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Protocols

Isolation of Milligram Quantities of Nuclear DNA from Tomato *(Lycopersicon esculentum*), A Plant Containing High Levels of Polyphenolic Compounds

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Abstract: We have developed a protocol for isolating milligram quantities of highly purified DNA from tomato nuclei. The protocol utilizes fresh seedlings or leaves without freezing. Tissues are treated with ethyl ether, thoroughly washed, and placed in a buffer containing the nuclear-stabilizing agent 2-methyl-2,4-pentanediol. Nuclei are liberated from tomato cells by homogenization in a Waring blender. The interaction of nuclear DNA with oxidized polyphenols is inhibited by compounds that adsorb polyphenols or prevent oxidation reactions. Chloroplasts and mitochondria are preferentially eliminated with Triton X-100. Nuclei are concentrated using a Percoll gradient and lysed with SDS. DNA is subsequently purified by RNase and protease digestions and phenol/chloroform extractions. The isolated DNA is essentially free of polyphenols and other major contaminants based upon its lack of coloration, A_{260}/A_{280} ratio, digestability with restriction enzymes, melting profile, and reassociation properties.

Like many other plant species, tomato *(Lycopersicon esculentum*) tissues contain high levels of tannins and other polyphenolic compounds. When cells are disrupted, these cytoplasmic compounds can come into contact with nuclei and other organelles (Loomis, 1974). In their oxidized forms, polyphenols covalently bind to DNA giving it a brown color and making it useless for most research applications (*e.g*., Katterman and Shattuck, 1983; Guillemaut and Maréchal-Drouard, 1992; our observations). One method commonly used to avoid problems with polyphenols involves freezing tissue during or prior to homogenization (*e.g.*, Katterman and Shattuck, 1983; Leutwiler et al., 1984). For

many species, however, homogenization of frozen tissue results in isolation of a mixture of nuclear, chloroplast, and mitochondrial DNA. Contamination of nuclear DNA with organellar DNA complicates library construction and screening, DNA hybridization studies, and research on DNA renaturation.

In our research we needed large quantities of highly pure tomato nuclear DNA. After trying published protocols and failing repeatedly to obtain DNA that was not contaminated with polyphenolic compounds and/or organellar DNA, we developed a new protocol that yields milligram quantities of polyphenol-free nuclear DNA from seedlings or leaves. We find that this DNA is extremely pure and well suited for use in molecular biology studies.

Solutions Required

extraction medium[1] *:* 1.0 M 2-methyl-2,4-pentanediol, 10 mM PIPES-KOH, 10 mM $MgCl_2$, 2% PVP, 10 mM sodium metabisulfite, 5 mM 2-mercaptoethanol, 0.5% sodium diethyldithiocarbamate, pH 6.0.

nuclear buffer[2] *:* 0.5 M 2-methyl-2,4-pentanediol, 10 mM PIPES-KOH, 10 mM $MgCl_2$, 0.5% Triton X-100, 10 mM sodium metabisulfite, 5 mM 2-mercaptoethanol, pH 7.0.

Notes

1. This medium is partially based on media described in Watson and Thompson (1986) and Couch and Fritz (1990). PVP adsorbs polyphenols thereby preventing their interaction with DNA (Loomis, 1974). 2-mercaptoethanol, sodium metabisulfite, and sodium diethyldithiocarbamate inhibit oxidation of polyphenols.
2. This medium is partially based on medium described in Watson and Thompson (1986).

Protocol

- Collect 200-600 g of tomato seedlings from which seed coats have been removed or a comparable mass of young leaves.[1] Immediately submerge harvested seedlings/leaves in ice water.
- Remove seedlings/leaves from ice water, and place them in 1000 mL of ice cold ethyl ether for three min.[2] Remove the seedlings/leaves from ether, wash 4 times in 4°C TE buffer (pH 7.0), and place in 3000 mL of ice cold extraction medium.

- Homogenize the mixture in a commercial Waring blender (high speed for 30 s), and squeeze the homogenate through muslin. Take the filtrate, and refilter through 20 µm nylon mesh.
- Measure the volume of the filtrate (usually about 3250 mL), and add Triton X 100 to a final concentration of 0.5%.[3]
- Centrifuge the mixture in 250 mL centrifuge bottles at 1200 x *g* for 20 min. Decant supernatants, and resuspend each pellet in 0.3 mL nuclear buffer. Combine all nuclear suspensions. To verify the purity of the nuclear suspension, mix a small drop of the suspension with an equal volume of 1% aqueous methylene blue on a microscope slide. Add a coverglass and examine by phase-contrast and bright-field microscopy.[4]
- Mix 15 mL of Percoll with 15 mL of nuclear buffer. Have this mixture and place each half in a separate 30-mL Corex siliconized glass centrifuge tube.
- Divide the nuclear suspension into two parts and gently layer each half onto one of the two beds of 50% Percoll (v/v). Centrifuge both tubes in a swinging bucket rotor at 650 x *g* for 60 minutes (4°C), discard the supernatants, and resuspend each pellet in 1 mL of nuclear buffer.
- Transfer the nuclear suspensions to a single polypropylene centrifuge tube. Add 20% aqueous SDS (w/v) to a final concentration of 2% (w/v). Mix contents of the tube by gentle inversion to lyse the nuclei.[5] Heat the tube containing the nuclear lysate in a water bath at 60°C for 10 min, cool to room temperature, and add 5 M sodium perchlorate (20°C) to a final concentration of 1 M.
- Spin the lysate in a swinging bucket rotor at 400 x *g* for 20 min to pellet starch grains. Transfer the supernatant to a sterile polypropylene tube using a 1000 µL plastic pipet tip from which the bottom third has been cut off. Perform all subsequent transfers of DNA-containing solutions using such modified pipet tips to minimize shearing of the DNA.
- Perform a DNA extraction by mixing the nucleic acid solution with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1).[6] To minimize shearing of DNA, use a test tube rocker (18 cycles/min for 30 min) rather than a shaker to mix the organic and aqueous phases. Centrifuge the mixture at 3000 x *g* in a swinging bucket rotor for 10 min. Transfer the upper aqueous phase into a new sterile poly-propylene tube. Perform a second extraction as described above.
- Dialyze the aqueous phase (containing nucleic acids) into TE (pH 7.0) at 4°C.
- After dialysis, add RNase T1 and RNase A to give final concentrations of 50 units/mL and 50 µg/mL, respectively.[7] Incubate at 37°C for 60 min.
- Make the solution 150 µg/mL Proteinase K, and incubate at 37°C for 60 min.
- Perform two additional phenol/chloroform/isoamyl alcohol extractions followed by an extraction using only chloroform.[8]

