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Protocols

RAPD Analysis in Flax: Optimization of Yield and Reproducibility using Klen*Taq* 1 DNA Polymerase, Chelex 100, and Gel Purification of Genomic DNA

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Abstract: We have developed an optimized RAPD analysis approach using the unusually heat-stable Klen*Taq*1 DNA polymerase. This enzyme is used in conjunction with a genomic DNA isolation method that includes a modified CTAB DNA isolation protocol, ethanol re-precipitation of resuspended nucleic acids from 2M NaCl, and Chelex 100 treatment. When needed, additional gel purification and isolation of high molecular weight DNA for use as a template in RAPD analysis is shown to remove amplification product ambiguity from within isolates of the same line as well as from between lines. This optimized RAPD analysis was used to define polymorphisms in lines of flax nearly isogenic for rust resistance at the *L* locus. It should also be useful for any plant species.

RAPD (random amplified polymorphic DNA), (Williams et al., 1990), arbitrarily primed PCR (AP-PCR), (Welsh and McClelland, 1990), and amplification fragment length polymorphisms (AFLPs) (Caetano-Annoles et al., 1991), are similar approaches to identifying polymorphisms among lines. We are using RAPD analysis to identify polymorphisms between the flax cultivar Bison and differential (D) lines that are nearly isogenic to Bison except for a selected introgressed region

Abbreviations: CTAB, hexadecyltrimethylammonium bromide; RAPD, random amplified polymorphic DNA.

containing a single rust resistance specificity at the *L* resistance locus. This approach uses: arbitrarily chosen oligonucleotide primers, 10 bases in length; the thermostable *Taq* DNA polymerase, or its cloned version, Ampli*Taq* DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) from *Thermus aquaticus*; and low-stringency annealing conditions to amplify sequences between primer target sites located at arbitrary positions throughout the genome. Amplification product differences between varieties, strains or species are resolved by electrophoresis.

In this paper we expand our preliminary description (Aldrich and Cullis, 1992) of changes in the RAPD approach, which reduce product variability from run to run and increase product yield. These include a modified CTAB DNA isolation protocol, additional purifications of genomic DNA after CTAB isolation and use of the enzyme Klen*Taq* 1. This enzyme is a truncated version of *Taq* DNA polymerase from *Thermus aqaticus* (Barnes, 1992). It is unusually heat stable, tolerating repeated exposure (20 cycles or more of PCR) to 98°C without diminution of the enzyme activity, and significant activity remains even after exposure to 99°C. Neither *Taq* DNA polymerase nor the truncated version of it, Stoffel fragment (Perkin Elmer Cetus), tolerate these treatments (product data sheet, AB Peptides, St. Louis, MO; W. Barnes, pers. comm.).

Materials and Methods

Isolation of Flax DNA

Flax genomic DNA was isolated from the leaves of young seedlings using a modification of the CTAB extraction method of Doyle and Doyle (1987). The major modifications are an increase in the amount of b-mercaptoethanol to 5% from 2%, the ethanol re-precipitation of resuspended total nucleic acid DNA from 2M NaCl to remove polysaccharides as described by Fang et al. (1992), and the use of Chelex 100 to remove heavy metal ions and possibly other contaminants (protocol of C. Bult, pers. comm.). Yields ranged between 100 and 500 µg of total nucleic acid per gram of leaf tissue. High-molecular-weight genomic DNA was also isolated from low-melting agarose using the method of Bult et al. (1992) if major variability was obtained within and among lines.

Reagents and buffers

CTAB extraction buffer: 2% hexadecyltrimethylammonium bromide, 1.4 M NaCl, 20 mM EDTA, 100 mM Tris, pH 8.0
β-mercaptoethanol
chloroform:octanol (24:1)

isopropanol, 100%
ethanol, 100%
wash buffer: 76% ethanol, 10 mM ammonium acetate
TE (1X): 1mM Tris, 0.1mM EDTA, pH 8.0

