Carborundum-dependent entrance of EcoRI restriction enzyme into plant cells and specific cleavage of genomic DNA

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In a basic research to determine the morpho-molecular interactions of plant tissues with EcoRI DNA restriction enzyme, it was demonstrated that this protein is capable of entering the sunflower and maize leaf cells using a plant tissue-abrading material and cleaving the genomic DNA at specific sites. This was inferred from the analysis of morphological patterns of EcoRI-treated leaf areas as well as using some molecular tests, including the cleavage pattern analysis of genomic DNA isolated from treated locations followed by ligation of cleaved fragments into EcoRI site of a DNA cloning vector system. The overall results indicated that the specific restriction of genomic DNA may happen following the entrance of EcoRI protein most likely into the nucleus of plant cells.

Keywords: Cleavage, EcoRI, Entrance, Interaction, Plant cell, Restriction enzyme

The entrance of bioactive proteins needed to express function in plant cells usually is limiting and sometimes labour-consuming step for basic research. Traditionally, this has been accomplished indirectly by transfecting transcriptionally active DNA into living cells where the gene is expressed and the protein is made by cellular machinery. Progress has been made in trying to deliver proteins into cells, thereby bypassing the DNA transfection step. The most popular means to deliver proteins into plant or animal cells include microinjection, electroporation, construction of viral fusion proteins, and use of cationic lipids 1-7. Plant cells, while superficially similar to animal cells in basic construction, have one main and fundamental difference with animal cells, which profoundly affects the permeability of these cells to exogenous larger molecules such as proteins. Due to the possession of thick, rigid and cellulose-based cell walls, in addition to impermeability of cellular membranes, the protein transduction across such barriers has become more restricted than animal system. A simple mechanism for the entry of the proteins into plant cells appears to be passive carriage through the breaches in the cell wall and cell membrane. Cell surface abrading materials have long been used as transfection agents to introduce plant viral/microbial proteins into plant tissues 8-11. This has simplified many experimental procedures related to protein action in plant system.

Type II DNA restriction endonucleases are generally bacterial proteineous enzymes recognizing specific target nucleotide sequence (referred as recognition site) in double-stranded DNA, that cause the breakage of internal bonds between specific nucleotides within these targets. They are a part of a bacterial restriction-modification (R-M) system, protecting the cell against foreign DNA 12,13. A number of R–M systems have been cloned, sequenced and over-expressed primarily in heterologous systems because of their application potential and commercial benefits 14-18. The EcoRI restriction endonuclease is a type II restriction enzyme that specifically recognizes and cleaves a well defined hexanucleotide palindrome (GAATTC). It is composed of a single polypeptide containing 277 amino acids and having molecular weight of 29 kDa that has been originally purified from a clinical strain of Escherichia coli RY13 19-21. EcoRI protein enters the yeast nucleus and cleaves its DNA at specific recognition sites when it is expressed as a foreign gene in yeast cells 22. Further, EcoRI protein interacts with native chromatin inside the
yeast nucleus and cleaves definition sites. Since, this protein does not contain specific signal peptide to direct its transport to the yeast nucleus, therefore it seemed that EcoRI protein may enter the nucleus through the nuclear pores\textsuperscript{22}.

Despite this information, there are no reports regarding the interactions of such bacterial restriction enzymes with higher eukaryotic cells. Therefore, the morpho-molecular responses of the leaf tissues of sunflower and maize plants to purified EcoRI restriction enzyme has been studied and it is demonstrated that this protein can enter the plant cells and restrict genomic DNA by the help of a plant tissue-abrading material.

Materials and Methods

Materials—The seeds of Helianthus annuus L and Zea mays L. plants and bacterial strain DH5$\alpha$ for plasmid transformation were obtained from Dr. B. Baghban (Department of Plant Breeding and Biotechnology, University of Tabriz, Tabriz). The pGEM-T easy vector system for cloning of EcoRI restricted fragments was purchased from Promega (Cat. No. A1360). Purified EcoRI restriction enzyme supplied in storage buffer containing [10 mM potassium phosphate (pH 7.4 at 25°C), 300 mM NaCl, 1 mM EDTA, 7 mM 2-mercaptoethanol, 0.2 mg/ml BSA, 0.15% Triton X-100 and 50% glycerol] was provided by Cinnagen Company (RE7707C, Lot. 00011581). AxyPrep Multisource Genomic DNA Miniprep Kit (Cat. No. AP-MN-MS-GDNA-50; Axygen Biosciences, USA) was used for plant DNA isolation and purification. Other chemicals used in this research work were of molecular biology grades.

