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TECHNICAL ADVANCE

Preparation of megabase-size DNA from plant nuclei

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Summary

A novel technique has been developed for the preparation of high molecular weight (HMW) DNA from plant nuclei. This technique involves physical homogenization of plant tissues, nuclei isolation, embedding of the nuclei in low-melting-point agarose microbeads or plugs, and DNA purification *in situ*. This technique is simple, rapid, and economical, and the majority of the DNA prepared is over 5.7 Mb in size. The genomic DNA content of the HMW DNA prepared by this technique is enriched by at least threefold and the chloroplast DNA content is reduced by over twofold relative to that prepared from plant protoplasts by existing methods. The DNA is readily digestible with different restriction enzymes and partial digestions of the DNA could be reproducibly performed. This method has been successfully used for the preparation of HMW DNA from a wide range of plant taxa, including grasses, legumes, vegetables, and trees. These results demonstrate that the DNA prepared by this technique is suitable for plant genome analysis by pulsed-field gel electrophoresis and for the construction of yeast and bacterial artificial chromosomes.

Introduction

Pulsed-field gel electrophoresis (PFGE, Schwarts and Cantor, 1984) and yeast and bacterial artificial chromosome cloning (Burke *et al.*, 1987; Shizuya *et al.*, 1992) techniques have allowed scientists to manipulate DNA in the megabase (Mb) size scale and efficiently perform large DNA fragment cloning (yeast artificial chromosome (YAC) cloning: Albertsen *et al.*, 1990; Burke *et al.*, 1987; Chartier *et al.*, 1992; Edwards *et al.*, 1992; Garza *et al.*, 1989; Grill and Somerville, 1991; Guzman and Ecker, 1988; Kleine *et al.*, 1993; Larin *et al.*, 1991; Libert *et al.*, 1993; Martin *et al.*, 1992; Ward and Jen, 1990; bacterial artificial chromosome (BAC) cloning: Shizuya *et al.*, 1992; Woo *et al.*, 1994; Zhang *et al.*, in preparation), chromosome walking (Putterill *et al.*, 1993; Rommens *et al.*, 1989; Zhang *et al.*, 1994), physical mapping (Ganal *et al.*, 1989; Wing *et al.*, 1994), and analysis of large genes or chromosomal regions (Cheung *et al.*, 1991; Ganal and Tanksley, 1989; Garza *et al.*, 1989). Because of the improved techniques for genome analysis and chromosome walking, it has become practical to physically bridge closely linked DNA markers on a genetic map (Ganal *et al.*, 1989; Wing *et al.*, 1994) and clone mutations mapped between specific markers (map-based cloning: Arondel *et al.*, 1992; Bent *et al.*, 1994; Giraudat *et al.*, 1992; Martin *et al.*, 1993; Rommens *et al.*, 1989).

An essential element for such investigations is the preparation of high molecular weight (HMW) DNA. Unlike conventional methods for DNA isolation, HMW DNA must be protected from physical shearing during preparation. In yeast and mammalian systems, HMW DNA is prepared by directly embedding whole cells in agarose plugs or microbeads (Overhauser and Radic, 1987; Schwartz and Cantor, 1984). Subsequent cell lysis, DNA purification, and manipulation are performed *in situ*.

In plant systems, however, cell walls make it much more difficult to prepare HMW DNA from plant tissues. To prepare HMW DNA from plant tissues, the cell walls must first be removed before the cells are embedded in agarose. Therefore, most of the widely used methods in plant systems for preparation of HMW DNA involve the isolation of protoplasts using cell wall hydrolyses (e.g. cellulase and pectinase) and subsequent embedding of the protoplasts in agarose (Cheung and Gale, 1990; van Daelen *et al.* 1989; Ganal and Tanksley, 1989; Honeycutt *et al.*, 1992; Wing *et al.*, 1993,1994). These methods are only successfully used for HMW DNA preparation of the plant species in which techniques have been developed for large-scale protoplast isolation. Since the types and amounts of cell wall hydrolyses and enzyme action conditions vary for different plant species, a method for protoplast isolation for one species often cannot be directly used for protoplast isolation for other species (e.g. for tomato HMW DNA preparation see Ganal and Tanksley (1989) and van Daelen *et al.* (1989); for wheat HMW DNA preparation see Cheung and Gale (1990)). In addition, protoplast isolation on a large scale is extremely costly and tedious. Furthermore, HMW DNA prepared from plant protoplasts contains a significant amount of chloroplast and mitochondria DNA (Martin *et al.*, 1992; Wing *et al.*, 1993; Woo *et al.*, 1994), which could potentially mislead chromosome walking studies using libraries constructed from such HMW DNA due to homology between some organellar and nuclear DNA sequences (Timmis and Scott, 1983).

Alternatives to protoplast isolation have been reported

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for HMW DNA preparation of wheat and rye (Guidet and Langridge, 1992). Guidet and Langridge (1992) directly embedded the crushed tissues of wheat and related species in agarose plugs. The HMW DNA prepared by this method was thus mixed with unlysed intact cells and tissue debris that may affect the access of restriction enzymes to DNA. Hatano *et al.* (1992) isolated nuclei from rice germ tissues and embedded the nuclei in agarose plugs. The quality of HMW DNA prepared by this method was greatly improved, however, it is very difficult to obtain a sufficient quantity of germ tissue from many species for HMW DNA preparation.

In this study, we have developed a novel method for preparing HMW DNA from plant nuclei. The principles of the method are to break plant cell walls physically, isolate nuclei, and embed the nuclei in either agarose plugs or microbeads. The isolation of nuclei in this procedure is general and simple, and the HMW DNA embedded in microbeads is as easy to manipulate as DNA in aqueous solution without significant shearing. This method has been tested in several divergent plant taxa, including grasses (wheat, sorghum, and maize), legumes (soybean and greenbean), vegetables (cauliflower, squash, eggplant, watermelon, and pepper), and trees (peach, walnut, and willow).