Protocol

- Starting with 3-week-old seedlings, harvest leaves and freeze immediately in liquid N_2. Use immediately, or freeze at -80°C until ready for use.[1]
- Grind 4 grams of leaves in liquid N_2 using a mortar and pestle pre-chilled to -20°C or - 80°C. Pour some liquid N_2 in just before adding the leaves.[2]
- Using a 2-cm-wide flexible rubber spatula, quickly transfer the pulverized leaves to a liquid N_2 pre-chilled, 50-mL conical tube (Falcon 2070)
- Quickly add 16 mL of preheated (65°C) 2% CTAB buffer containing 5% v/v β-mercaptoethanol, stir with a glass rod to mix, and place tube at 65°C.[3]
- Incubate at 65°C for 1, 2, or more hrs.[4]
- Extract with an equal volume of chloroform:octanol (24:1) and centrifuge the sample for 10 min at room temperature at 1000- to 1500xg to separate phases. Repeat.
- Remove the supernatant and precipitate with 2/3 volume of isopropanol.
- Collect the precipitated nucleic acids and wash twice with wash buffer.[5]
- Air-dry the pellets and resuspend in TE.[6]
- Bring the dissolved nucleic acids to 2M NaCl and re-precipitate using 2 volumes of ethanol.[7]
- Wash the pellet twice using 80% ethanol, air-dry the pellets and resuspend in TE.[8]

Notes

1. Using leaves of seedings instead of older plants reduces nucleic acid contamination by plant metabolites that interfere with solubilization of precipitated nucleic acids.
2. We routinely use 4 grams of leaves. Smaller amounts may also be used, but the ratio of buffer to leaves should always be 4:1 vol/wt or greater.
3. Moving quickly at this stage is critical to getting DNA that is of high molecular weight. To aid in minimizing time spent doing this step, pre-measure the 16 mL of 2% CTAB buffer in a 50-mL conical tube. Add 836 µL of β-mercaptoethanol (5% v/v) and place the tube in a 65°C water bath until ready for use. Use of 5% instead of the 2% used in the Doyle and Doyle (1987) procedure produced nucleic acid pellets that were not nearly as brown. Adding the pre-warmed, pre-measured CTAB buffer to the frozen leaf tissue contained in the pre-chilled conical tube saves precious time in bringing the tissue from -80°C

to 65°C as rapidly as possible resulting in higher molecular weight DNA.
4. Longer incubations result in higher yield and cleaner, whiter pellets. We have successfully incubated overnight for convenience.
5. CTAB is soluble in ethanol and residual amounts are removed in this step.
6. Large pellets are most easily dissolved by adding 150 µL of TE, placing the tube at 65°C for 10 minutes, removing the TE containing the dissolved nucleic acids to a new tube, and repeating the process until all the pellet has gone into solution.
7. During ethanol precipitation of nucleic acids from 2M NaCl, polysaccharides remain dissolved in the ethanol (Fang et al.,1992). The freer the nucleic acids are from contaminants, the easier the pellet is to resuspend. We do two such precipitations if the pellet obtained from the first ethanol precipitation from 2 M NaCl is hard to resuspend. The pellet obtained from the second precipitation usually goes into solution very easily.
8. Washing in 80% ETOH removes residual NaCl and CTAB.

Chelex 100 Treatment of Nucleic Acid

The heavy-metal chelating resin, Chelex 100, has been used to increase the product yield in PCR (Singer-Sam et al., 1989). Our protocol for removing heavy-metal and possibly other contaminants from plant nucleic acid solutions was adapted from one provided by Carol Bult, Smithsonian Institute (personal communication, manuscript in preparation).

Reagents and solutions required

5% w/v suspension of Chelex 100 in sterile distilled water[1]

Protocol

- Pellet the beads from 500 µL of a well-suspended solution of 5% Chelex 100 in sterile distilled water contained in a 0.5-mL microfuge tube by microcentrifugation for 1 minute. Discard the supernatant.
- Add 250 µL of DNA at 10-50 ng/µL to the pelleted beads and mix well.[2]
- Heat in a thermocycler at 100°C for 15 minutes.[3]
- Pellet the beads by microcentrifugation for 2 minutes.
- Remove the supernatant to a new tube and remove residual beads by microcentrifugation for 2 minutes. Transfer the supernatant to a new tube. Recovery is 200 to 225 µL.[4]

Notes

1. Chelex 100 is available from BioRad Laboratories, 200 Alfred Nobel Dr., Hercules, CA 94547, 1-800-424-6723. The catalog number is 1432 832.
2. If the starting nucleic acid solution is more dilute than desired, it can be added directly to the dry beads, which will absorb water. The recovered volume will be less, but the solution will be more concentrated.

