

[29] Detecting Differential Expression of Parental or Progenitor Alleles in Genetic Hybrids and Allopolyploids

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Abstract

Three assays useful for detecting specific RNA transcripts are primer extension, S1 nuclease protection, and reverse-transcription–cleaved amplified polymorphic sequence (RT-CAPS) analysis. All three of these techniques are used routinely for gene expression analyses and allow insights not possible by RNA blot (northern blot) hybridization. In this chapter, we describe how the primer extension, S1 nuclease protection, and RT-CAPS methods can be used to discriminate one or more parental or progenitor alleles in hybrids or allopolyploids. We discuss the rationale for using the different techniques and provide examples of the data generated.

Introduction

A molecular understanding of genetic or epigenetic traits in diploid hybrids and allopolyploids (polyploid hybrids) requires measuring the expression state of alleles inherited from the different parents or progenitor species. Indeed, uniparental gene expression is the basis for a number of intriguing epigenetic phenomena, including gametic imprinting ([Brannan and Bartolomei, 1999](#); [Sleutels *et al.*, 2000](#)), mammalian X-chromosome inactivation ([Avner and Heard, 2001](#); [Huynh and Lee, 2001](#)), and nucleolar dominance ([Pikaard, 2000a,b](#); [Reeder, 1985](#)).

Typically, transcripts from orthologous alleles are too similar in sequence to be discriminated from one another using RNA blot hybridization. Therefore, more precise methods are needed. The primer extension, S1 nuclease protection, and reverse-transcription–cleaved amplified polymorphic sequence (RT-CAPS) assays can all be designed to provide the needed precision, because all three methods can exploit relatively minor sequence differences among allelic RNAs. Primer extension ([Boorstein and Craig, 1989](#)) is useful for discriminating allele transcripts that share regions of sequence identity interrupted by insertions/deletions in the region near the transcription initiation site. S1 nuclease protection

(Berk and Sharp, 1977) is useful for discriminating transcripts that may be identical in size but have at least several contiguous nucleotides that are polymorphic. RT-CAPS is a variation of the CAPS method used for generating polymerase chain reaction (PCR)-based markers for genetic mapping (Konieczny and Ausubel, 1993). This method exploits one or more restriction endonuclease site polymorphisms following reverse transcription of RNA into first-strand complementary DNA (cDNA) and subsequent PCR amplification. All three techniques require knowledge of the allele sequences to design the appropriate primers or probes. Different alleles can only be discriminated if there is sequence variation between them. The type of sequence differences among the alleles then dictates which methods are viable options. Examples of primer extension, S1 nuclease protection, and RT-CAPS data are presented based on published (Chen and Pikaard, 1997; Lewis and Pikaard, 2001) and unpublished studies of nucleolar dominance, the epigenetic phenomenon that describes the expression of only one parental set of ribosomal RNA (rRNA) genes in a genetic hybrid (Pikaard, 2000a; Reeder, 1985; Viegas *et al.*, 2002).

Primer Extension Assay for Detection of Allele-Specific Transcripts

Primer extension is a method typically used to map the 5' end(s) of an RNA, thus defining the transcription start site and providing initial evidence for where the promoter is located within a cloned gene. The method uses a single-stranded DNA primer that is designed to hybridize to the RNA at a position downstream of the transcription start site (defined as +1), preferably within about 200 bp of +1. One then adds RNA-dependent DNA polymerase (reverse transcriptase [RT]) and deoxyribonucleotide triphosphates. RT catalyzes the sequential addition of deoxyribonucleotides to the 3' hydroxyl group supplied by the oligonucleotide. The oligonucleotide thus acts as a primer for the synthesis of first-strand cDNA, which continues to extend until the 5' end of the RNA template is reached. As a consequence of this reaction, the relatively short (25–40 nt) primer becomes extended into a longer DNA molecule. By labeling the primer radioactively at its 5' end, both the initial primer and the final extended product can be resolved and visualized after denaturing gel electrophoresis and exposure to x-ray film or a PhosphorImager screen. The size of the extended product corresponds to the precise distance from the labeled nucleotide to the 5' end of the RNA. This size can be estimated by comparison to end-labeled size markers run on the same gel. Better yet is to perform a dideoxynucleotide sequencing reaction using the 5' end-labeled primer and genomic DNA as template. In this manner, one generates a sequencing ladder that is run on the gel adjacent to the primer

extension product. The fragment in the sequencing ladder that matches the size of the extension product defines the precise nucleotide that corresponds to +1. In addition to being useful for mapping transcription start sites, primer extension results are quantitative so long as the primer is in excess of the RNA to which it hybridizes, which is easily accomplished. Thus, the amount of radioactive primer extension signal on the gel is directly proportional to the abundance of the target RNA.