Results

In most of the widely used methods, plant HMW DNA is prepared from protoplasts (see the Introduction). In this study, a novel technique was developed for the preparation of HMW DNA from plant nuclei. Plant tissues were homogenized either by blending with a kitchen blender or by grinding in liquid nitrogen with a mortar and pestle; nuclei were isolated from the homogenate and embedded in low melting point (LMP) agarose plugs or microbeads, and HMW DNA was purified in the agarose.

The intactness of nuclei

To determine the intactness of nuclei prepared by the two homogenization methods, tomato leaves were homogenized by grinding in liquid nitrogen or blending with a kitchen blender. Nuclei were isolated from the homogenate, stained with DAPI, observed, scored, and photographed under an epi-fluorescence microscope. The nuclei prepared with the liquid nitrogen method are shown in Figure 1. Fifty-seven percent (114/200) of 200 nuclei prepared by the blending method and 95% (235/242) of 242 nuclei prepared by the liquid nitrogen method were observed to be intact in shape under the microscope. These results demonstrate that both homogenization methods could yield a suitable amount of intact nuclei for the preparation of HMW DNA.

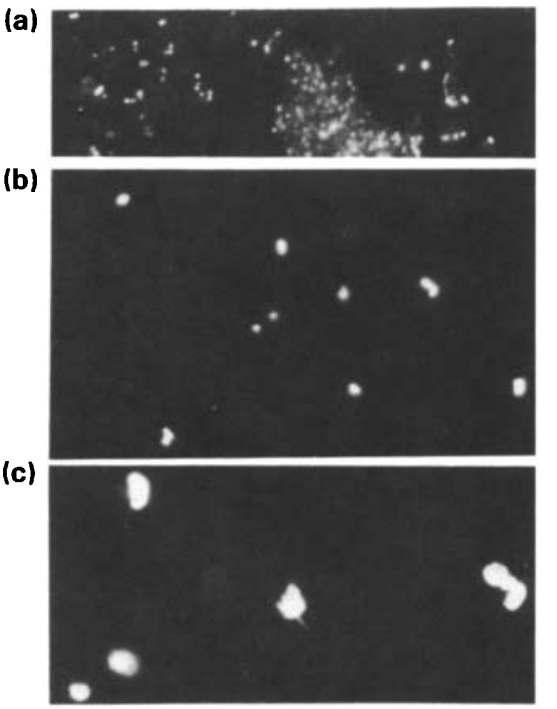

Figure 1. Tomato (Heinz 1706) nuclei isolated from homogenate prepared by the liquid nitrogen method, stained with DAPI, and photographed under 10 × (a), 16 × (b) and 63 × (c) phase-contrast objective lenses of a fluorescence microscope.

The quantity and quality of HMW DNA

HMW DNA was prepared from different plant species by our technique. To test the quantity and quality of the HMW DNA, HMW DNA prepared from tomato was evaluated in size, restriction enzyme digestibility, yield, and genomic and chloroplast DNA content.

Size. Uncut HMW DNA was analyzed by PFGE using *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* chromosomes as molecular weight markers. Two PFGE conditions were used to resolve DNA fragments ranging from 1500 to 5700 kb (Figure 2a) and from 240 to 1200 kb (Figure 2b). After electrophoresis, the gel was stained with ethidium bromide and destained in water overnight. While a light smear of DNA was observed in the region of the gel between 220 kb and 2000 kb, the majority of the DNA is still retained in the wells. This result indicated that the majority of DNA prepared by our technique is larger than 5.7 Mb in size.

To compare the two methods for homogenization of plant tissue, by blending fresh tissue with a kitchen blender and by grinding frozen tissue in liquid nitrogen with a mortar and pestle, the DNAs prepared by both methods were subjected to PFGE (Figure 2). No significant difference in sizes of the HMW DNAs prepared by the two methods was observed on the ethidium bromide-stained

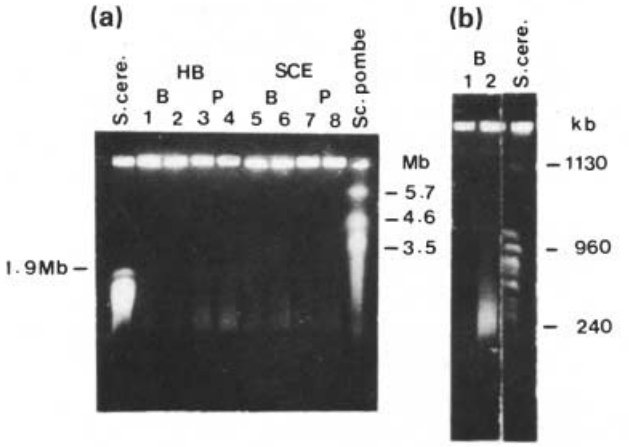

Figure 2. The size of tomato HMW DNA tested by PFGE.
HB indicates that 1 × HB buffer without β-mercaptoethanol was used to embed the nuclei into LMP agarose and SCE indicates that SCE buffer was used to embed the nuclei into LMP agarose. 'B' denotes agarose microbeads and 'P' denotes agarose plug. Odd numbers indicate that the DNA was isolated from the homogenate prepared by grinding in liquid nitrogen with a mortar and pestle and even numbers indicate that the DNA was isolated from the homogenate prepared by blending with a kitchen blender. 'S. cere.' indicates *S. cerevisiae.*
(a) The tomato HMW DNA prepared by this technique was subjected to PFGE using the CHEF Mapper (Bio-Rad, USA). The conditions of PFGE are 0.6 % chromosomal grade agarose gel, 0.5 × TAE buffer (1 × TAE: 40 mM Tris-acetate, 2 mM EDTA, pH 8.0), 120° angle, 30 min pulse time, and 1.5 V cm^{-1} voltage gradient, and the gel was run for 72 h at 14°C.
(b) The HMW DNA was subjected to PFGE in an 1% agarose gel and 0.5 × TBE (1×: 89 mM Tris-borate, 2 mM EDTA, pH 8.0) using the CHEF (CBS Scientific HEX CHEF 6000) set with the MJ Research Programmable Power Inverter for 48 h at 85 sec pulse, 150 V, and 13°C.