3. Heating at 100°C to denature the DNA was reasoned to help strip the DNA from heavy metals, polyphenolics and polysaccharides (C. Bult, pers. comm.).
4. Removal of residual beads is important because degradation of DNA can occur if all the beads are not removed (C. Bult, pers. comm.).

Gel purification of Total Genomic DNA

Isolation of high molecular weight DNA from low-melting agarose was performed essentially as described by Bult et al., 1992.

Reagents and buffers

ethidium bromide (10 mg/mL)
FMC GTG SeaPlaque agarose
RNAse A
1X TBE: 0.089 M tris-borate, 0.089 M boric acid, 0.002 M EDTA

Protocol

- Treat the nucleic acid solution with RNAse A to a final concentration of 10 μg/mL and incubate 30 min at 37°C.[1]
- Load 5 μg/well and electrophorese at 50 volts for 1.5 hours in 0.75% FMC GTG SeaPlaque agarose dissolved in 1X TBE containing 200 ng ethidium bromide/mL.
- After electrophoresis, wash the gel 3X, 10 min per wash, in distilled water to leach the borate out of the gel.
- Excise the band of high-molecular-weight DNA and place the gel slice in a microfuge tube.
- Melt the gel slice for 10 min at 65°C, dilute 1:1 with sterile distilled water, and store at 4°C.
- Estimate DNA concentration by comparison to λ DNA of known concentration using gel electrophoresis in 1.6% agarose.

Notes

1. We added an RNAse treatment because it removed enough RNA so that the ethidium bromide fluorescence was due to DNA alone, and the higher molecular weight DNA band was more defined.

RAPD assay

Reagents and buffer per reaction

DNA, 25 ng
primer, 0.2 μM[1]
dNTPs, 0.1 mM each
AB peptides buffer: PC2, 1X[2]
Klen*Taq* 1 polymerase, 6 units[3]
water to 15 μL
mineral oil[4]
thin-walled 0.5-mL reaction tubes[5]

Protocol

This protocol is a modification of the RAPD analysis described by Williams et al. (1990).

- DNA at a concentration of 25 ng/10 µL in water was introduced under 50 µL mineral oil contained in 0.5-mL thin-walled reaction tubes. The tubes were microcentrifuged briefly,[6] heated at 95°C[7] in a MJ Research thermocycler for 5 minutes, brought to 85°C and held there during addition of the master mix. Master mix (15 µL), containing all other reaction components, was added under the oil to the DNA solution for a final reaction volume of 25 µL. The amplification program was a brief additional denaturation at 94°C, 10 sec; primer annealing at 35°C, 1 min; primer extension at 65°C,[8] 2 min; ramp, 1°C/3 sec, 1 cycle; denaturation at 94°C, 1 sec, 92°C, 30 sec; annealing at 35°C, 1 min; extension at 65°C, 2 min, ramp, 1°C/3 sec; 39 cycles followed by 4°C hold.[9]
- RAPD products were size-separated by electrophoresis in 1.6% FMC GTG agarose in 0.5X TBE at 8 V/cm.[10]

Notes

1. The primers were 10-mers obtained as a set of 100 from Dr. John Carlson, The University of British Columbia, Biotechnology Laboratory, 6174 University Blvd., Vancouver, B.C. V6T 1W5.
2. Constituents of the PC2 AB Peptides buffer supplied with the enzyme for Klen*Taq* 1 are: 20 mM Tris-HCl pH 8.55, 16 mM ammonium sulfate, 2.5 mM $MgCl_2$, and 150 µg/mL bovine serum albumin.
3. Klen*Taq* 1 is available at 30U/µL, $2/µL from AB Peptides, 4000 Laclede Ave., St. Louis MO 63108. (800) 383-3362.
4. Squibb mineral oil is available from a local drug store at $4.50 per 500 mL.
5. Thin-walled tubes with frosted lids for ease in identifying samples are available from Midwest Scientific, P.O. Box 458, Valley Park, MO 63088, USA.
6. Microcentrifugation of the samples will bring floating aqueous sample under the oil. Any further floating is prevented by keeping the samples at 4°C in the thermocycler until ready to heat to 95°C.
7. Heating the samples to 95°C and bringing the temperature to 85°C for addition of master mix is a modification of the "hot-start" method of Hosta and Flick (1992). Doing a hot-start eliminated product formation in absence of template with all primers tested. Amplification product yield was also enhanced.
8. The optimal temperature for Klen*Taq* 1 is 65°C (W. Barnes, pers. comm.).
9. Care was taken to fill each space in the thermocycler to avoid well-to-well variability (Van Leuven, 1991).
10. Among several brands tested, FMC GTG agarose consistently produced the sharpest bands. It is available from Midwest Scientific.