Leaf treatments—The seeds of sunflower and maize plants were germinated and grown in small pots under natural day and night regimes without any supplementary conditions at laboratory temperature (28°-30°C). All the plants at fourth leaf stage were chosen for experimental assay. Treatments carried out locally on the fourth leaf of each test plant were as follows: (i) group I: the leaves treated with “1x EcoRI reaction buffer R” This buffer composed of 10 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 100 mM KCl and 0.1 mg/ml BSA, (ii) group II: the leaves treated with “EcoRI storage buffer” at concentrations of 1x, 0.1x, 0.01x and 0.001x. The composition of this buffer has been given under materials above, (iii) group III: the leaves treated with “1U EcoRI enzyme in 1x reaction buffer R”. This mixture was prepared by removing storage buffer through ethanol precipitation of enzyme EcoRI and the subsequent resuspending in 1x reaction buffer R (this method was described by Dr. M. Lebendiker, Protein Purification Faculty, Wolfson Centre for Applied Structural Biology, Hebrew University of Jerusalem). The activity of ER was tested and recovered by 60% in vitro,\textsuperscript{23} (iv) group IV: the leaves treated with “1U EcoRI enzyme in carborundum dusted reaction buffer R” and (v) group V: the leaves treated with “previously heated (at 80°C for 2 h) EcoRI enzyme in carborundum-dusted reaction buffer”. The leaves were treated locally and for every spot 10 $\mu$l of each sample was applied. Seven locations were treated on each leaf and three leaves were considered as replicates to realize each assay. Overall “7 × 3” locations were taken for each treatment. The treated areas were observed for morphological appearance and photographed. The scores of morphological changes were rated using indexes as shown in the Fig.1.

Genomic DNA extraction and analysis—The treated areas of each leaf were cut gently, weighted to 0.1 g and fine powdered using liquid $N_2$. The powder was homogenized in DNA extraction buffer provided by AxyPrep Multisource Genomic DNA Miniprep Kit, for purifying genomic DNA using silica gel columns. All steps of isolation and purification including phase-partition, filtration, binding, washing and elution were followed according to the plant tissue spin protocol of the kit. Isolated genomic DNA solution (5 $\mu$l) from each location was electrophoresed on 1% agarose gel using TBE-running buffer and photographed.

Cloning of restricted fragments—To clone the EcoRI restricted fragments into EcoRI site of pGEM-T easy vector system, this plasmid DNA was isolated from a blue colony of DH5$\alpha$ strain of Ecoli using alkaline lysis method\textsuperscript{24}. The isolated plasmid was restricted with EcoRI enzyme and used for ligating of DNA isolated from treated locations, using T$_4$ DNA ligase as described in

![Fig. 1—Indexing of morphological changes. The scores of morphological changes appeared in the treated locations were indexed as follows: (1) green area, (2) appearance of death spots, (3) growing death area, (4) completely death location, and (5) falling down of the completely death area.]
protocol. Five ligation reactions were separately carried out for DNA samples isolated from different treated areas (see leaf treatments). A control ligation reaction was also considered for the EcoRI-digested DNA sample, named ED, isolated from non-treated green areas. The ligation reactions were separately followed by bacterial transformation into E. coli strain DH5α and plated in ampicilnine, X-gal and IPTG containing media. The plates were first observed for the presence of transformed bacteria and then screened for the recombinants. The recombinant colonies were analyzed for the presence of inserts in EcoRI site(s) of their plasmids by EcoRI-specific restriction analysis on 1% agarose gel.

Results and Discussion
The morpho-molecular interactions of EcoRI DNA restriction enzyme with sunflower and maize leaf tissues are presented in this communication. Since the optimal temperature for EcoRI in vitro activity ranges between 25°-37°C, the plants were grown in laboratory conditions at temperature 28°-30°C (Fig. 2a) and used for treatment assay at fourth leaf stage. Time-course scoring of morphological changes in the treated areas showed that all the locations treated with EcoRI storage buffer treatment group II and those with EcoRI enzyme in carborundum-dusted reaction buffer treatment group IV were changed to similar necrotic-type death areas on the leaves of both plants. Where as, the locations treated with reaction buffer treatment group I and those with EcoRI enzyme in reaction buffer treatment group III and heated samples treatment group V did not result in death responses on the leaves of both plants (Fig.2b-j). The scores of morphological responses of
all treated areas were rated by indexing from 1 to 5, as described under Materials and Methods. Comparison of the indexes between all treatments showed significant differences among them. In case of treatments group II (storage buffer concentration ≥ 0.1x) and group IV, it increased from 1 to 4 in a month and finally reached to 5 in both experimental plants. However, in treatments group I, III and V, it was found that the final morphological score index remained at 1 and did not increase (Table 1). Similarly, the analysis of morphological indexing for treatment group II at different concentrations showed that the score was same as for group IV, at the concentrations of ≥ 0.1x, but it did not increase more than 2 in 0.01x and did not reach 1 at concentration of 0.001x (Table 2).