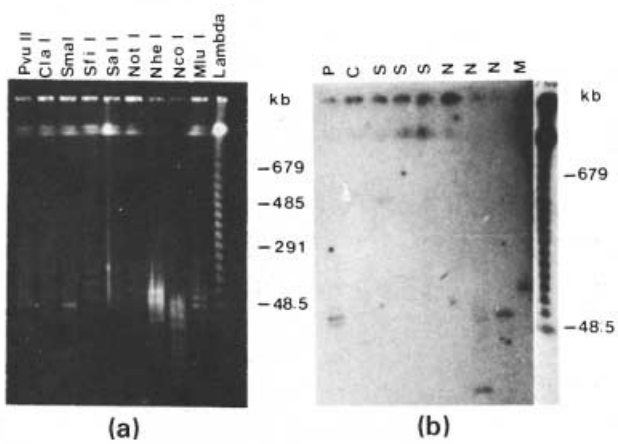

Figure 3. Digestion of tomato HMW DNA with different restriction enzymes for genome analysis.
(a) The ethidium bromide-stained pulsed-field gel of tomato HMW DNA digested with different restriction enzymes. (b) The autoradiograph of HMW DNA blotted from the gel shown in (a) and hybridized with a single-copy tomato DNA clone TG523B. The digested DNA was fractionated in an 1% agarose gel and 0.5 × TBE using the CHEF (CBS Scientific HEX CHEF 6000) set with the MJ Research Programmable Power Inverter for 40 h at 150 V and 11°C with A, 100 sec; B, −0.05 sec; C, 100 sec; D, −0.05 sec; E, 175 times; F, −0.01 sec; and G, −0.01 sec.

gel. On the same gel, the HMW DNAs embedded in agarose plugs and microbeads were also tested. The DNA embedded in agarose plugs had no significant difference in size from that embedded in agarose microbeads.

In our previous studies (Wing *et al.*, 1993, 1994), we embedded tomato protoplasts in agarose plugs or microbeads using SCE buffer (1 M sorbitol, 0.1 M sodium citrate, 60 mM EDTA, final pH 7.0 adjusted with concentrated KOH). To determine whether the buffers used for embedding nuclei in agarose influence the size of the HMW DNA, the SCE buffer was used to replace 1 x HB without β-mercaptoethanol to embed the nuclei in agarose plugs and microbeads. As shown in Figure 2a, no significant difference was observed between the DNAs embedded by using the SCE and 1 x HB buffers.

Digestion of HMW DNA for genome analysis by PFGE. To determine the quality of the HMW DNA in terms of restriction and physical mapping, tomato DNA embedded in microbeads was digested with nine restriction enzymes, including frequent cutters such as *Nco*I and *Nhe*I, a rare cutter such as *Not*I, and methylation-sensitive cutters such as *Sma*I and *Mlu*I, and subjected to PFGE. On the PFGE gel (Figure 3a), several restricted bands were observed on a smeared background for each restriction pattern except for *Not*I which recognizes an 8 bp nucleotide sequence. It was also noted that while the restriction fragments of the DNA generated with most of the enzymes were larger than 50 kb, those with *Nco*I as well as *Nhe*I were smaller than 50 kb. The DNAs retained in the wells restricted with *Nco*I and *Nhe*I were significantly fewer than those restricted with the other enzymes. These results confirm the results shown in Figure 2 and indicate that the DNA prepared by our method is sensitive to restriction enzymes.

To confirm the digestion of the HMW DNA on the PFGE gel (Figure 3a) and its potential uses for plant genome analysis by PFGE, the DNA was Southern blotted and hybridized with two tomato single-copy probes TG523B (a subclone of TG523 in Wing *et al.*, 1994) (Figure 3b) and RPD443 (Wing *et al.*, 1994). As shown in Figure 3b, single hybridized bands were generated when the DNA was cleaved with *Cla*I, *Sma*I, *Nco*I, and *Mlu*I, and multiple hybridized bands were generated when the DNA was cleaved with *Pvu*II and *Nhe*I. The sizes of the hybridized bands for different restriction enzymes ranged from about 10 kb in the *Nhe*I pattern to 600 kb in the *Sma*I pattern. In the restriction patterns of *Not*I, *Sal*I, and *Sfi*I, TG523B hybridized to the DNA fragments in the 1000 kb sized compression zones of the lanes. A similar result was obtained when the DNA on the blot was hybridized with RPD443 (not shown). These results agree with our previous results in which tomato HMW DNA prepared from protoplasts was Southern blotted and hybridized with TG523 and RPD443 (Wing *et al.*, 1993b) and indicate that the

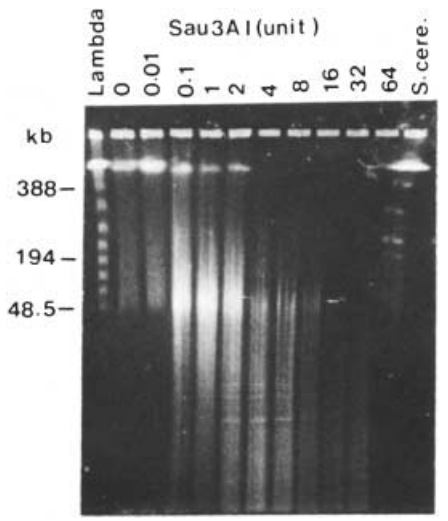

Figure 4. Partial digestion of tomato HMW DNA with *Sau*3AI. HMW DNA embedded in 50 µl of microbeads was digested with a series of amounts of *Sau*3AI for 30 min and subjected to PFGE in an 1% agarose gel and 0.5 × TBE using the CHEF-Mapper in DRIII (Bio-Rad, USA) for 22 h at 40 sec pulse, 150 V, and 13°C.