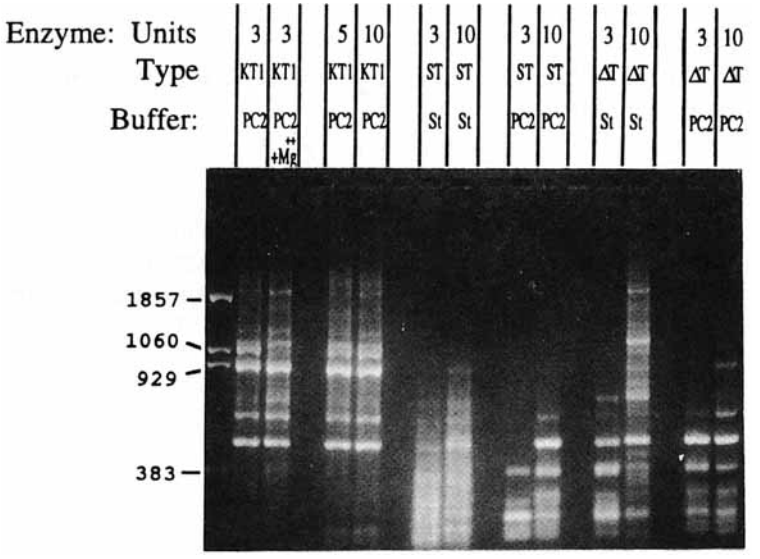

Fig. 1. Comparison of three truncated *Taq* DNA polymerases in RAPD reactions using genomic DNA from cultivar 'Bison'. Klen*Taq* 1 (KT1) amplification products (10 μL) obtained using buffer PC2 provided with the enzyme from AB Peptides, lanes 2, 5, and 6; and with buffer PC2 containing an additional 2.5 mM $MgCl_2$ for 5 mM, lane 3. Stoffel fragment used with buffer St provided from Perkin Elmer Cetus, lanes 8, 9; and with buffer PC2, lanes 11 and 12. Δ*Taq* polymerase version 2 used with buffer St, lanes 14 and 15; and with buffer PC2, lanes 17 and 18. Lanes 4, 7, 10, 13 and 16 are controls lacking template DNA. Primer 140 is GTCGCATTTC. Lane 1, pBR322 cleaved with *Bst*NI.

Results and Discussion

In our initial attempts to optimize RAPD analysis in flax, we noted that amplification product yields using Stoffel fragment of *Taq* DNA polymerase were often much greater, although of somewhat lower molecular weight, compared to those from the full-length enzyme, Ampli*Taq* (both enzymes from Perkin Elmer Cetus). This truncated version of *Taq* DNA polymerase is more heat stable and has a broader magnesium optimum than the native or cloned *Taq* DNA polymerase (Anonymous, 1991). However, we often noted a lack of reproducibility in amplification product yield from reaction to reaction regardless of what primer, DNA isolation procedure (i.e., CTAB or CsCl gradient-purified DNA), enzyme or DNA concentration was used. This lack of reproducibility prompted

