SHORT COMMUNICATION

A simple agarose gel-based method to detect homology in DNA molecules using denaturation/renaturation approach

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A simple agarose gel-based method for evaluating the homology between two DNA molecules has been developed. This procedure involves a simple *in vitro* assay where gene-targeting plasmid DNA vector and PCR amplified genomic fragment of the desired species are first denatured by heat treatment and later mixed in a solution to achieve gradual renaturation. The renatured DNA molecules are then resolved in an agarose gel to evaluate the degree of homology between desired DNA molecules.

Keywords: chloroplast; gene targeting; DNA homology; agarose gel; denaturation; renaturation.

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Introduction

High expression of foreign genes has been achieved by integrating genes in chloroplast genomes of plants by two homologous recombination events [1, 2]. The plasmid DNA vectors have been constructed with foreign genes, flanked by locus-specific chloroplast DNA sequences, in order to insert the foreign genes at predetermined loci in the plant plastid genome [3, 4]. The gene-targeting sequences differ from species to species. Therefore, vectors constructed to target one plant species either do not work for another species or exhibit low transformation events. To test whether a plasmid DNA vector constructed to target one plant species can be used for another species, either the sequence of the targeting fragment should be available for comparison or a membrane hybridization-based assay has to be performed to evaluate the DNA homology. Both of these strategies are cumbersome and require sophisticated instrumentation, skilled work force, and high cost. Kinetics of DNA renaturation has been used in prokaryotes and eukaryotes to demonstrate genetic relationship between different organisms [5, 6]. These studies utilize UV absorption assays, which complicate the analysis, especially when studying a mixture of cloned smaller DNA molecules. The purpose of this study was to develop a simple and cost-effective agarose gel-based method for detecting homology among DNA molecules using denaturation/renaturation approach.

Materials and methods

A 2.1 kb chloroplast genomic DNA fragment flanking the 16S rRNA and tRNA genes of

Nicotiania tobacum was amplified using the forward primer 5'-CCTCCGTGGAAAGGATGATTCA CGG-3' and the reverse primer 5'-CAGTACCTC GACGTGACATGAGCGTG-3'. The PCR reaction was carried out in a final volume of 50 µl containing 100 µM each of the four dNTPs, 5 pM of each primer, 20 ng of genomic DNA, and 0.3 units of Taq DNA polymerase (Bangalore Genei Pvt. Ltd., Bangalore, Karnataka, India). Each reaction tube was loaded with 20 µl of mineral oil. The PCR amplification was performed in a Robocycler (Stratagene/Agilent, Santa Clara, CA, USA) which was programmed for 1 cycle of 5 min at 95°C followed by 37 cycles of 1 min at 94°C, 1 min at 58°C, and 2 min at 72°C. The last extension cycle was programmed at 72°C for 7 min. The PCR product was tested in 1% agarose gel for a single band of expected size prior to its purification. Subsequently, the PCR product was purified using QIAquick PCR purification kit (Qiagen, Germantown, MD, USA). An equal amount (330 ng) of the PCR product was mixed in 10 µl reaction volume with 9.3 kb plasmid DNA vector pMS65LBgus containing two foreign genes (*aad*A and *gus*A) flanked by approximately 1.0 kb homologous (to PCR product) DNA sequence of tobacco chloroplast genome on each side [4, 7]. Next, 2.0 µl of 10× PCR buffer (100 mM Tris (pH 9.0), 500 mM KCL, 15 mM $MgCl_2$ and 0.1 % gelatin) was added to this sample mix, which was subsequently diluted with water to a final volume of 20 µl in a PCR tube. Finally, after overlaying with 10 µl of mineral oil, the PCR reaction mix was heated to 95°C for 10 min to denature the double-stranded molecules, followed by incubation at 55°C for 45 min to slowly renature the molecules in a PCR machine (Robocycler). The specific controls only included either DNA vector or PCR product alone. Each of these two controls were heated similarly for 10 min at 95°C to denature and were quickly chilled on ice to prevent renaturation. At the end of the incubation, 3.0 µl of the gel loading dye was added and the samples were electrophorized in a 0.8% agarose gel using 0.5x TBE (89 mM Tris borate, 2 mM EDTA, pH 8.3) buffer at 70 Volt for an hour. The gel was stained with ethidium bromide and visualized under UV light. Subsequently, the gel was processed for Southern blotting using a DIG-labeling kit (Boehringer Mannheim, Mannheim, Baden-Wurttemberg, Germany) as described earlier [8]. The 2.1 kb PCR product was used as a DNA probe for hybridization.