Primer extension can be used as an assay to discriminate alleles of two progenitors if the sequences of the two RNAs are different enough that two primers can be designed, each specific for the allele of only one progenitor. However, we find primer extension to be most useful when the sequences of the progenitors' alleles are very similar but have insertions/deletions relative to one another. In this latter scenario, a single primer is used, but distinct primer extension products are generated. The use of a single primer is ideal, because there is no need to worry about possible differences in the specific activity or hybridization efficiency of two different primers.

Figure 1 provides an example of the use of primer extension to map the transcription start sites for rRNA genes of *Brassica oleracea* and *Brassica nigra* and to show that only *B. nigra* rRNA genes are expressed in *Brassica carinata*, the allotetraploid hybrid of *B. oleracea* and *B. nigra*. The rRNA genes of the two progenitor species are very similar in sequence, but at a location 87 bp downstream of the transcription start site, *B. oleracea* has 10 bp inserted relative to *B. nigra* (see diagram in Fig. 1). A 30-nt primer corresponding to positions 158–187 (numbered relative to the *B. nigra* start site) was designed because there are only two nucleotide differences between *B. nigra* and *B. oleracea* in this interval. Both polymorphic nucleotides are located near the 5' end of the primer where they are unlikely to interfere with hybridization near the 3' end of the primer where the extension reaction takes place. Indeed, this primer works with equal efficiency for primer extension of both *B. oleracea* and *B. nigra* RNA but yields a longer extension product with *B. oleracea* RNA, as expected (Fig. 1, compare lanes 5 and 7). Side-by-side sequencing reactions performed using the primer with genomic DNA clones of the promoter regions for the two species shows that the sequences at the transcription start sites of *B. oleracea* and *B. nigra* are identical (shown to the side of the photo). Primer extension of RNA isolated from the allotetraploid hybrid *B. carinata* shows that only *B. nigra* rRNA gene transcripts are detected (Fig. 1, lane 6). Collectively, the data indicate that *B. nigra* rRNA genes are expressed and *B. oleracea* rRNA genes are silenced in *B. carinata*, an example of the epigenetic phenomenon known as *nucleolar dominance*.

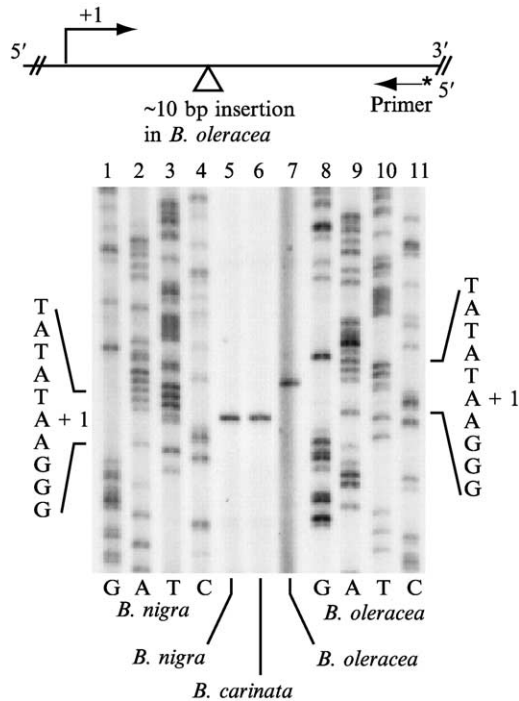


FIG. 1. Use of the primer extension assay to reveal nucleolar dominance in *Brassica carinata*, the allotetraploid hybrid of *Brassica nigra* and *Brassica oleracea*. The diagram illustrates the fact that a single end-labeled primer can hybridize to ribosomal RNA (rRNA) transcripts of either *B. nigra* or *B. oleracea*. Because of an estimated 10-bp insertion in *B. oleracea* relative to *B. nigra*, extension of the primer by reverse transcriptase results in primer extension product that is longer for *B. oleracea* (compare lanes 5 and 7). Primer extension of RNA isolated from *B. carinata* reveals abundant rRNA transcripts from the *B. nigra* genes, but no *B. oleracea* rRNA transcripts can be detected (lane 6). The same end-labeled primer was used to generate sequencing ladders from promoter DNA clones (lanes 1–4, 8–11) allowing the transcription initiation sites (+1) to be accurately mapped to the same sequence motif. Reprinted, with permission, from [Chen and Pikaard \(1997\)](#).