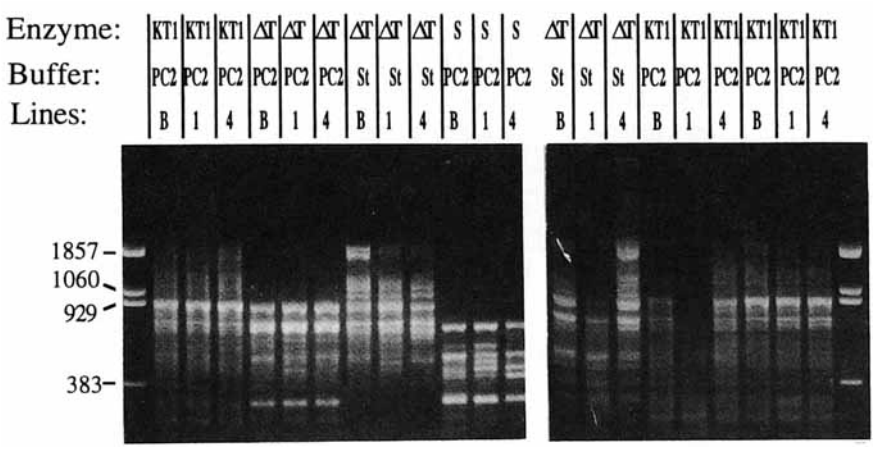

Fig. 2. Comparison of Klen*Taq* 1, Stoffel fragment and Δ*Taq* polymerase, version 2. Effect of ethanol re-precipitation of genomic DNA from 2 M NaCl followed by Chelex 100 treatment on RAPD amplification product yield (10 μL/lane) using primer 147 (GTGCGTCCTC). *Left.* Amplification products from Bison (B), D1, and D4 using CTAB protocol followed by reprecipitation from 2M NaCl followed by Chelex 100 treatment. *Right* Amplification products from genomic DNA isolated using only CTAB protocol, first 6 lanes; last 3 are replicates of the first 3 lanes in gel A for closer comparison. Enzymes were used at 3 units/reaction. Abbreviations of enzymes and buffers are described in Fig. 1.

us to try two other truncated *Taq DNA* polymerases, Klen*Taq* 1 (AB Peptides, St. Louis, MO) and Δ*Taq* polymerase version 2 (U.S. Biochemicals, Cleveland, OH) to assess their use in RAPD analyses.

An example of the performance of Klen*Taq* 1, Δ*Taq* polymerase version 2 and Stoffel fragment in the RAPD assay using genomic DNA from Bison and primer 140 (GTCGCATTTC) is shown in Fig. 1. In this experiment, genomic DNA was isolated using the CTAB protocol but without ethanol reprecipitation from 2M NaCl or Chelex 100 treatment. We also compared enzyme performance in AB Peptides buffer (PC2) as well as Stoffel buffer (St) provided with the Stoffel fragment from Perkin Elmer Cetus. Both buffers contain 2.5 mM Mg^{++}. Only Klen*Taq* 1 was able to make fragments greater than about 1.8 kb at 3 units/reaction. Δ*Taq* polymerase version 2 used with buffer St also was able to make amplification products of greater than 1.8 kb, but only if 10 units were used, and then only with St buffer. Interestingly, the amplified DNA fragment profile produced using this enzyme was different from that of Klen*Taq*

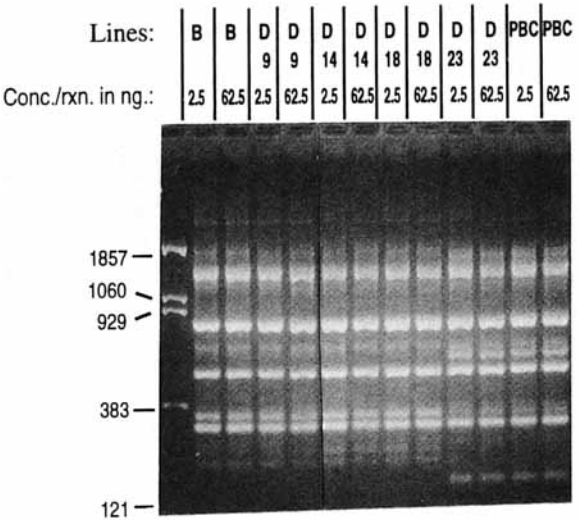

Fig. 3. Effect of genomic DNA concentration on RAPD product yield. Genomic DNAs from cultivars 'Bison' (B) and 'Pale Blue Crimped' (PBC), and differential lines (D) were adjusted to 2.5 or 62.5 ng and used in RAPD reactions using Klen*Taq* 1 (6 units/reaction) and primer 491 (TCCTGTCAAG). Each lane contains 10 μL amplification product.

1. Klen*Taq* 1 at 3, 5 and 10 units per reaction and 2.5 mM Mg^{++} produced similar fragment profiles. When Klen*Taq* 1 at 3 units and Mg^{++} at 5 mM/reaction was used (lane 3), a band at about 1 kb was not made (compare to lane 2, 3 units enzyme and 2.5 mM Mg^{++}).

