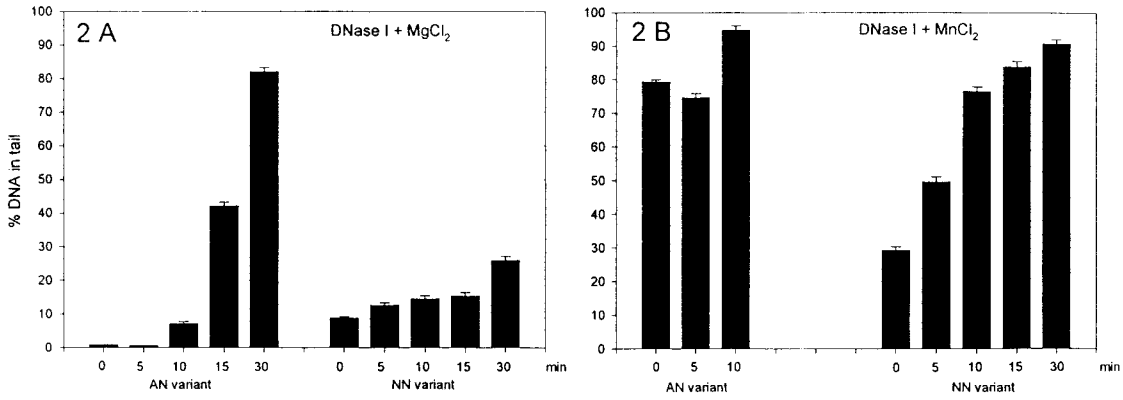

Fig. 2. Results of a representative experiment for detection of SSBs after DNase I treatment in the presence of $MgCl_2$ (**A**) and for detection of DSBs after DNase I treatment in the presence of $MnCl_2$ (**B**).

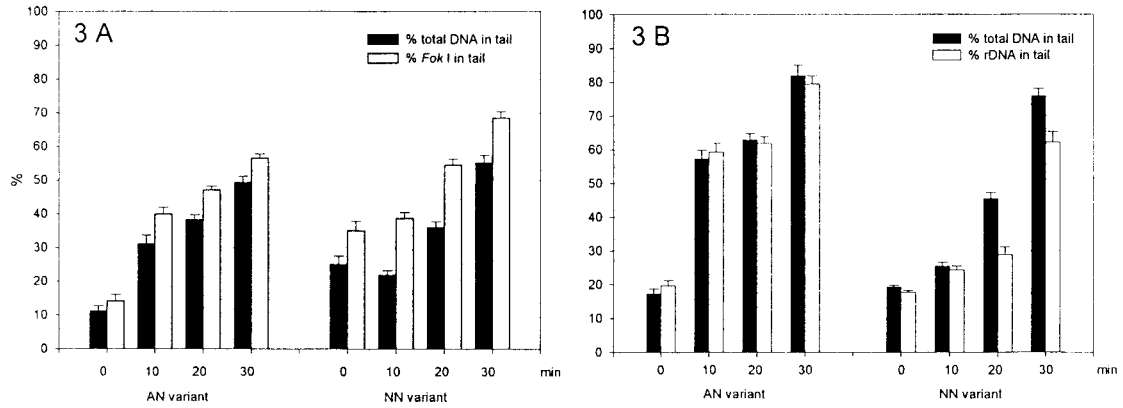

Fig. 3. Results of a representative experiment for comparison of the mean percentage of DNA and of specific FISH signals in comet tails after *Fok*I digestion of *V. faba* nuclei, AN or NN comet assay and hybridization of the comets with either the *Fok*I sequence (**A**) or rDNA-specific sequences (**B**). Bars indicate standard error.

have shown that the extent of migration increases with exposure time (from 31.7 to 82.7% DNA in tail after exposure times of 10 and 60 min, respectively) and with the concentration of Mn^{2+}-ions (33.5% DNA in tail after 0.1 mM to 93.6% after 5 mM $MnCl_2$ treatment for 30 min). A reduction to less than 10% DNA in tail was achieved when nuclei were incubated in ice-cold buffer containing 10 mM $MnCl_2$. These data show that the experimental conditions, in addition to the substance to be tested, may strongly influence the results obtained by use of the comet assay.

Detection of Single-Strand Breaks (SSBs) vs. Double-Strand Breaks (DSBs) in Dependence on Alkaline Denaturation

Under neutral conditions (NN), comet formation (i.e., migration in electric fields of DNA out of the nuclei embedded in gels) is considered to be due predominantly to DSBs. Alkaline unwinding of DNA before electrophoresis (AN) may cause free single-stranded DNA fragments at SSB positions, which also migrate and contribute to tail formation. When electrophoresis is run under alkaline conditions (AA protocol, not applied in our experiments), comet formation is usually even more pronounced, possibly the result of transformation of "alkali-labile sites" into SSBs (for instance, see Klaude et al., 1996; Angelis et al., 1999).

