JARQ 33, 223–226 (1999)

Identification of DNA Polymorphism Induced by X-Ray and UV Irradiation in Plant Cells

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Abstract

Angelonia protoplasts were irradiated with X-rays or UV to obtain a half survival rate. To evaluate the DNA damage, total genomic DNA was prepared from protoplasts irradiated with X-rays or UV and nonirradiated control protoplasts, respectively. An aliquot of DNA was subjected to polymerase chain reaction (PCR) with a decamer arbitrary primer in the presence of radiolabeled nucleotides under annealing conditions of low stringency. Randomly amplified PCR products were analyzed using a denaturing polyacrylamide gel. All the DNA bands detected by autoradiography were compared. The number of DNA bands detected in one lane was approximately ～150. The results indicate that the arbitrarily amplified PCR method is very sensitive for monitoring the DNA polymorphism caused by X-ray and UV irradiation. This novel procedure, designated as RI (radioisotope incorporated)-RAPD, could be applied to detect precise DNA polymorphism in plant cells.

Discipline: Biotechnology
Additional key words: RI-RAPD, *Angelonia salicariefolia*

Introduction

Randomly amplified polymorphic DNA (RAPD) analysis has been widely used to identify genetic polymorphism in many organisms[11,12]. Using arbitrary decamer primers, polymerase chain reaction (PCR) is performed under the conditions of low stringency annealing temperature. PCR products are conventionally separated on agarose gel, and stained with ethidium bromide (EtBr). This method is very simple and rapid. However, the resolution of detailed and complex DNA bands in agarose gel is limited. Another improvement was achieved by introducing silver staining[2] or digoxigenin (DIG)-based[10] methods into RAPD. Since the DNA bands observed in an agarose gel or an acrylamide gel are sometimes indistinct, the reproducibility of the RAPD methods has been doubtful.

We are currently examining the effects of X-ray and UV irradiation on mesophyll protoplasts of *Angelonia salicariefolia* to inactivate nuclear DNA. Protoplasts whose nuclear DNA is partly inactivated by the application of irradiation techniques are subsequently utilized for asymmetric plant cell hybridization[1,4].

To identify the DNA polymorphism induced immediately after the exposure of protoplasts to X-rays and UV, we applied an improved RAPD analysis procedure for the genomic DNA prepared from control and irradiated protoplasts. A conventional RAPD analysis of DNA from the 3 different protoplasts (control, X-ray-irradiated, and UV-irradiated) revealed only slight differ-

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ences, while significant differences between the control and irradiated protoplasts were detected by the novel procedure. Here we describe the protocols for an improved RAPD method which could be applied in general.

Materials and methods

1) X-ray and UV irradiation of protoplasts

Mesophyll protoplasts from leaf segments of *Angelonia salicariefolia* L. were cultured in liquid MS16 medium as previously described[7]. Protoplasts ($\sim 5\times10^4$ cells/mL) were irradiated with X-rays (60 kVp, 4 mA, dose of 15 K) or UV (2.5 mW/cm^2/s, dose of 25 mW/cm^2 at 254 nm). Under these conditions, the cell division frequency of the irradiated protoplasts was reduced to half, compared to that of control cells after 7 days of culture (unpublished data). Immediately after the exposure to X-rays and UV, protoplasts were frozen and kept at –70°C until the preparation of DNA.

2) DNA preparation

Total genomic DNA from protoplasts was prepared according to the method of Comczynski[3] for simultaneous DNA and RNA extraction from tissues or cultured cells. We found that this single step procedure which is very simple and rapid can be used to prepare reasonable amounts of total genomic DNA from protoplasts. The guanidium isocyanate/phenol solution (ISOGEN™) was obtained from Nippon Gene.

3) Polymerase chain reaction (PCR)