Results and discussion

DNA moves randomly in a solution, and single-stranded DNA molecules encounter one another by chance. If their sequences are complementary to each other, they come together to form a duplex (a phenomenon called renaturation), which later extends like a zipper. In this study, we observed that when heat denatured single-stranded plasmid DNA vector and PCR product are mixed and slowly renatured at 55°C for 45 min, a band of about 7.0 kb (arrow marked in figure 1b, lane 3) was seen, in addition to 9.3 kb and 2.1 kb double stranded and single stranded bands. This band is putatively the hybrid molecule between the single stranded (ss) plasmid DNA and ssPCR product, as it did not appear in control lanes 1, 2, 4, and 5. We observed that this hybrid band resolved into two separate bands when the gel was run for an extended time (data not shown). The plasmid vector DNA contains a 2.1 kb of tobacco chloroplast fragment, which is interrupted in the middle by insertion of a 3.0 kb foreign DNA comprising of *aad*A and *gus*A genes with their signal sequences [7]. Thus, the foreign genes are flanked on each side by approximately 1.0 kb of the chloroplast sequence, which is used to target the chloroplast specific genomic loci to produce a transgenic line through homologous recombination. With this design of mixing partners, there could be three theoretical possibilities for hybrid formation between plasmid DNA and the PCR product (Figure 2). Since B and C conformations in figure 2 are similar, except for the complementary sequence annealing sites, they would resolve only at one position in an agarose gel. However, conformation A in figure 2 makes a stem and loop between plasmid DNA vector and the PCR

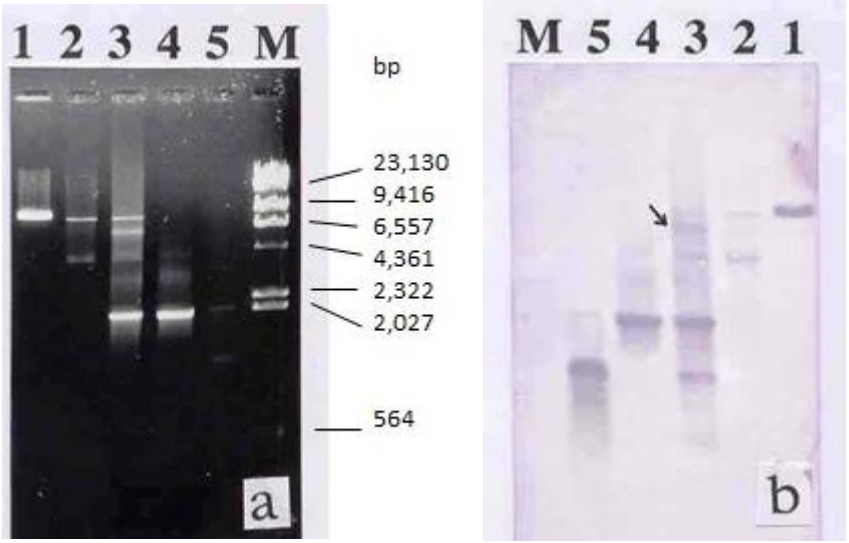

Figure 1. Analysis of DNA homology between a linearized plasmid and a chloroplast genome PCR product. Panels **a** and **b** show agarose gel and its corresponding Southern blot, respectively. **Lane 1 and 4**: double-stranded, *Not* I restriction enzyme digested plasmid DNA vector pMS65LBgus and 2.1 kb PCR product from chloroplast genomic DNA, respectively. **Lanes 2 and 5**: single-stranded (denatured) vector DNA and the PCR product, respectively. **Lane 3**: mixture of denatured single-stranded DNAs from both the plasmid vector and the PCR amplified product after their slow renaturation at 55°C. **M**: λ *Hind* III digested DNA molecular weight marker.