Solutions Needed

- A. primer extension reaction mix: 20 mM Tris-HCl (pH 8.7), 10 mM MgCl₂, 5 mM DTT (added just before use), 50 μg/ml actinomycin D, 0.5 mM each dNTP (dATP, dCTP, dGTP, TTP), 2–5 units/μl MMLV RT.

- B. Formamide gel-loading dye: 80% formamide (deionized ultrapure), 10 mM NaOH (to help degrade RNA), 10 mM EDTA, 0.5 mg/ml xylene cyanol FF dye, 0.5 mg/ml bromophenol blue dye.

Procedure

Primer extension uses a radioactively labeled primer, so take the appropriate precautions for handling radioisotopes. These include wearing gloves, a lab coat, and eye protection and keeping your work behind a radiation shield at all times. Keep a radiation monitor (Geiger counter) at your side and check your hands and pipetter often to ensure they do not become contaminated. As for all assays involving RNA, use sterilized tubes, pipette tips, and solutions to prevent introduction of RNases into the reactions.

1. Radioactive 5' end labeling of the primer:

To a 1.7-ml or 0.5 ml plastic Microfuge tube add the following:

5 μ l water (distilled and/or Milli-Q treated)

1 μ l of primer DNA (\sim 50–70 ng; 25–30 nt long)

2 μ l 660 mM Tris-HCl pH 7.5

2 μ l 100 mM DTT

2 μ l 100 mM MgCl₂

2 μ l 10 mM spermidine

1 μ l T4 polynucleotide kinase (\sim 5 units)

5 μ l gamma-labeled ³²P-ATP (10 μ Ci/ μ l; 6000 Ci/mmol)

Incubate labeling reaction at 37° for 60 min. Heat reaction at 65° for 15 min to inactivate the kinase. Remove unincorporated ³²P-ATP by centrifugation through a Sephadex G50 spin column (these are often marketed for removing unincorporated nucleotide triphosphates from DNA sequencing reactions). Alternatively, one can use ethanol precipitation using ammonium acetate, rather than sodium acetate, as the salt according to standard methods ([Sambrook and Russell, 2001](#)).

2. In a fresh Microfuge tube, add the RNA to be tested, dissolved in sterile water (10–40 μ g depending on the abundance of the transcript). Add about 10⁵ cpm (\sim 1 ng) of labeled primer. Ethanol precipitate the RNA and primer together by adding 1/10 volume of 3 M sodium acetate pH 5.2 and 2.5 volumes of cold (–20°) ethanol. Incubate on ice 5–10 min, then centrifuge at 14,000g for 15 min. Wash the pellet with 70% ethanol and air-dry the pellet.

3. Resuspend the RNA and primer in 8 μ l TE (10 mM Tris, 1 mM EDTA), pH 8.4 by pipetting up and down repeatedly. Add 2 μ l of 1.25 M

KCl and mix. Spin tube briefly in a microcentrifuge to collect all of the solution at the bottom of the tube.

4. Bring a water bath to boiling. Turn off the gas and put the tube containing the RNA and primer into the bath to denature the RNA and primer. After about 1–2 min, transfer the tube to a 42° waterbath for 30 min to allow the primer and RNA to anneal and hybridize.

5. Add 24 μl of primer extension reaction mix. Mix the reaction and spin briefly in a microcentrifuge to collect all of the solution at the bottom of the tube.

6. Incubate 1 h at 42° to allow primer extension (cDNA synthesis) to occur.

7. Add 70 μl TE, pH 8.0. Precipitate nucleic acids by addition of 50 μl 7.5 M ammonium acetate and 375 μl cold (–20°) ethanol. Incubate on ice 5–10 min and then centrifuge at 14,000g for 15–20 min. Wash pellet with 70% ethanol and air-dry.

8. Dissolve pellet in 6–8 μl of formamide gel-loading dye. Place tubes into a boiling waterbath just after turning off the heat and incubate for 5 min. Chill tubes on ice.

9. Load sample onto a 6–8% polyacrylamide, 8 M urea sequencing gel (Sambrook and Russell, 2001). As controls, load a small aliquot of the labeled primer and load end-labeled size markers or dideoxynucleotide sequencing reaction products.

10. Run the gel until the bromophenol dyes have migrated the desired distance, then pry apart the gel plates, transfer the gel to filter paper, and dry, using a vacuum gel dryer.