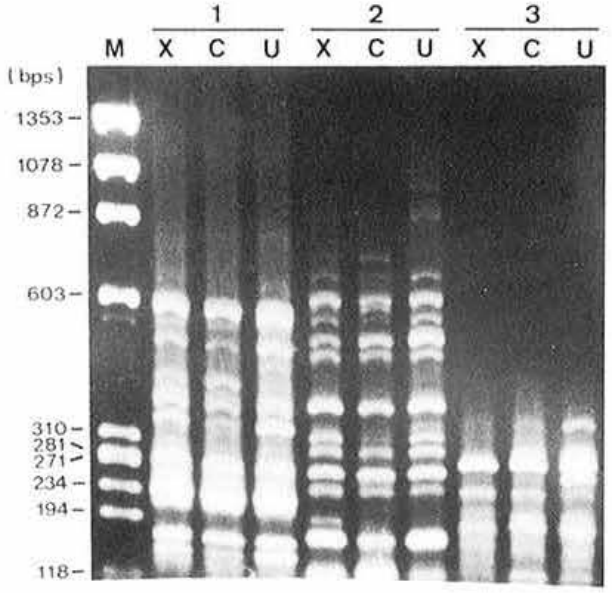
Three decamer oligonucleotides, designated as RA1, RA3, and RA5 whose sequences were 5'-GTCTGACGGT-3', 5'-CGATCGAGGA-3', and 5'-AAGCAGCAAG-3', respectively, were used as RAPD primers[8]. The standard PCR solution (20 μL) was composed of 100 ng of template DNA, 20 μM of primer, 200 μM of dNTP, 10 μCi of [α-^{32}P]dCTP (Amersham, 3,000 Ci/mmol), 2.5 units of *Tth* Polymerase (Toyobo) in the recommended buffer. PCR was carried out in an IWAKI thermal sequencer (TSR-300) and the conditions were as follows; 94°C denaturation for 30 s, 42°C annealing for 60 s, and 72°C extension for 30 s with 40 cycles, and another 72°C extension for 5 min. For DNA standard molecular markers, ϕ×174/*Hae*III digests and DNA sequencing ladder (M13 DNA as a template) were used for an agarose gel and a denaturing polyacrylamide gel, respectively.

4) Gel electrophoresis

Following 1.5% agarose gel electrophoresis in TBE buffer, the PCR products were visualized by staining in EtBr solution (1 μg/mL) and photographed. For denaturing gels, a 6% polyacrylamide gel (acrylamide : BIS=19 : 1) containing 42% urea was used in Tris borate buffer. The size of the denaturing gel was 200 mm (width)×600 mm (length)×0.35 mm (thickness). Electrophoresis was carried out for 8 h at 1,500 V, the gel was dried on 3MM paper (Whatman) subsequently, and autoradiographed overnight without the use of an intensifying screen.

Results and discussion

Fig. 1 shows the PCR products derived from the X-ray irradiated (X), the control (C), and the UV-irradiated protoplasts (U), using 3 different primers (RA1, RA3, and RA5). In lane 1 using a primer RA1, some extra DNA bands were



Detection of damage of protoplasts by conventional RAPD

Lane 1: primer RA1, X: X-ray treatment,
2: primer RA3, C: control,
3: primer RA5, U: UV treatment.

Fig. 1. Conventional RAPD pattern

PCR products derived from X-ray (X)-, UV-irradiated (UV), and control (C) protoplasts using arbitrary primers, RA1, RA3 and RA5, were electrophoresed in a 1.5% agarose gel and stained with EtBr.
(M) indicates ϕ×174/*Hae* III digests.

observed in the X-ray (X)- and UV (U)-irradiated cells compared to the control (C). Similar results were also obtained using RA3 (Fig. 1, lane 2) and RA5 (Fig. 1, lane 3), respectively. These results indicated that irradiation with X-rays and UV led to DNA rearrangements in the portion of genomic DNA.

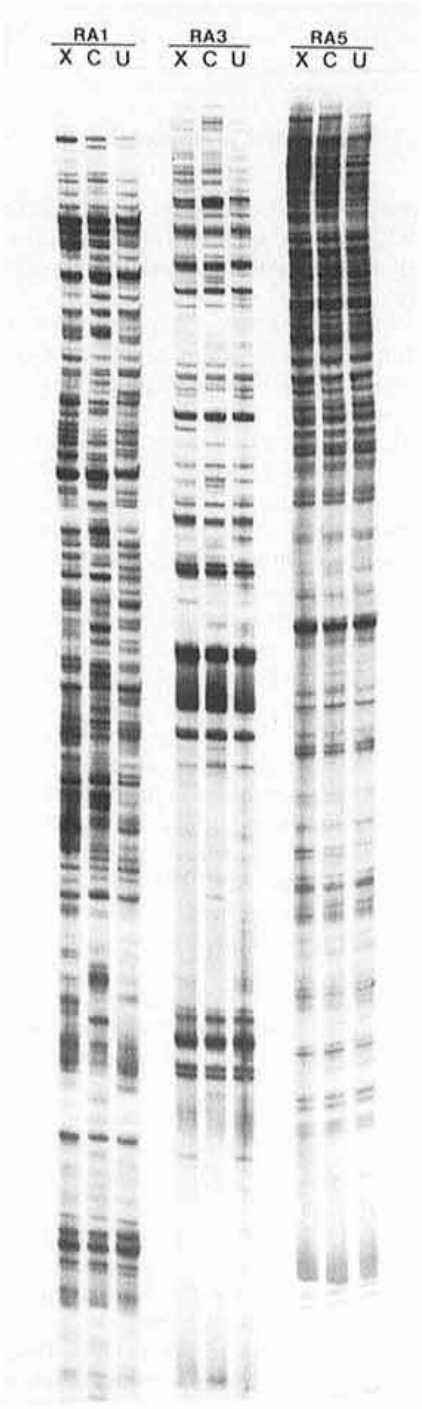


Fig. 2. RI-based RAPD pattern
PCR products derived from X-ray (X)-, UV (U)-irradiated, and control (C) protoplasts using arbitrary primers, RA1, RA3 and RA5 were electrophoresed in a 6% denaturing gel and autoradiographed over-night.