Other primers were tested with these enzymes with the results that Klen*Taq* 1 consistently gave the most reproducible products especially when the genomic DNA was ethanol re-precipitated from 2 M NaCl followed by Chelex 100 treatment. Results with primer 147, GTGCGTCCTC, are shown in Fig. 2 as an example. In this experiment, 25 ng of genomic DNA from the lines Bison, D1 and D4 were used per RAPD reaction with Klen*Taq* 1, Δ*Taq* polymerase version 2 and Stoffel fragment at 3 units/reaction in either buffers PC2 or St. In addition, we also show the effect of CTAB isolation of DNA followed by ethanol reprecipitation from 2M NaCl and Chelex 100 treatment on product yield and reproducibility. A comparison of panels A and B show that for the combinations Klen*Taq* 1, PC2 buffer (panel A, lanes 2-4 and panel B, lanes 4-9) and Δ*Taq* polymerase version 2, St buffer (lanes 7-9, panel A, and 1-3, panel B), product yield and ability to make higher molecular weight products is increased with the additional purification steps. There was no line to line variability only when the Klen*Taq* 1 and buffer PC2 combination was used with genomic DNAs that were ethanol re-precipitated from 2M NaCl and Chelex 100 treated after CTAB isolation. Interestingly, the Δ*Taq*

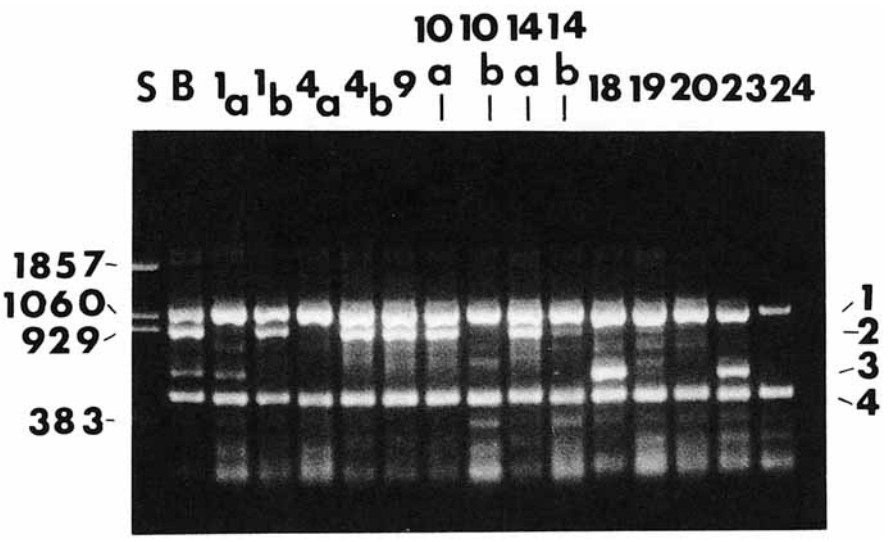

Fig. 4. Comparison of Klen*Taq* 1 primer 140-generated RAPD amplification products from different D-lines and from different isolates of the same lines. Samples for RAPD analysis were adjusted to 25 ng/reaction for genomic DNAs and 6 units of Klen*Taq* 1/reaction, and 10 µL of amplification product was loaded per lane. Numbers above lanes refer to different D lines, and **a** and **b** subscripts refer to isolates from the same line but from plants grown at different times (*a* = growth in February and *b* = growth in March or April, 1992). Lane B, Bison. Lane S, pBR322 cleaved with *Bst*NI. Key amplification products 1-4 are marked for comparison.

polymerase version 2 and buffer PC2 combination (panel A, lanes 4-6) was not as able to make higher molecular weight products as the Δ*Taq* polymerase version 2 and buffer St combination (panel A, lanes 6-9). Neither Klen*Taq* 1 nor Stoffel fragment made products with this primer when buffer St was used with genomic DNAs from either isolation protocol (data not shown). These results prompted a revised protocol that included the ethanol re-precipitation from 2M NaCl and Chelex 100 treatment.

In our initial RAPD screens of genomic DNAs obtained using the revised CTAB protocol, we used 3 units of Klen *Taq* 1 per reaction. Occasionally product variability occurred in all lines with a given primer with this amount of enzyme. We reasoned that because the D lines differed from Bison mainly by only a segment containing the resistance gene, when a primer made different products for all lines conditions were not optimal for that primer. Since this variability was usually