11. Expose the dried gel to X-ray film or a PhosphorImager screen to obtain an image analogous to that shown in Fig. 1.

S1 Nuclease Protection Assay for Detecting Allele-Specific Transcripts

The S1 nuclease protection assay allows accurate estimations of transcript abundance and has the ability to distinguish RNA transcripts that sometimes differ by only a few nucleotide substitutions (Berk and Sharp, 1977). In this method, equal aliquots of RNA are hybridized with radioactively 5' end-labeled DNA probes specific for the parental/progenitor alleles to be assayed. The probes are typically made from genomic clone restriction fragments spanning the transcription start site (as diagrammed in Fig. 2), but long oligonucleotides (typically >50 nt) can also be used. The DNA probes are designed so they include short regions, often only several nucleotides long, where the nucleotide sequence is different in the alleles to be discriminated. A probe molecule that hybridizes to a

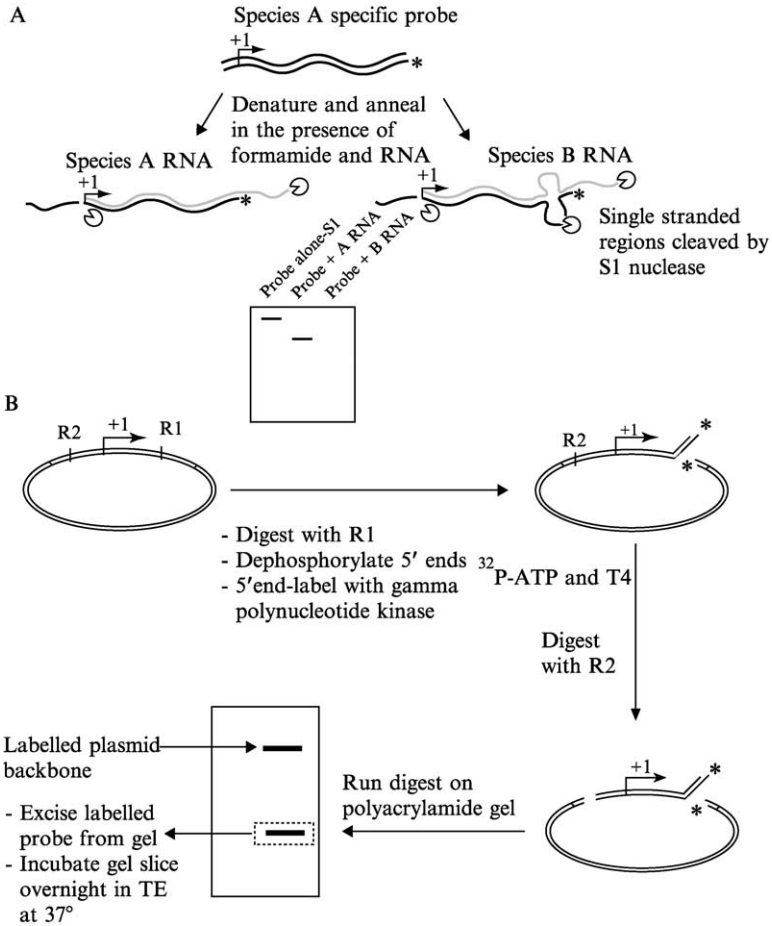


FIG. 2. The S1 nuclease protection assay and probe preparation. (A) Diagrammatic representation of the specificity of the S1 nuclease protection assay. A double-stranded DNA fragment from species A is 5' end-labeled on the bottom strand (*), complementary to the RNA, at a position of about 50–300 bp downstream from the transcription start site (+1). The probe is then denatured in the presence of formamide and RNA from species A or species B. Upon hybridization with RNA from species A, a perfect match between the probe and RNA occurs from the 5' end of the RNA to the labeled end of the probe. Single-stranded portions of the DNA probe extend upstream of the RNA 5' end, and single-stranded RNA extends beyond the labeled end of the probe. S1 nuclease digests these single-stranded nucleic acids, leaving the RNA–DNA duplex intact. As a result, the initial labeled strand of the probe becomes shorter and migrates faster on a denaturing sequencing gel, depicted at the bottom of panel A. By contrast, hybridization of the probe from species A with RNA from species B, which does not result in a perfect hybrid, results in looped out single-stranded regions that are digested with S1 nuclease to produce labeled fragments not detected on the subsequent gel.

transcript for which it is an exact match forms a perfectly base-paired RNA–DNA duplex that is resistant to digestion by S1 nuclease (Fig. 2A). By contrast, hybridization of the probe to a homologous but nonidentical RNA transcribed from a different allele will form a duplex that leaves single-stranded loops at sites of sequence mismatch. These single-stranded regions are cleaved by S1 nuclease (Fig. 2A). Placement of the 5' radioactive end-label near such regions of nonhomology ensures that any resulting labeled digestion products are small enough that they migrate off the bottom of a sequencing gel.