The predominant mode of DNA hydrolysis of bovine pancreatic DNase I (induction of SSBs vs. DSBs in covalently closed circular DNA) depends on the type of activating divalent metal cations present in the reaction buffer. The proportion of DSBs among the breakage events is about 4% in the presence of Mg^{2+}-ions and about 30% in the presence of Mn^{2+}-ions [Campbell and Jackson, 1980]. We have treated *V. faba* nuclei with DNase I activated by Mg^{2+} and Mn^{2+}, respectively, and then processed according to the AN and the NN protocol to see whether the relative proportions of SSBs and DSBs are reflected by different intensities of comet formation under neutral and alkaline conditions. In the case of the AN protocol, the control values were low in relation to values obtained after 30 min DNase I treatment in the presence of $MgCl_2$ (0.8 vs. 82% DNA in tail for the experiment shown in Fig. 2A, left). Under neutral

TABLE II. Mean Percentage of DNA in Comet Tails (± SE) for Various Treatments[a]

Probe	Protocol	Treatment time (min) 0	10	20	30
		*Fok*I treatment			
*Fok*I elements	AN	24.0 ± 1.4	46.5 ± 1.7	54.6 ± 1.5	66.9 ± 1.3
		12.5 ± 0.8	52.3 ± 1.6	61.6 ± 1.7	74.2 ± 1.3
		1.7 ± 0.3	83.3 ± 1.6	78.7 ± 1.3	
		2.9 ± 0.5	87.3 ± 1.5	85.0 ± 1.3	
		17.7 ± 0.9	18.8 ± 0.6	49.5 ± 1.1	70.8 ± 1.2
		2.8 ± 0.2	14.4 ± 0.6	42.8 ± 1.4	69.1 ± 1.6
		11.1 ± 1.7	30.9 ± 2.7	38.1 ± 1.6	49.2 ± 1.9[b]
		14.0 ± 2.0	39.9 ± 2.1	47.0 ± 1.1	56.5 ± 1.3
	NN	16.1 ± 0.8	61.3 ± 2.0	74.9 ± 1.2	76.1 ± 1.3
		22.9 ± 1.0	71.3 ± 1.4	80.7 ± 0.8	83.9 ± 0.9
		17.8 ± 0.7	22.1 ± 0.8	39.2 ± 1.2	65.7 ± 1.4
		20.3 ± 0.7	34.9 ± 1.3	58.0 ± 1.6	81.7 ± 1.5
		24.9 ± 2.6	21.8 ± 1.4	36.0 ± 1.8	55.1 ± 2.2[b]
		35.0 ± 2.8	38.6 ± 1.7	54.4 ± 1.8	68.4 ± 1.9
rDNA	AN	20.5 ± 1.6	65.4 ± 1.3	68.4 ± 1.1	78.1 ± 1.4
		13.4 ± 1.1	56.0 ± 1.9	54.7 ± 2.0	72.8 ± 1.9
		17.2 ± 1.6	57.3 ± 2.6	62.8 ± 1.8	81.9 ± 3.3[b]
		19.6 ± 1.7	59.2 ± 2.6	61.8 ± 2.0	79.4 ± 2.5
	NN	19.3 ± 0.5	25.4 ± 1.1	45.4 ± 1.8	75.9 ± 2.2[b]
		17.7 ± 0.6	24.3 ± 1.2	28.7 ± 2.3	38.0 ± 3.2
		23.2 ± 4.0	24.3 ± 2.9	39.9 ± 2.8	
		21.7 ± 4.0	25.0 ± 2.4	35.0 ± 2.9	
		*Eco*RI treatment			
*Fok*I elements	AN	7.2 ± 0.5		57.2 ± 1.1	57.5 ± 1.8
		4.4 ± 0.4		22.3 ± 0.8	27.8 ± 1.0
	NN	31.2 ± 0.8	38.9 ± 1.0	61.0 ± 1.1	69.1 ± 1.3
		47.0 ± 0.9	44.0 ± 1.1	56.2 ± 1.0	60.9 ± 1.2
rDNA	AN	6.8 ± 0.6	10.5 ± 0.8	49.0 ± 1.6	62.1 ± 1.7
		13.0 ± 1.1	12.6 ± 0.9	40.6 ± 1.4	50.2 ± 1.5
	NN	23.2 ± 1.0	37.7 ± 1.0	46.8 ± 1.6	
		28.2 ± 1.1	18.5 ± 1.0	25.4 ± 1.2	
TTTAGGG	AN	6.2 ± 0.8	44.4 ± 1.4	58.8 ± 2.1	82.9 ± 0.9
		1.4 ± 0.3	16.4 ± 1.4	42.3 ± 2.1	68.0 ± 1.6
	NN	29.1 ± 1.0	50.7 ± 0.9	78.6 ± 1.0	74.7 ± 1.3
		18.8 ± 1.3	25.3 ± 1.2	73.4 ± 2.1	57.1 ± 3.2
		DNase I treatment (+$MgCl_2$)			
*Fok*I elements	AN	17.7 ± 0.7	79.1 ± 0.9	93.1 ± 1.1	94.6 ± 1.4
		28.7 ± 0.8	39.0 ± 0.8	79.8 ± 1.1	87.2 ± 1.4
		7.2 ± 1.3	13.4 ± 1.0	42.8 ± 1.2	73.3 ± 0.9
		11.7 ± 1.4	18.6 ± 1.0	37.4 ± 0.9	57.0 ± 1.0
		27.1 ± 1.0	28.6 ± 1.0	48.9 ± 1.1	57.3 ± 1.1
		23.7 ± 1.0	23.7 ± 1.0	39.7 ± 1.1	46.6 ± 1.1
		17.3 ± 0.9	25.2 ± 0.8	38.3 ± 1.0	52.3 ± 1.6
		20.1 ± 0.8	23.6 ± 0.7	34.1 ± 0.8	42.1 ± 1.6
	NN	29.7 ± 0.9	41.0 ± 1.0	56.4 ± 0.9	65.2 ± 1.1
		41.9 ± 1.1	49.7 ± 1.0	62.6 ± 1.2	72.6 ± 0.9
rDNA	AN	2.6 ± 0.9		56.2 ± 1.1	
		2.4 ± 0.8		56.0 ± 1.2	
		9.9 ± 1.1		52.7 ± 1.8	
		14.4 ± 1.2		42.6 ± 2.7	
		18.2 ± 1.3	24.5 ± 1.2	28.0 ± 1.1	48.2 ± 1.4
		21.9 ± 1.3	28.0 ± 1.3	31.6 ± 1.2	47.8 ± 1.4
	NN	31.6 ± 1.3	34.0 ± 2.0	54.2 ± 2.4	68.6 ± 2.0
		33.0 ± 1.4	30.3 ± 2.1	54.5 ± 2.5	66.4 ± 2.1