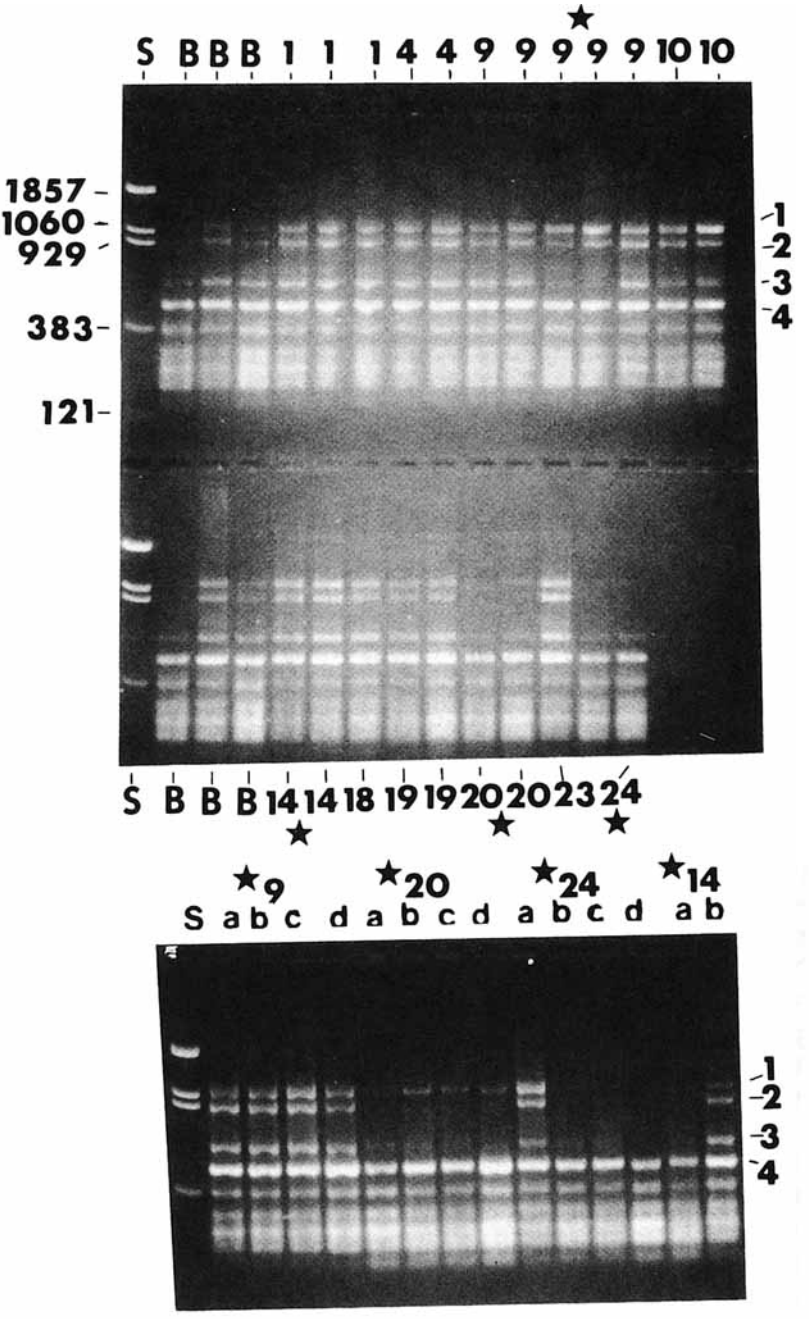
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Fig. 5 (opposite page). **Effect of gel purification of genomic DNA on RAPD amplification product variability.** *Top gel*: Klen*Taq* 1 and primer 140-generated RAPD product profiles from genomic DNAs isolated using CTAB, ethanol reprecipitation from 2M NaCl, and electrophoretic purification in low-melting agarose. Gel-purified high-molecular-weight DNA samples for RAPD analysis were adjusted to 25 ng/reaction and 6 units of Klen*Taq* 1/reaction; 10 μL of amplification product was used per lane. Lanes: S, standard, pBR322 cleaved with *Bst*NI, and B, Bison. Numbers above other lanes refer to D lines. Replicate lanes: products from different isolations. *Bottom gel*: Further purification of selected DNAs from the low-melting agarose. The genomic DNAs from the samples to the right of the stars, top gel, were ethanol-precipitated after β-agarase treatment or were treated with Chelex-100. Genomic DNA samples were adjusted to 2.5 μg/100 μL before ethanol precipitation or Chelex 100 treatment. Amplification products (10 μL) from ethanol-precipitated samples brought to 2.5, 25, or 100 ng per reaction before RAPD analysis are shown in lanes a, b, and c, respectively. Lane d: 10 μL of amplification products from 25 ng/reaction of Chelex-100-treated DNA. D14, top gel, is starred because, after storage of the genomic DNA in the low melting agarose, bands 1–3 were not produced on subsequent RAPD analysis (compare D14, star, top gel with D14, star, lane a, bottom gel). D14, star, lane b, bottom gel: 10 μL of amplification products of 25 ng of Chelex 100-treated genomic DNA from D14. Key amplification products 1–4 are marked for comparison.