To identify a RAPD profile more precisely, we adopted a radioisotope-incorporated PCR (RI-based PCR) method. Fig. 2 shows one example of the autoradiograph of the PCR products. The order of samples was the same as that in Fig. 1. The DNA bands observed in the X-ray (X)- and UV (U)-irradiated protoplasts were distinct from those of the control (C). The sizes of the DNA bands were estimated to be in the range of ~100 to ~500 bp by comparison with the sequence ladder of M13 (data not shown). The total number of bands in each lane shown in Fig. 2 was determined (Table 1), and found to be larger than that shown in Fig. 1, presumably due to the higher resolution in polyacrylamide denaturing gels.

Approximately 120 to 160 DNA bands were detected in the range of ~100 to ~500 bp in each lane. The total number of DNA bands was not significantly different among the primers, especially between RA1 and RA5. However, the number of bands that disappeared or the appearance of new DNA bands depended on the primer used (Table 1). It should be noted that PCR products less than 100 bp could not be detected easily by autoradiography because the amount of [α-^{32}P] dCTP incorporated into the PCR products was very small. In brief, the present study revealed that (1) DNA rearrangement in protoplasts by X-rays and UV could be effectively monitored by RI-based PCR, (2) a denaturing polyacrylamide gel is superior to an agarose gel for the resolution of PCR products in the range of ~100 to ~500 bp, and (3) some primers enabled to detect DNA damage more effectively than others.

Hall et al.[5] examined the effects of X-rays and UV on DNA isolated from a protoplast culture of sugar beet (*Beta vulgaris*). They showed a significant fragmentation of nuclear DNA by pulsed-field gel and conventional agarose gel electrophoresis. They were the first to demonstrate the DNA damage by X-rays and UV at the molecular level. However, only large DNA fragments were analyzed in that study. To detect the DNA damage caused by UV irradiation, Stapleton et al.[9] measured pyrimidine dimers using a specific antibody. Since their method was based on immunoreactive detection and was quantitative, only

Table 1. DNA bands detected by RI-based RAPD

Primer	Total DNA bands			DNA bands appearing in (+) or disappearing in (–)			
	C[a]	X[b]	U[c]	X		U	
RA1	159	157	150	+23	–25	+14	–23
RA3	126	138	132	+22	–10	+22	–16
RA5	153	153	152	+0	–0	+1	–2

The numbers of DNA bands detected in Fig. 2 are indicated.
a): C; Control. b): X; X-ray-irradiated protoplasts. c): U; UV-irradiated protoplasts.

the total amount of pyrimidine dimers induced by UV-irradiation could be measured. Our present work focused on the detailed and complex changes at the nucleotide level resulting from X-ray and UV irradiation of protoplasts, and the use of powerful tools (conventional RAPD, Fig. 1, and RI-based PCR, Fig. 2) to monitor such DNA rearrangements.

The length of the PCR products indicated in Figs. 1 and 2 seems to be relatively shorter than that of the products obtained in previous studies[11,12], which may be due to the shorter extension time (30 s) than in conventional methods (for example, 120 s in Refs. 11, 12). However, our procedure was effective for detecting polymorphic DNA bands. By using 20 mer sequence derived from the bacteriophage T3 promoter region, Kubota et al.[6] reported the detection of γ-ray-induced DNA damage in the Japanese medaka. They obtained DNA-fingerprinting of fish genomic DNA, but the numbers of PCR products were still small, presumably due to the longer length of the arbitrary primer used in their study.

In conclusion, RI-based PCR is a very sensitive method to detect a very low DNA polymorphism. This method is rather laborious and requires the use of radioisotope-labeled compounds in contrast to conventional RAPD analysis. However, the system consisting of a denaturing polyacrylamide enables to detect a difference of even one nucleotide. When conventional RAPD is not adequate for distinguishing a DNA band profile of PCR products, RI-based RAPD method would be a suitable alternative to detect polymorphic DNA.

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(Received for publication, February 3, 1999)