conditions (NN), comet formation observed after the same treatment was much less pronounced. Incubation for 30 min in buffer yielded 8.8% and in DNase I, 26% DNA in tail for the experiment shown in Figure 2A (right). The effect observed for the NN variant, in addition to the high control values, may be caused by the small amount of DSBs that are

TABLE II. *Continued.*

Probe	Protocol	Treatment time (min) 0	10	20	30
			DNase I treatment (+$MnCl_2$)		
*Fok*I elements	AN	54.2 ± 1.0	87.5 ± 1.1	95.4 ± 0.9	—[c]
		41.5 ± 0.8	78.8 ± 1.2	95.7 ± 0.7	
	NN	35.4 ± 1.1	84.5 ± 0.7	94.5 ± 0.3	94.0 ± 0.3
		50.9 ± 1.1	89.7 ± 0.4	97.6 ± 0.2	96.4 ± 0.2
rDNA	AN	58.9 ± 1.1	91.5 ± 1.3	96.6 ± 0.6	—[c]
		65.2 ± 0.8	89.9 ± 1.3	96.6 ± 0.5	
	NN	36.7 ± 0.8	80.0 ± 0.9	92.6 ± 0.4	—[c]
		40.2 ± 0.9	81.3 ± 0.8	92.6 ± 0.4	
		DNase I treatment (+$MgCl_2$, +$MnCl_2$) without FISH (Fig. 2)			
		0 min	10 min	15 min	30 min
+$MgCl_2$	AN	0.8 ± 0.1	7.0 ± 0.8	42.1 ± 1.1	82.0 ± 1.2
	NN	8.8 ± 0.4	14.5 ± 0.9	15.4 ± 1.0	26.0 ± 1.2
+$MnCl_2$	AN	79.4 ± 0.5	94.8 ± 1.3	—[c]	—[c]
	NN	29.3 ± 1.0	76.5 ± 1.4	83.8 ± 1.6	90.8 ± 1.3

[a] Each experiment represents pooled data from two separate gels (first row) performed with the endonucleases *Fok*I, *Eco*RI and DNase I on nuclei of *Vicia faba* under AN or NN conditions. Each second row shows the corresponding data for FISH signals obtained with *Fok*I elements, rDNA, or the telomeric sequence TTTAGGG.
[b] Data used in Fig. 3.
[c] No measurable comets because of high degree of DNA damage.

induced by DNase I in the presence of $MgCl_2$ and by complementation of closely adjacent SSBs.