eliminated when 6 units of enzyme were used per reaction, we routinely use 6 units of enzyme/reaction. We also explored the effect of DNA concentration on product yield and reproducibility. We noted that with Stoffel fragment and Δ*Taq* polymerase version 2, DNA concentrations greater than about 50 ng/reaction sometimes resulted in smearing of the higher molecular weight fragments, and too little DNA (2.5 ng) resulted in lack of production of higher molecular weight bands (data not shown). In contrast, the amount of product formed using Klen*Taq* 1 was usually insensitive to the amount of genomic DNA used in the range of 2.5 to 250 ng/reaction. In Fig. 3, DNA concentrations of 2.5 and 62.5 ng/reaction were used with Klen*Taq* 1 at 6 units/reaction and primer 491, TCCTGTCAAG. Although some of the major bands are present at higher yield with 62.5 ng DNA, the product profiles are identical within a line. Line D23, which was made by back-crossing cultivar Pale Blue Crimped to Bison 8 times, each time selecting for rust resistance, shows polymorphisms that are also present in Pale Blue Crimped. The retention of the same polymorphisms in D23 suggests that they may be linked to the rust resistance gene.

Primer 140, GTCGCATTTC, initially looked promising relative to producing major band polymorphisms with Klen*Taq* 1, Stoffel fragment or Δ*Taq* polymerase version 2 with DNAs from 11 lines. However, when

this primer was used with DNAs from lines grown in the greenhouse at different times, we noted major band variability within and among lines. The variability obtained using any of these three enzymes was not removed by additional organic extractions, ethanol precipitation from 2M NaCl, then Chelex 100 treatment, CsCl gradient centrifugation or column purification using Sepharose CL-6B. Fig. 4 shows the within and among line variability of bands 1 to 3 using Klen*Taq* 1 with DNAs isolated using the CTAB protocol followed by ethanol re-precipitation from 2M NaCl then Chelex 100 treatment. The variability within and among lines was largely removed when the DNAs were gel purified using low melting agarose (Fig. 5). Sometimes, bands 1 to 3 were under-represented even with this treatment as with some of the gel purified samples from lines D9, D20 and D24. The DNA from these lines was removed from the low melting agarose by using β-agarase (New England Biolabs, Beverly, MA) followed by ethanol precipitation according to the manufacturer's instructions. DNAs retained in the low melting agarose were also Chelex 100 treated as described in the legend to Fig 5. These two modes of purification removed the band variability in all but one line. We also noted that storage of the DNA in the low melting agarose can inhibit product formation. For example, D14, top gel, had bands 1 to 3. After storage in low melting agarose, bands 1 to 3 were not produced (bottom gel, D14a). This inhibition is removed with Chelex 100 treatment as shown in lane D14b, bottom gel.

Summary

Optimization of RAPD analysis was accomplished using Klen*Taq*1 DNA polymerase in conjunction with our modified CTAB protocol, ethanol re-precipitation from 2M NaCl and Chelex 100 treatment of the genomic DNA. Because electrophoretic purification of DNA is tedious, we only use this this procedure when there is ambiguity in RAPD product formation within and between lines as observed with primer 140. Using nearly isogenic lines was extremely valuable in conducting this study. Because the lines should contain very few polymorphisms, when "too many" showed up with a given primer, we were able to use this primer to adjust conditions so that ambiguities were eliminated. Using 6 units of enzyme removed many ambiguities and aided in formation of longer products, supporting the observation that excess enzyme is harmless and may lead to more efficient amplification, especially of longer PCR products (Barnes, 1992). If product yield was low

with a given primer, it was increased by increasing the time of extension to as long as 12 minutes. Sometimes given primers produced a smear of products. In some cases, lowering the extension time and increasing the number of cycles produced clear individual bands. This optimized procedure for RAPD analysis should be useful for any plant species.

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