HMW DNA prepared by our technique is suitable for plant genome analysis by PFGE.

Partial digestion of HMW DNA for YAC and BAC cloning. Most of the YAC and BAC libraries available were constructed from partially digested HMW DNA (e.g. Albertsen *et al.*, 1990; Grill and Somerville, 1991; Martin *et al.*, 1992; Shizuya *et al.*, 1992; Woo *et al.*, 1994). Therefore, the ability to reproducibly partially digest HMW DNA is important to evaluate the potential uses of the DNA prepared by this technique for YAC and BAC cloning. HMW DNA embedded in microbeads was digested with a series of amounts of *Sau*3AI (Figure 4) or *Eco*RI (data not shown), respectively, and subjected to PFGE. As shown in Figure 4, the amount of DNA retained in the wells was gradually reduced when the amount of *Sau*3AI was increased from 0.01 to 64 units per 50 µl of microbeads. In the compression zone of the lanes, the amount of DNA digested with 0.01 units significantly increased relative to that in the undigested lane (0.0 unit). When 0.1 or more units of *Sau*3AI were used, the DNA in the compression zone was significantly reduced as the amount of enzyme was increased and completely disappeared when 4.0 units of the enzyme were used. A similar result was observed for the partial digestion of the tomato HMW DNA with *Eco*RI (data not shown).

Yield. To estimate the concentration of HMW DNA from nuclei embedded in LMP agarose microbeads, tomato HMW DNA prepared from nuclei (this study) and protoplasts (Wing *et al.*, 1993) was digested with *Hae*III, fractionated by standard agarose gel electrophoresis, and stained with the FluorKit™. The digested HMW DNA was compared with a series of dilutions of *Hae*III-digested conventional tomato DNA of known concentration. To load approximately equal amounts of HMW DNA prepared by both methods, preliminary data had shown that 10 µl of nuclei microbeads were approximately equivalent to 60 µl of protoplast microbeads in total amount of DNA. The intensities of stained DNA fluorescence images at the same positions in each lane were measured using a FluorImager. The concentrations of HMW DNA were estimated by comparing their fluorescence intensity values with those of the dilution series of conventional DNA. The approximate concentration of HMW DNA from nuclei was 1.85 µg total DNA in 10 µl of microbeads and that of HMW DNA from protoplasts was 2.05 µg total DNA in 60 µl of microbeads, equivalent to 0.34 µg DNA in 10 µl of microbeads.

Genomic and chloroplast DNA content. To determine the content of genomic and chloroplast DNA in the HMW DNA prepared by this technique relative to that in the HMW DNA prepared by the protoplast technique, an identical agarose gel was prepared from the *Hae*III-digested DNAs used for estimation of the HMW DNA concentrations (Figure 5a). The DNA was blotted and hybridized with a tomato genomic DNA probe TG523B (Wing *et al.*, 1994; Figure 5b) and a barley chloroplast DNA probe pBHP134 (Figure 5c), respectively. Both probes hybridized to a single band on the Southern blot (Figure 5b and c). The intensities of hybridized bands were measured with a Computing Densitometer. The hybridization intensity of HMW DNA prepared by the nuclei method was 3.28-fold higher than that prepared by the protoplast method when TG523B was used as a probe and 2.35-fold lower than that prepared by the protoplast method when pBHP134 was used as a probe. These results indicate that HMW DNA prepared by the nuclei method is 3.28 times more enriched in genomic DNA content and 2.35 times reduced in chloroplast DNA content than HMW DNA prepared by the protoplast method.

Universality of the method

Since most of the currently used techniques for plant HMW DNA preparation involve protoplast isolation using cell wall hydrolyses (e.g. Cheung and Gale, 1990; van Daelen *et al.*, 1989; Ganal and Tanksley, 1989; Honeycutt *et al.*, 1992), a method used for one plant species often can not be directly used for other plant species. In our procedure, plant cell walls are broken physically and nuclei are isolated from the homogenate. Therefore, it should be possible to apply this technique to different plant species. To determine the potential uses of this technique for HMW DNA prepara-