When nuclei were exposed for 30 min to DNase I in the presence of Mn^{2+} using the NN protocol, 90.8% DNA was found in tail, whereas the controls showed 29.3% in tail (Fig. 2B, right). With the AN protocol (in the experiment shown in Fig. 2B [left]), after just 10 min of DNase I treatment, 94.8% of the DNA was already in tail and after 20 min, DNA was no longer detectable in the gel. The controls showed 54.2 to 79.4% DNA in tail. The heavy damage of nuclei after DNase treatment in the presence of Mn^{2+} under AN conditions is obviously caused by a combination of DNase I–mediated breakage and the effects of Mn^{2+}-ions.

FISH with Specific DNA Probes on Comets

Besides DNase I, the restriction endonucleases *Eco*RI (GAATTC) and *Fok*I (GGATG$(N)_{9-13}$) were used to investigate comet formation after DSB induction in plant nuclei. *Eco*RI has previously been found to digest *V. faba* chromosomes uniformly in euchromatic but less efficiently in heterochromatic regions [Schubert, 1990], whereas *Fok*I digested the DNA of euchromatin moderately but nearly completely the DNA of those heterochromatic regions (~10% of the genome), which are composed omainly f a 59-bp tandem repeat, the *Fok*I element, characterized by a *Fok*I-recognition site [Fuchs et al., 1994]. As expected, both enzymes led to comet formation under AN as well as under NN conditions (Fig. 3 and Table II).

We have compared the distribution of FISH signals obtained with specific probes (*Fok*I repeat, rDNA, telomere repeat) on comets after treatment with DNase I, *Fok*I, and *Eco*RI, respectively. Since comets caused by the *Fok*I enzyme should be based on DSBs at the enzyme-specific recognition sites, which are clustered at the heterochromatic loci of *Fok*I elements, we expected a predominance of *Fok*I-element-mediated FISH signals in tails as compared to heads of *Fok*I-mediated comets. In six of seven experimental series, a clear bias of signal distribution in favor of tails increasing with treatment time was observed after *Fok*I treatment (Figs. 1 and 3A; Table I). Since efficient digestion with *Fok*I produces a lot of short mono- and oligomeres of the element, which would be expected to be lost from the gels during electrophoresis, the obtained figures certainly underestimate the proportion of *Fok*I elements in tail. Contrary to the *Fok*I elements, rDNA probes revealed in four experimental series a random distribution or occasionally an underrepresentation of FISH signals in tails of *Fok*I-mediated comets (Fig. 3B and Table I). This was to be expected, since within 4834 bp of *V. faba* rDNA sequences (accessions X17535, X16615) 14 *Fok*I-recognition sites were found.

*Eco*RI, on the other hand, does not cleave *Fok*I elements. Therefore, it is expected that FISH signals obtained for this probe should be underrepresented in tails. This was the case after 20 and 30 min of treatment for both experimental series (Table I). The underrepresentation of FISH signals for rDNA in tail at all but one experimental point after *Eco*RI treatment (Table I) is in line with the rare occurrence of recognition sites in rDNA (no *Eco*RI sites were found within the above-mentioned 4834 bp of rDNA). Clustering of FISH signals in comet heads was also expected for the telomere-specific probe because there are no *Eco*RI-recog-

nition sites within this sequence. Although telomere repeats caused only weak FISH signals on comets, in agreement with expectations, fewer signals occurred in tails than in heads of *Eco*RI-mediated comets (Table I). For both restriction endonucleases, which cause only DSBs, the FISH results were similar for AN and NN variants of the comet assay (Table I).

On DNase I–mediated comets, FISH signals obtained with rDNA probes were randomly distributed in four independent experimental series (Table I).

In conclusion, the results from this study have shown that SSBs, as well as DSBs, can be detected in plant nuclei following the AN protocol for the comet assay. Following the NN protocol, mainly DSBs are detectable, but the control values are unfavorably higher.

The distribution of FISH signals obtained with specific DNA probes can be measured simultaneously with the distribution of DNA between heads and tails of comets. The proportion of DNA elements from specific chromosome domains in comet heads and tails corresponded to the expectation based on the distribution of cleavage sites for specific endonucleases. This is additional evidence that the observed comets were caused by enzyme-specific DSBs within the *Vicia faba* genome. Therefore, the combination of the comet assay with FISH will allow comparison of the involvement of specific chromosomal domains in DNA breakage, repair, and aberration formation after mutagen treatment.

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