Interestingly, the results of morphological analysis between treatments group I, III and IV indicated that EcoRI protein could enter the plant cells and produce the death phenomenon. The results also demonstrated that this protein itself was not able to overcome the plant cell wall and membrane impediments and enter the cells, without the help of carborundum powder, a material affecting the permeability of the cell walls and membranes. In addition, comparison of morphological responses between treatments group IV and V strongly suggested the role of EcoRI enzyme in creation of death phenomenon in treated areas. But, how the EcoRI protein entered the nucleus of test plant cells remains to be investigated.

The morphological experiments were followed by two molecular tests designed to reliably predict the results. The first experiment was carried out by studying the genomic DNA patterns of different treated locations in both test plants. Genomic DNAs were isolated from different treated locations (treatments group II, III and IV) and were analyzed on 1% agarose gel, along with two control samples including DNA isolated from non-treated areas and EcoRI-digested DNA isolated from non-treated green areas. The electrophoresis result showed that DNA patterns of all locations were different from each other. It depicted the cleavage of genomic DNA in locations treated with storage buffer and EcoRI enzyme in carborundum-dusted reaction buffer as compared to intact DNA isolated from non-treated locations as well as locations treated with EcoRI enzyme in reaction buffer (Fig. 3). But, the cleavage pattern of DNA from locations of treatment group IV interestingly differed from that of treatment group II, showing most similarity to EcoRI-digested control DNA. This experiment enabled us to predict that EcoRI protein most likely enters the nucleus of plant cells to degrade the genomic DNA. The probability of this event is by nature higher, when the EcoRI protein enters the nucleus of cells. This is consistent with the reports of early investigations done on the entrance of EcoRI enzyme into yeast nucleus22. The obvious

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The scores of morphological changes were rated using indexes shown in Fig. 1.

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The scores of morphological changes were rated using indexes shown in Fig. 1.

Fig. 3—(a): Maize (1: DNA isolated from non-treated green areas; M: EcoRI-HindIII double digested lambda DNA marker; 2: DNA isolated from locations treated with EcoRI enzyme in 1x reaction buffer; 3: DNA isolated from locations treated with carborundum-dusted EcoRI; 4: EcoRI-digested DNA isolated from non-treated green areas; 5: DNA isolated from locations treated with EcoRI storage buffer). (b): sunflower (1: DNA isolated from locations treated with EcoRI storage buffer; 2: DNA isolated from locations treated with carborundum-dusted EcoRI; 3: EcoRI-digested DNA isolated from non-treated green areas; 4: DNA isolated from locations treated with EcoRI enzyme in 1x reaction buffer; M: marker DNA; 5: DNA isolated from non-treated green areas).
difference between DNA cleavage patterns of treatments group II and group IV was interesting and underscored that degradation of genomic DNA in storage buffer-treated areas is most probably related to the activation of deoxy-ribonuclease enzymes (DNases) during cell death process.

The second experiment was also carried out, in order to further demonstrate that the cleaved DNA fragments from locations treated with EcoRI enzyme in carborundum-dusted reaction buffer were reliably EcoRI-digested. For this purpose, five separate ligation reaction tests were considered using DNA samples of different treatments and control sample (EcoRI-digested) in both test plants. Ligation reaction were followed by bacterial transformation and screening for the presence of recombinants. Analysis of transformed bacteria on five different test plates showed that out of 14, 15, 15 and 15 transformants in the plates belonging to treatments group I, II, III and V no recombinant colonies were observed in sunflower plant. But, the presence of recombinant bacteria in the plates of treatment group IV and control sample were detected 4 out of 13 and 6 out of 17 respectively (Fig. 4). In maize, 4 out of 17 clones in the plates related to treatment group IV and 4 out of 13 clones in the plates of control sample were found to be recombinants (Fig. 4). Data presented in the figure are related to the means of two replicates. The recombinants were not only detected by white-blue screening but also further confirmed by EcoRI-specific restriction analysis of their transformed plasmids. Eight recombinant colonies were randomly picked up from the plates of treatment group IV for plasmid isolation and restriction analysis. The presence of inserts in the plasmids has been shown in Fig. 4. According to this experiment, it is very likely that the EcoRI protein acts on genomic DNA after entering the nucleus of the cells and causes cell death phenomenon. Since, degraded DNA fragments isolated from locations treated with storage buffer were not clonable, it can be concluded that these fragments were most likely cleaved by deoxy-ribonuclease enzymes activated during cell death phenomenon induced by EcoRI storage buffer.

Conclusions

Based on the morphological analysis, EcoRI enzyme may enter the plant cells by the help of caborundum powder and then proceed the death phenomenon in the treated areas. As a result of molecular detections, EcoRI protein definitely enter the plant cells and most probably to the nucleus to cleave the cellular DNA at specific sites.

Acknowledgement

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References