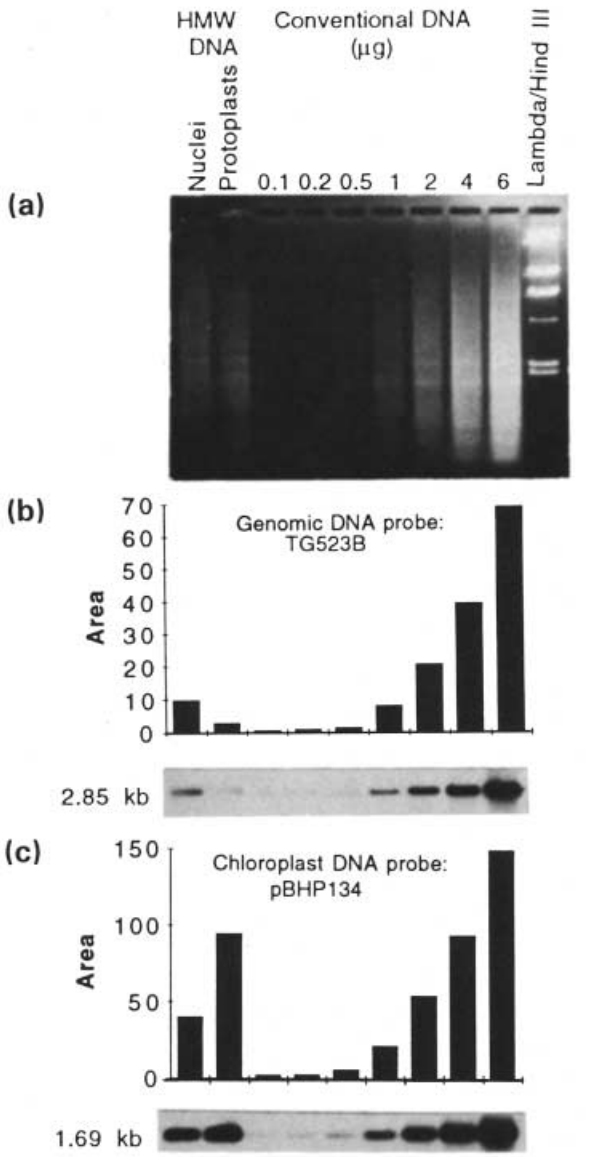

Figure 5. Content of genomic and chloroplast DNA in tomato HMW DNA, prepared by the present technique, relative to those in tomato HMW DNA prepared from protoplasts (Ganal and Tanksley, 1989; Wing *et al.*, 1993).
The HMW DNA was prepared by the nuclei and protoplast techniques, and the conventional tomato DNA was isolated as described by Bernatzky and Tanksley (1986). The DNA was digested with *Hae*III and fractionated on a 0.8% standard agarose gel. To make approximate equal amounts of the HMW DNA loaded per lane, 10 μl of nuclei HMW DNA microbeads and 60 μl of protoplast HMW DNA microbeads were loaded.
(a) The ethidium bromide-stained gel of the *Hae*III-digested HMW DNA prepared by both techniques (lanes 1–2 from left), and a series of amounts of the *Hae*III-digested DNA isolated by the conventional method (lanes 3–9 from left). Note the amount of HMW DNA relative to those of conventional DNA loaded in the gel.
(b and c) The hybridization and hybridization intensity of the DNA with a tomato genomic DNA clone TG523B (b) and a barley chloroplast DNA clone pBHP134 (c). The numbers in (a) indicate the amount (μg) of conventional DNA loaded per lane.

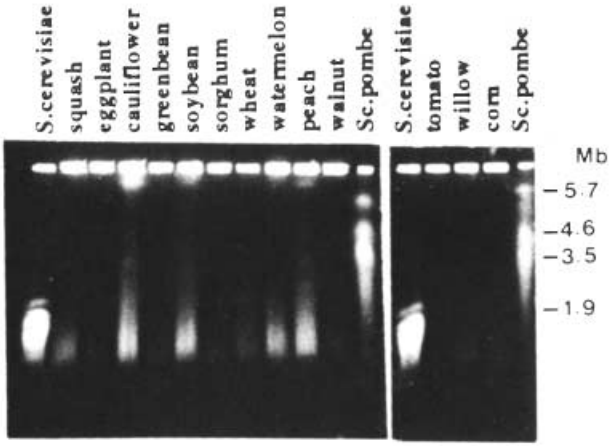

Figure 6. PFGE analysis of HMW DNA prepared from widely divergent plant taxa.
The tomato HMW DNA analyzed for size, restriction, yield, and genomic and chloroplast DNA content was used as a control. The scientific names of the species were indicated in Experimental procedures. The DNAs were subjected to PFGE as described in Figure 2(a).

tion from different plant species, HMW DNA was isolated from a wide range of plant taxa and analyzed by PFGE (Figure 6). The HMW DNA of these plant species had a similar quality to that of tomato as analysed above (see Figure 2). A majority of the DNA of each species was larger than 5.7 Mb in size.

To determine if the DNA could be used potentially for physical mapping and YAC and BAC cloning, HMW DNA from the different plant species was digested with *Eco*RI and subjected to PFGE. Over 95% of the DNA from these species was cleaved into fragments of smaller than 50 kb (Figure 7). These results further confirm the results shown in Figure 6 and demonstrate that the technique developed in this study is suitable for HMW DNA preparation from widely divergent plant taxa for genome analysis.

Discussion

The HMW DNA prepared by this method is suitable for genome analysis

For the analysis of plant genomes by PFGE and for YAC and BAC cloning, an important step is the preparation of HMW DNA. The intactness of nuclei prepared by this technique indicates that this technique can be used to prepare nuclei for HMW DNA isolation from plants (Figure 1). Pulsed-field gel electrophoresis of the DNA demonstrates that the majority of the DNA prepared by this technique is larger than 5.7 Mb in size. Digestion of tomato HMW DNA with different restriction enzymes and subsequent Southern blot hybridization show that the HMW DNA is sensitive to different restriction enzymes and suitable for physical mapping. Satisfactory partial digestions of

