Protection of Megabase-Sized Chromosomal DNA from Breakage by DNase Activity in Plant Nuclei

A prerequisite for constructing a physical map that spans an entire plant chromosome is isolation of intact chromosome-sized DNA. Two methods are commonly used to isolate megabase (Mb)-sized DNA from plant materials, i.e., from protoplasts (1) or from nuclei (3,5). Isolated protoplasts or nuclei are embedded in low-melting-point agarose to form either plugs or microbeads. DNA molecules are isolated and manipulated in situ to protect them from physical shearing. Rice and Arabidopsis DNA molecules isolated by these methods have been used for the construction of yeast artificial chromosome (YAC) or bacterial artificial chromosome (BAC) libraries (3,5). However, most DNA molecules that migrated into gels were less than 2.5 Mb in size. Furthermore, these previous studies did not show whether or not the chromosomeal DNA that remained in the sample well was intact in the presence of a buffer that contained magnesium ions but without added restriction enzymes.

We have used both the protoplast method (1) and the nuclei method (3,5) in attempts to isolate intact chromosomeal DNA molecules from Arabidopsis, rice, corn and tomato plants, but we found large amounts of broken DNAs, presumably due to the presence of endogenous DNase activity. To find conditions by which this DNase activity can be inhibited while preserving the activity of several common restriction enzymes used in the manipulation of chromosome-sized DNA, we tested several potential DNase inhibitors and different combinations of two inhibitors. We found that the combination of 160 mM L-lysine HCl plus 4 mM EGTA fulfills both requirements.

Figure 1 shows the result of a Southern blot hybridization analysis of Arabidopsis chromosomal DNA embedded in agarose plugs after pulsed-field gel electrophoresis (PFGE) (4) using a CHEF® Mapper System (Bio-Rad, Hercules, CA, USA). Arabidopsis nuclei were isolated, embedded and treated according to a commonly used method (5). Briefly, our nuclei isolation method was based on the published procedures (3,5) but modified as follows: 20 g of leaves were ground into fine powder in liquid nitrogen, which was transferred into ice-cold wash buffer [homogenization buffer (HB; 10 mM Tris-HCl, 80 mM KCl, 10 mM EDTA, 1 mM spermidine, pH 9.4, 0.5 M sucrose) plus 0.5% Triton® X-100 and 0.15% β-mercaptoethanol], mixed well and filtered through cheesecloth and Miracloth (Calbiochem-Novabiochem, La Jolla, CA, USA) and centrifuged at 1800×g for 25 min. After washing in wash buffer, the pellet was resuspended in 400 µL of HB and mixed with 450 µL of 1% low-melting-point agarose in HB at 42°C. The mixture was made into 10 plugs (each containing 85 µL). The plugs were incubated in lysis buffer (0.5 M EDTA, pH 9.0, 1% sodium lauryl sarcosine, 0.1 mg/mL proteinase K) at 55°C for 24 h, followed by treatment with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) plus 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for 1 h, three times. The plugs were finally kept in 0.5 M EDTA, pH 8.0, at 4°C. PFGE was carried out using 0.6% SeaKem® Gold Agarose Gel (FMC BioProducts, Rockland, ME, USA) in 0.5× TBE (0.045 M Tris-boric acid, 0.001 M EDTA, pH 8.0) at 14°C. The pre-run PFGE conditions were 2.2 V/cm with switch time 1.5 to 22.5 min over 10 h. The resolution PFGE conditions were 1.5 V/cm with switch time 15 to 90 min over 48 h. The probe was 32P-labeled Arabidopsis ribosomal DNA (rDNA). The PFGE pre-run described was done to remove the chloroplast and mitochondrial DNA and any broken nuclear DNA. After the pre-run PFGE, the agarose plugs were removed from the wells, equilibrated in appropriate buffers and incubated at 37°C overnight under one of several different conditions, which include: Mg++-containing REAct4 buffer (Life Technologies, Gaithersburg, MD, USA) plus KpnI (Figure 1, lane 3), REAct 4 buffer only (lane 4) and TE only (lane 5). Following this incubation, each agarose plug was again subjected to PFGE but for a longer time for resolution of DNA molecules. The gel was stained with ethidium bromide, exposed to UV light, and the DNA fragments were transferred onto a Nytran® Membrane (Schleicher & Schuell, Keene, NH, USA) and hybridized with 32P-labeled Arabidopsis rDNA (rDNA, including intragenic
spacer, 18S, 5.8S and most of 26S rDNA). The result indicated that, in the presence of a 
Mg\(^{++}\)-containing buffer but without any added enzyme, a large proportion of the Arabidopsis DNA molecules were broken into fragments smaller than 2 Mb in size, as detected by Southern blot hybridization (Figure 1, lane 4). With the addition of Kpnl (lane 3), the result was similar. In contrast, if the Mg\(^{++}\)-containing REACT 4 buffer was replaced with the TE buffer, no broken DNA fragments were detected (Figure 1, lane 5). We interpret this to mean that when MgCl\(_2\)-containing buffer was added, endogenous DNase activity in the nuclei preparation cleaved chromosome-sized Arabidopsis DNA into smaller fragments.