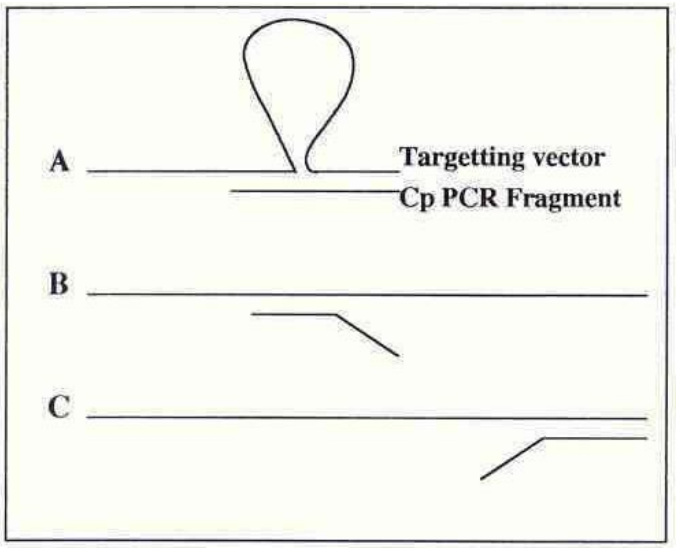

Figure 2. Diagrammatic representation of the possible renaturation routes of the chimeric plasmid DNA targeting vector and the chloroplast genome PCR amplified fragment. **A**, **B**, and **C** represent three different possible conformations as described in the text.

product due to interruption of homologous sequence by *aad*A and *gus*A gene and, therefore, can appear at a different position in agarose gels. This argument is supported by Monckton and Jeffrey's earlier study, where they have distinguished minisatellite isoallels in agarose gels [9].

To assess the degradation and see invisible bands in agarose gels, we performed Southern hybridization of agarose gel using a 2.1 kb chloroplast genome PCR fragment as a probe. As shown in the figure 1b, all the bands appearing in agarose gel (Figure 1a) except the marker lane were hybridized as expected, since 2.1 kb DNA

sequence is represented in all the bands. It should be noted that even the lowest ssDNA band of PCR product that is less visible in agarose gels (Figure 1a, lane 5) shows a clear signal in Southern blots, of course with significant degradation. Formamide has been reported to lower denaturation temperature substantially and thereby reducing DNA degradation. However, since formamide presence prevents renaturation of DNA strands, it was not used. Renaturation was observed at all the temperatures from 37°C to 60°C, but we chose 55°C stringent temperature to allow renaturation of only highly complementary molecules. Renaturation increased with an increase in incubation time, but beyond 2 hours, no substantial increase in renatured hybrid molecules was observed. It has been reported earlier that the rate of renaturation of PCR products is higher as compared to bigger molecules i.e., genomes [6, 10].

To test the general usefulness of the approach, we also investigated renaturation of plasmid DNA vector and the heterologous PCR products from two different plant species, *Camellia sinensis* and *Valeriana wallichii*. Our preliminary results show different degrees of hybrid band formation in agarose gel, indicating that these plants have corresponding homologous fragment (data not shown) and thus the designed same plasmid DNA vector could be used to target these species.

In conclusion, this simple agarose gel-based denaturation/renaturation approach can be used to test the homology of plasmid DNA targeting vectors with the corresponding sequences in various plant species and the possibility of using the same vectors for different species.

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