- Transfer the aqueous phase to a small siliconized beaker, and add 3M sodium acetate (pH 5.2) to a final concentration of 0.15 M. Mix by gently swirling the beaker.
- Layer two volumes of ice cold 100% ethanol on top of the nucleic acid solution, and place in the freezer (-20°C) for 15 min. Spool DNA from the interface using a glass rod.[9] Allow the DNA to dry.
- Redissolve the DNA in a buffer of choice.

Notes

1. See Peterson and Stack (1997a) for detailed protocol on growing, harvesting, and rapidly removing seed coats from tomato seedlings.
2. Treatment of tissue with ethyl ether dissolves waxes and cutins and makes cells more fragile. Consequently, nuclei are more easily freed from cells, and the yield of nuclei is increased (Hamilton et al., 1972; Watson and Thompson, 1986).
3. Addition of Triton X-100 lyses chloroplasts and mitochondria but leaves nuclei intact.
4. Nuclei will stain deep blue while starch grains remain unstained. No other recognizable organelles *(e.g.,* mitochondria, chloroplasts) should be observed.
5. The solution should become viscous at this point due to release of DNA from the nuclei.
6. These techniques can be found in Sambrook et al. (1989).
7. Each RNase was dissolved in TE buffer, boiled for 10 min to destroy any contaminating DNases, mixed with an equal volume of glycerol, and stored at -20°C. Glycerol stocks of RNase A and RNase T1 were 10,000 µg/mL and 10,000 units/mL, respectively.
8. If a protein layer still appears at the organic/aqueous interphase, additional chloroform extractions should be performed until no visible protein layer is observed.
9. The DNA can also be precipitated by making it 0.15 M sodium acetate, mixing the solution thoroughly with 2 volumes of ethanol, and spinning the resulting mixture in an ultracentrifuge at 32,000 rpm for 30 min at 4°C. The supernatants are decanted and the DNA pellets allowed to dry.

Results

Approximately 2-3 mg of DNA is obtained from 250 g of seedlings or 400 g of leaf tissue. When spooled out of solution the DNA is clear or white, *i.e.*, there is no visible coloration. The A_{260}/A_{280} ratio of the DNA (adjusted for light scatter at 320 nm) ranges from 1.8-1.93. The DNA is of high molecular weight with a mean length >13 kbp as determined by gel electrophoresis. The isolated tomato DNA is completely digestable with 1 unit of *HindIII* or *BamHI* per µg of DNA and shows no visible RNA contamination as determined by agarose gel electrophoresis. The DNA exhibits normal denaturation and reassociation properties as determined by melting experiments and C_0t analysis (see Peterson and Stack, 1997b,c).

Discussion

In an attempt to isolate pure nuclear DNA from tomato, we tried several published nuclear DNA isolation protocols. The protocol that provided the greatest yield of nuclei (Watson and Thompson, 1986) resulted in polyphenol contamination. Other protocols designed to prevent contamination of nuclear DNA by polyphenols resulted in highly reduced yields of nuclei *(e.g*., Couch and Fritz, 1990). A nuclear isolation protocol designed for *Gossypium* (Katterman and Shattuck, 1983) did not work for tomato because it resulted in premature rupture of tomato nuclei and subsequent contamination of nuclear DNA with organellar DNA. Consequently, we developed a new protocol for isolating tomato nuclear DNA which maximizes yield while simultaneously combating polyphenol contamination. Because we were interested in isolating large quantities of DNA, we chose to use phenol/chloroform extractions to purify DNA rather than more expensive methods involving CTAB or cesium chloride. The DNA we obtained appeared to be as pure as DNA isolated with other purification techniques (*e.g*., Katterman and Shattuck, 1983; Watson and Thompson, 1986; Couch and Fritz, 1990; Guillemaut and Maréchal-Drouard, 1992). Because different plants can vary considerably in the number and types of secondary compounds they produce, it is unlikely that any one technique for isolating contaminant-free nuclear DNA will ever be developed (see Loomis, 1974). However, it is likely that our tomato DNA isolation protocol can be used to isolate nuclear DNA from a variety of other plant species.

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