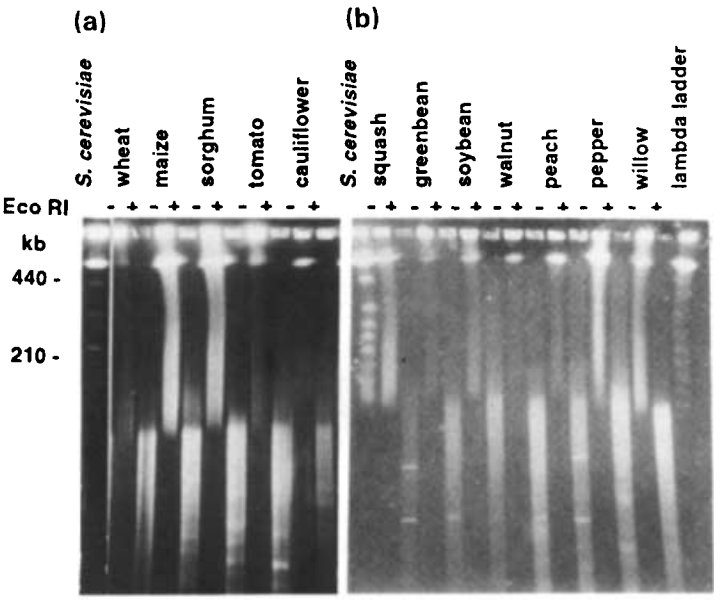

Figure 7. Digestibility of HMW DNA prepared from widely divergent plant species.
The DNA was digested with 40 units of *Eco*RI at 37°C for 3 h (a) and overnight (b), and subjected to PFGE with the CBS Scientific HEX CHEF 6000 set with the MJ Research Programmable Power Inverter under the following conditions: 1% agarose gel, 0.5 × TBE, 14°C, and 40 sec pulse time for 24 h. '+' DNA incubated with *Eco*RI; '−' DNA incubated without *Eco*RI.

the HMW DNA with *Sau*3AI and *Eco*RI were also obtained. *Sau*3AI is compatible with *Bam*HI and thus can be used for YAC and BAC cloning of large DNA segments into the *Bam*HI sites of the vectors pYAC41 (Grill and Somerville, 1991), pBAC108L (Shizuya *et al.*, 1992), and pBeloBAC II (Shizuya *et al.*, unpublished). *Eco*RI is often used for YAC cloning of large DNA segments into the *Eco*RI sites of the vectors pYAC4 (Burke *et al.*, 1987) and pJS97 and pJS98 (McCormick *et al.*, 1990). Therefore, the HMW DNA prepared by this technique is suitable for YAC and BAC cloning.

The nuclei technique is simple, economical, and yields HMW DNA that is less contaminated with chloroplast DNA

The current widely used methods for the preparation of HMW DNA involve the isolation of protoplasts using cell wall hydrolyses (e.g. Cheung and Gale, 1990; Ganal and Tanksley, 1989; Honeycutt *et al.*, 1992; van Daelen *et al.*, 1989; Wing *et al.*, 1993), which is extremely time-consuming and costly. In the method reported here, plant cell walls are broken physically instead of releasing protoplasts from the manually sliced plant leaves with cell wall hydrolyses. Therefore, our method is much more economical in terms of labor, time, and cost. While the intactness of nuclei prepared by the liquid nitrogen method was much higher than that prepared by the blending method, no significant difference in size was observed between the HMW DNA prepared by both the liquid nitrogen and blending methods. This indicates that either of these two homogenization methods can be used for plant nuclei isolation. Since it has been demonstrated that DNA embedded in agarose microbeads is more readily digestable than that embedded in agarose plugs in our previous study (Wing *et al.*, 1993), it is recommended that nuclei used for HMW DNA preparation are embedded in microbeads.

As described above, 1.85 μg of tomato HMW DNA could be embedded in 10 μl of agarose microbeads when HMW DNA was prepared by the nuclei technique. Since 2 ml microbeads were prepared from 20 g tomato fresh leaves, the yield of total HMW DNA by this technique was 18.5 μg per gram fresh leaf tissue. Additional investigations are needed to determine how much HMW DNA can be prepared per unit of tissue and how much HMW DNA can be embedded per unit of agarose microbeads.

Martin *et al.* (1992) and Wing *et al.* (1993) showed that 6–8% of the tomato YAC clones and Woo *et al.* (1994) showed that 14% of the sorghum BAC clones constructed from the protoplast DNA originated from chloroplast DNA. This means that more clones are needed to be generated and screened in order to have an equal chance of isolating a particular nuclear DNA clone. Additionally, the presence of YAC or BAC clones originated from chloroplast DNA in a YAC or BAC library can potentially mislead a chromosome walk because some nuclear DNA sequences are homologous to some organellar DNA sequences (Timmis and Scott, 1983). The enriched content of genomic DNA and the reduced content of chloroplast DNA in HMW DNA prepared by this new technique (Figure 5) suggest that a YAC or BAC library constructed from HMW DNA prepared by the nuclei technique will have a lower proportion of clones derived from chloroplast DNA (Zhang *et al.*, in preparation).

The method developed for HMW DNA preparation in this study is applicable to a wide range of plant taxa

While the current widely used methods for plant HMW DNA preparation involve the isolation of protoplasts, the conditions for isolating protoplasts from different plant species vary (from tomato, see Ganal and Tanksley, 1989 and van Daelen *et al.*, 1989; from wheat, see Cheung and Gale, 1990). This indicates that a method for protoplast isolation of one species often may not be used directly for protoplast isolation for other plant species. The technique reported here has been used successfully for HMW DNA preparation from a wide range of plant species. Furthermore, HMW DNAs prepared from tomato, rice, and wheat by this technique have been successfully used for the construction of two complete rice BAC libraries (Zhang *et al.*, in preparation), and partial tomato and wheat BAC libraries (Zhang and Wing, unpublished). We have also used this technique successfully with an additional buffer to prepare cotton HMW DNA (Zhao *et al.*, 1994). All these results demonstrate that the technique developed in this study is applicable to HMW DNA preparation from widely divergent plant taxa.