We also tested for DNase activity in

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**Figure 2.** (A) Effect of L-lysine-HCl plus EGTA on DNase activity in Arabidopsis nuclei. The substrate used for testing DNase activity was \(\lambda\)DNA. Lane 1, 1-kb DNA ladder used as size marker; lane 2, \(\lambda\)DNA (0.55 \(\mu\)g) + I-PpoI buffer; lane 3, \(\lambda\)DNA + I-PpoI buffer + Arabidopsis nuclei; lane 4, \(\lambda\)DNA + I-PpoI buffer + Arabidopsis nuclei + 160 mM L-lysine-HCl + 4 mM EGTA; lane 5, \(\lambda\)DNA + I-PpoI buffer + Arabidopsis nuclei + 200 mM L-lysine-HCl + 4 mM EGTA; lane 6, \(\lambda\)DNA + I-PpoI buffer + Arabidopsis nuclei + 160 mM L-lysine-HCl + 6 mM EGTA. I-PpoI buffer consists of 2 mM MgCl\(_2\), 25 mM 2-(N-cyclohexylamino)-ethanesulfonic acid, 25 mM 3-(cyclohexylamino)-propanesulfonic acid, pH 10.0, 1 mM dithiothreitol (DTT). (B) Effect of DNase inhibitors (160 mM L-lysine-HCl plus 4 mM EGTA) on activity of I-PpoI and SmaI. Lane 1, 1-kb DNA ladder; lanes 2–4, incubation was at 37\(^\circ\)C for 2 h; lane 2, 1 \(\mu\)g of a plasmid containing an I-PpoI recognition sequence (I-PpoI plasmid); lane 3, I-PpoI plasmid + 50 U of I-PpoI; lane 4, I-PpoI plasmid + 50 U of I-PpoI + DNase inhibitors; lanes 5–8, 1 \(\mu\)g of pGHNC5 (a Smal site-containing plasmid) was incubated at 37\(^\circ\)C for 2 h; lane 5, pGHNC5; lane 6, pGHNC5 + 10 U of Smal; lane 7, pGHNC5 + 10 U of Smal + DNase inhibitors; lane 8, pGHNC5 + 20 U of Smal + DNase inhibitors. (C) Effect of DNase inhibitors (160 mM L-lysine-HCl plus 4 mM EGTA) on the activities of several other restriction enzymes (10 U of each used). Lane 1, 1-kb DNA ladder; lane 2, pGHNC5 control (containing the restriction sites for the following enzymes). Lanes 3–12 show pGHNC5 DNA that was digested with the following enzymes in the presence of DNase inhibitors except as indicated: lane 3, SalI; lane 4, ScaI; lane 5, PstI; lane 6, BamHI (no DNase inhibitors); lane 7, BamHI; lane 8, Hinfl (no DNase inhibitors); lane 9, HindIII; lane 10, NotI; lane 11, KpnI (no DNase inhibitors); lane 12, Kpnl.
rice, tomato and corn leaves and also found DNase activity in the nuclei preparation of these plants. In addition, we found that the older the leaf tissue, the higher the DNase activity. For example, the DNase activity was much higher in nuclei prepared from 28-day-old rice leaves than from 14-day-old rice leaves (data not shown).

Magnesium ions are required for activity of most DNases including restriction endonuclease, and EDTA is known to be an inhibitor of many DNases. However, EDTA also inhibits the activity of restriction endonucleases and an Intron-Encoded Endonuclease, I-Ppo (Promega, Madison, WI, USA). To find chemicals that inhibit the endogenous DNase activity but do not inhibit the activities of restriction endonucleases or I-Ppo used for DNA manipulation, we have tested several potentially useful DNase inhibitors, including L-lysine-HCl (2) and EGTA. We found that the combination of 160 mM L-lysine-HCl and 4 mM EGTA can fulfill both requirements described above. We showed that, in the presence of I-Ppo buffer, when λDNA was incubated in a 30-µL reaction volume with 5 µL Arabidopsis nuclei (equivalent to 9% of the amount in an agarose plug) at 37°C overnight, the λDNA was broken down into smaller fragments as shown by the smear after agarase gel electrophoresis (Figure 2A, lane 3). The breakage of λDNA was inhibited by adding 160 mM L-lysine-HCl (pH 6.0) plus 4 mM EGTA (pH 6.0) (lane 4). This result suggests that Ca++-dependent DNase is an important source of endogenous nuclease activity. Increasing the amount of L-lysine-HCl to 200 mM (lane 5) or EGTA to 6 mM (lane 6) did not change the result. On the other hand, either 160 mM L-lysine-HCl or 4 mM EGTA alone, or less than 160 mM of L-lysine-HCl or less than 4 mM of EGTA in this combination gave less inhibitory effect on the DNase activity (data not shown). When the I-Ppo buffer was replaced with REACT 4 buffer (containing 5 mM MgCl₂) or REACT 2 buffer (containing 10 mM MgCl₂), similar results were obtained.