Experimental procedures

Plant materials

Plant leaves or whole plants of all species were collected from the field, except for wheat whose leaves were collected from plants grown in a greenhouse. The growth stages of plant species are: flowering greenbean (*Phaseolus vulgaris*), wheat (*Triticum aestivum*), and pepper (*Capsicum annuum*), fruiting eggplant (*Solanum melongena*), squash (*Cucurbita maxima*), watermelon (*Citrullus vulgaris*), soybean (*Glycine max*), and walnut (*Juglans regia*), ripening tomato (*Lycopersicon esculentum*) and maize (*Zea mays*) (the top part of the leaf was yellow and the bottom part was green, the green part of the leaf was collected), and postharvested cauliflower (*Brassica oleracea* ssp. *botrytis*, regrowth leaves), sorghum (*Sorghum bicolor*, regrowth shoots), and peach (*Prunus persica*). Willow (*Salix babylonica*) is an adult tree. The tissues were either frozen in liquid nitrogen and stored in a −70°C freezer or kept fresh on ice before use.

Reagents

(i) Homogenization buffer (HB) (10×) stock: 0.1 M trizma base, 0.8 M KCl, 0.1 M EDTA, 10 mM spermidine, 10 mM spermine, final pH 9.4–9.5 adjusted with NaOH. The stock is stored at 4°C.

(ii) HB (1×): 1 × HB plus 0.5 M sucrose. The resultant 1 × HB was stored at 4°C. Before use, β-mercaptoethanol was added to 0.15%.

(iii) HB (1×) plus 20% Triton X-100: Triton X-100 was mixed with 1 × HB without β-mercaptoethanol to 20%. The solution was stored at 4°C.

(iv) Wash buffer (1 × HB plus 0.5% Triton X-100): it was prepared by mixing 1 × HB without β-mercaptoethanol with Triton X-100 and stored at 4°C. Before use, β-mercaptoethanol was added to 0.15%.

(v) Lysis buffer: 0.5 M EDTA, pH 9.0–9.3, 1% sodium lauryl sarcosine, and 0.1 mg ml^{-1} proteinase K. The proteinase K powder was added just before use.

(vi) Enzymes and other chemicals: restriction enzymes were purchased from New England Biolabs (USA), Promaga (USA), and Boehringer (USA), and proteinase K was purchased from Boehringer (USA). Other chemicals were purchased from Sigma (USA).

Preparation of intact nuclei

For homogenization of the plant tissue, two methods were used.

For fresh tissue, about 20 g of the tissue were washed with tap water, and if necessary, cut into suitable pieces for homogenization with a kitchen blender (Osterizer 10 Speed Blender). The tissue was homogenized in 200 ml of ice-cold 1 × HB plus β-mercaptoethanol in the kitchen blender at speed 4 or 'puree' for about 30 sec. The homogenate was filtered into an ice-cold 250 ml centrifuge bottle through two layers of cheesecloth and one layer of miracloth by squeezing with gloved hands. Five milliliters of 1 × HB plus 20% Triton X-100 were added (the final concentration of Triton X-100 was 0.5%). The contents of the centrifuge bottle were mixed gently and incubated on ice for 20 min.

For frozen tissue, about 20 g of the tissue were ground into powder in liquid nitrogen with a mortar and pestle. The powder was immediately transferred into an ice-cold 500 ml beaker containing 200 ml ice-cold 1 x HB plus β-mercaptoethanol and 0.5% Triton X-100. The contents were gently stirred with a magnetic stir bar for 10 min on ice and filtered into an ice-cold 250 ml centrifuge bottle as above.

The homogenate, prepared by either of the above two methods, was pelleted by centrifugation with a fixed-angle rotor at 1800 ***g*** at 4°C for 20 min. The supernatant fluid was discarded and approximately 1 ml of ice-cold wash buffer was added. The pellet was gently resuspended with the assistance of a small paint brush soaked in ice-cold wash buffer. Finally, an additional 30 ml of the ice-cold wash buffer were added. To remove the particulate matter remaining in the suspension, the resuspended nuclei were filtered into a 50 ml centrifuge tube through two layers of miracloth by gravity. The content was centrifuged at 57 ***g*** at 4°C for 2 min to remove intact cells and tissue residues. The supernatant fluid was transferred into a fresh centrifuge tube and the nuclei were pelleted by centrifugation at 1800 ***g*** at 4°C for 15 min in a swinging bucket centrifuge. The pellet was washed one to three additional times by resuspension in wash buffer followed by centrifugation at 1800 ***g*** at 4°C for 15 min. After the final wash, the pelleted nuclei were resuspended in a small amount (about 1 ml) of 1 × HB without β-mercaptoethanol, counted with a hemocytometer under a phase-contrast microscope, brought to approximately 10^7-10^8 nuclei ml^{-1} with the addition of the 1 × HB without β-mercaptoethanol (we usually resuspended the nuclei prepared from 20 g of tissue in 1 ml of 1 × HB without β-mercaptoethanol), and stored on ice.

Embedding the nuclei in agarose plugs and microbeads

The nuclei were prewarmed to 42°C in a water bath (about 5 min) before being embedded in agarose. To embed the nuclei in agarose plugs, the nuclei were mixed with an equal volume of 1% low melting point (LMP) agarose (BRL, USA) in 1 × HB without β-mercaptoethanol using a Pipetman. The agarose was melted in

boiling water and kept at 42°C. The mixture was aliquoted into ice-cold plug molds on ice with the same Pipetman (100 µl plug^{-1}). When the agarose was completely solidified, the plugs were transferred to 5–10 volumes of lysis buffer.