Next, we tested the effect of 160 mM L-lysine-HCl plus 4 mM EGTA on the digestion of plasmid DNA by several restriction endonucleases and I-Ppo. We found that 160 mM L-lysine-HCl plus 4 mM EGTA did not inhibit I-Ppo activity (Figure 2B, lanes 3 and 4), because all of the superhelical form of the plasmid (represented by the lower band in lane 2) was converted to the slow-migrating linear form shown in lanes 3 and 4. Smal activity was slightly inhibited by 160 mM L-lysine-HCl plus 4 mM EGTA, because there was a small amount of superhelical form present (lower band in lane 7), as compared with lane 6 where all the superhelical DNA was converted to the slow-migrating linear form (upper band). Slight inhibition of Smal activity was partly overcome by doubling the amount of the enzyme (lane 8). We also found that 160 mM L-lysine-HCl plus 4 mM EGTA did not inhibit the activity of SalI, SacI, PstI (Figure 2C, lanes 3–5) and BamHI (lanes 6 and 7), but partly inhibited the activities of HindIII and NotI (lanes 8–10, the upper bands represent the nicked circular forms of the plasmid DNA). Interestingly, this DNase inhibitor combination did not inhibit the activity of KpnI from Life Technologies (Figure 2C, lanes 11 and 12), but strongly inhibited the activity of KpnI from Boehringer Mannheim (Indianapolis, IN, USA), and the inhibition was caused by 160 mM L-lysine-HCl.

In the next experiments, we tested the effect of DNase inhibitors (160 mM L-lysine-HCl plus 4 mM EGTA) on DNase activity using Arabidopsis chromosome-sized DNA. Results in lanes 1–4 of Figure 3 show that in the presence of the Mg²⁺-containing REACT 4 buffer alone, some Arabidopsis chromosomal DNA was broken (Figure 3, lane 3, lower band) as compared with lane 1, in which the plug was incubated in TE buffer. When DNase inhibitors were added (Figure 3, lanes 2 and 4), degradation of chromosomal-sized DNA was largely inhibited because much less broken DNA was detected by Southern blot hybridization. Lanes 5–8 show the results by using REACT 2 buffer and EcoRV. In the presence of REACT 2 buffer, which contains 10 mM MgCl₂, a large amount of broken DNA was produced that migrated away from the plug during PFGE (lane 5).
breakage of large DNA was significantly inhibited by DNase inhibitors (lane 6). Results in lane 7 indicate that the Arabidopsis chromosomal DNAs were largely digested by EcoRV. Lane 8 shows that not only did the DNase inhibitors not inhibit the activity of EcoRV, but they seemed to support a faster digestion of the chromosomal DNA by this enzyme.

In conclusion, we have shown that the combination of 160 mM L-lysine-HCl plus 4 mM EGTA results in strong inhibition of DNase activity present in the Arabidopsis nuclei preparations, but these two compounds did not inhibit the activity of Ppol and the restriction enzymes EcoRV, Sall, SacI, BamHI, PstI and KpnI. However, the activities of Smal, HindIII and NotI were slightly inhibited by these DNase inhibitors. We also tested DNase activity in nuclei isolated from two monocots (rice and corn) and another dicot tomato. The results were similar to those found in Arabidopsis nuclei in that the DNase activity was inhibited by 160 mM L-lysine-HCl plus 4 mM EGTA. Thus, we believe that combining these two compounds can play a protective role in the isolation and manipulation of large chromosomal-sized DNA molecules from a wide range of plant materials. In principle, protection of chromosomal DNAs from digestion by endogenous DNases allows one to isolate large megabase-sized DNAs for overlapping large contigs and facilitates the construction of YAC and BAC libraries for use in obtaining larger insert sizes more efficiently.

REFERENCES


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Detection of Plasmid-Encoded gusA Gene in GUS-Positive Escherichia coli

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The β-glucuronidase gene (gusA) of Escherichia coli has been developed as an efficient and single most important reporter gene system for the analysis of gene expression in transformed plants (2). In the presence of β-glucuronide substrate, the expression of plasmid-encoded gusA gene can be measured as a blue color in the resultant colonies because of the lack of intrinsic activity of the gus gene in plants, fungi and in most bacteria. However, most of the laboratory strains of E. coli contain GUS activity encoded in their genome, which makes plasmid-encoded gusA gene detection relatively difficult. To express the plasmid-encoded gusA gene in E. coli, usually an E. coli host in which gusA locus is deleted from the chromosome is used (3,6). Unfortunately, not many such gusA deletion host strains are available to the scientific community. To our knowledge, only two such E. coli strains, SO200 (2) and