To embed the nuclei in agarose microbeads, the procedure developed by Wing *et al.* (1993) was followed with a few modifications. The above prewarmed nuclei suspension was mixed with an equal volume of 1% LMP agarose in 1 × HB without β-mercaptoethanol kept in a 42°C water bath and poured into a prewarmed 500 ml flask. Twenty milliliters of prewarmed light mineral oil at 42°C were added. The contents of the flask were shaken vigorously for 2–3 sec and immediately poured into an ice-cold 500 ml beaker on ice. The beaker contained 150 ml ice-cold 1 × HB without β-mercaptoethanol which was vigorously being stirred with a magnetic stir bar. The solution was stirred for 10 min on ice and agarose microbeads formed. The beads were harvested by centrifugation at 900 ***g*** at 4°C for 15–20 min in a swinging bucket centrifuge. In the case that the microbeads were hardly pelleted, a centrifugation at higher force (up to 1800 g) could be performed to pellet the beads, which would not damage the beads or the HMW DNA embedded in the beads. The supernatant fluid was discarded and the pelleted microbeads were resuspended in 5–10 volumes of lysis buffer.

The plugs and the microbeads were incubated in the lysis buffer for 24 h at 50°C with gentle shaking. The plugs and the beads were washed once in 0.5 M EDTA, pH 9.0–9.3 for 1 h at 50°C, once in 0.05 M EDTA, pH 8.0 for 1 h on ice, and stored in 0.05 M EDTA, pH 8.0, at 4°C.

Observation of intact nuclei

Two microliters of 20 µg ml^{-1} DAPI (4′,6-diamidino-2-phenylindole) in 1 × HB buffer were mixed with 198 µl of tomato nuclei prepared as above in a 0.5 ml microcentrifuge tube in the dark. After the mixture was incubated on ice for 1–2 min, about 10 µl of the stained nuclei suspension were dropped on a glass slide, gently covered with a glass cover slip, and observed under an Olympus AH-2 epi-flourescence microscope equipped with phase contrast objective lenses. Photographs were taken with Fuji 1600 ISO 35 mm color film.

Digestion of DNA embedded in agarose microbeads

Before use, agarose microbeads containing HMW DNA were washed three times in 10–20 volumes of ice-cold TE (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, pH 8.0) plus 0.1 mM phenylmethyl sulfonyl fluoride (PMSF) and three times in 10–20 volumes of ice-cold TE on ice, 1 h each wash. The washed beads can be stored at 4°C for a month without significant degradation. Fifty microliters of the microbeads were pipetted into a sterile microcentrifuge tube with a cut-off pipet tip and equilibrated with 0.5 ml 1 × restriction enzyme buffer plus 2 mM spermidine. After the mixture was incubated on ice for 30 min, the supernatant fluid was replaced with 0.5 ml fresh 1 × restriction enzyme buffer plus 2 mM spermidine and the beads were incubated for an additional 30 min. Approximately 70% of the supernatant fluid was removed and a suitable amount of restriction enzyme was added. For partial digestions, a series of enzyme dilutions were added and for complete digestions, approximately 5 units of enzyme per microgram of DNA were used. Before digestion, the reaction mixture was incubated on ice for 30 min to allow the enzyme to access the DNA in the agarose beads and then transferred to the recommended temperature for enzyme activity. For partial digestions, the beads were incubated for 30 min and for complete digestions, the beads were incubated for at least 3 h. The reactions were stopped by adding 1/10 volume of 0.5 M EDTA, pH 8.0.

PFGE analysis and Southern blot hybridization

The digested HMW DNA in agarose microbeads was loaded into an agarose gel and the beads were sealed in position with the same agarose as used for the gel. Pulsed-field gel electrophoresis was performed as described in the figure legends. The DNA was stained in ethidium bromide, photographed, and nicked with 60 mJoules of UV light (254 nm) using the GS Gene Linker (Bio-Rad, USA). Blotting of the DNA on to Hybond-N+ membrane (Amersham, USA) was performed in 1.5 M NaCl, 0.4 M NaOH for 40 h. ^{32}P-labeled DNA probes were prepared according to Feinberg and Vogelstein (1984) and Southern blot hybridization was performed according to Bernatzky and Tanksley (1986).

Preparation of HMW DNA from tomato protoplasts

Tomato protoplasts and HMW DNA were prepared according to Ganal and Tanksley (1989) and Wing *et al.* (1993).

Estimation of HMW DNA yield and genomic and chloroplast DNA content in the HMW DNA

Conventional-size tomato DNA, used as a control to measure the yield of total HMW DNA prepared as above, was isolated according to Bernatzky and Tanksley (1986) and quantified on an agarose gel using lambda DNA as a control. To estimate the yield of HMW DNA, the HMW DNA prepared by the standard nuclei method as described above and the standard protoplast method (Wing *et al.*, 1993) and a series of amounts of the conventional DNA were digested with 40 units of *Hae*III at 37°C for 3 h. Approximately equal amounts of the digested HMW DNA and a series of amounts of the digested conventional DNA were fractionated on a 0.8% standard agarose gel and stained with the FluorKit^{TM} as described by the manufacturer (Molecular Dynamics, USA). The fluorescent image of the DNA in each lane of the gel was measured using the FluorImager with the Volume Integration of ImageQuaNT^{TM} 4.1 software (Molecular Dynamics, USA).

To estimate the content of genomic and chloroplast DNA in the HMW DNA, an identical agarose gel was prepared using the *Hae*III-digested HMW DNA and conventional DNA as above. After electrophoresis, the gel was stained with ethidium bromide and photographed. The DNA on the gel was blotted on to the Hybond-N+ Membrane (Amersham, USA) and hybridized with a tomato genomic DNA probe TG523B (Wing *et al.*, 1994) and a barley chloroplast DNA probe pBHP134 as previously described (Bernatzky and Tanksley, 1986). The intensities of hybridized bands on the autoradiograph of the Southern blot were measured using the Computing Densitometer with ImageQuant^{TM} 3.0 software (Molecular Dynamics, USA).

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