DNA probes used for S1 nuclease protection are designed such that they begin upstream of the transcription start site and extend 50–300 nucleotides downstream of the start site. Upon hybridizing to RNA initiated at the start site, the DNA probe overhangs the 5' end of the RNA and the RNA extends beyond the downstream end of the probe (see Fig. 2A). S1 nuclease cleaves these single-stranded DNA and RNA overhangs, and any single-stranded bubbles within a non-perfect hybrid but does not digest the double-stranded regions. As a result, the initial labeled DNA probe becomes shorter in a successful S1 nuclease protection assay because of digestion of the single-stranded DNA that extends upstream of the RNA's 5' end.

We employ two methods for generating probes for the S1 nuclease protection assay. The simplest method is to use long (55–70 nt) gel-purified DNA oligonucleotides as probes. These oligos are designed to be complementary to the RNA and extend upstream of the transcription start site by about 10 nt, leaving about 45–60 nt to form a DNA–RNA hybrid that is quite stable. The oligos are 5' end-labeled using T4 kinase and can be used directly in the hybridization reaction, although gel purification is generally a good idea to eliminate smaller oligonucleotides in the synthesis reaction that will also hybridize to the target RNA. Despite the ease of labeling oligonucleotides to generate probes, our preferred method is to use restriction

(B) Probe preparation from a genomic DNA fragment spanning the promoter region, cloned within a plasmid. Based on the known sequence of the genomic clone and plasmid, two restriction sites (R1 and R2) are chosen which, ideally, are unique in the plasmid clone. Site R1 is typically located about 50–300 bp downstream from the transcription start site (+1). The plasmid is linearized at site R1, dephosphorylated, and then 5' end-labeled on both the top and bottom strands (*). The plasmid is then cut at site R2, which liberates the desired promoter fragment from the remainder of the plasmid. The promoter fragment is labeled only on the bottom strand, which is complementary to the RNA strand. The desired promoter fragment is then gel purified and allowed to diffuse out of a gel slice into TE for use in the S1 nuclease protection assay.

fragments that include the transcription start site and are isolated from genomic clones in plasmid vectors (see Fig. 2B). We find that the longer probes generated from a cloned fragment tend to generate cleaner results than from a synthetic oligonucleotide.

To make probes from cloned genomic DNA, the plasmid is first cut at a restriction endonuclease site typically chosen to be 50–300 bp downstream from the transcription start site (see Fig. 2B). Ideally, this site is unique in the clone and leaves a 5' overhang (sticky end). The cut DNA is then dephosphorylated with alkaline phosphatase and is 5' end-labeled using T4 polynucleotide kinase and γ -³²P-ATP. The plasmid is then cut with a second restriction enzyme at a site upstream of the transcription start site, thus liberating the labeled fragment from the plasmid, which is also labeled. The desired fragment that includes the transcription start site is then gel purified, using a 5% non-denaturing polyacrylamide gel (Fig. 2B). Although this probe fragment is double stranded, only the strand complementary to the RNA is labeled. If desired, one can isolate only this labeled strand after NaOH denaturation of the duplex and loading onto a long, strand-separating gel (Sambrook and Russell, 2001). However, we typically find the use of strand-separating gel electrophoresis to be unnecessary because by denaturing the double-stranded DNA fragment in the presence of the RNA to be tested and allowing hybridization to take place in the presence of a high concentration of formamide, RNA–DNA hybrid molecules are favored over the DNA–DNA duplexes that would result from reannealing of the probe fragment. Resulting RNA–DNA hybrids are then treated with S1 nuclease and the digestion products are resolved on a sequencing gel and visualized by autoradiography. As with the primer extension assay, end-labeled size markers are run on the gel as controls adjacent to the S1 digestion products. Alternatively, an end-labeled primer (25–30 nt) whose labeled 5' end corresponds precisely to the labeled 5' end of the S1 probe can be used to generate a dideoxynucleotide sequencing ladder. The sequence in the ladder corresponding to the size of the S1-protected probe fragment defines the transcription start site.

An example of the use of the S1 nuclease protection assay to discriminate rRNA transcripts from two parents of a hybrid is shown in Fig. 3. rRNAs of *A. lyrata* and *A. thaliana* are highly conserved in sequence but have occasional nucleotide substitutions, including short intervals of 2–3 polymorphic nucleotides. Thus, the S1 nuclease protection assay can be used to discriminate and quantify the relative expression levels of parental rRNA genes in F1 hybrids of *A. thaliana* and *A. lyrata*. Using probes derived from PCR-generated genomic clones, one specific for *A. thaliana* and one specific for *A. lyrata*, the experiment revealed that F1 hybrids

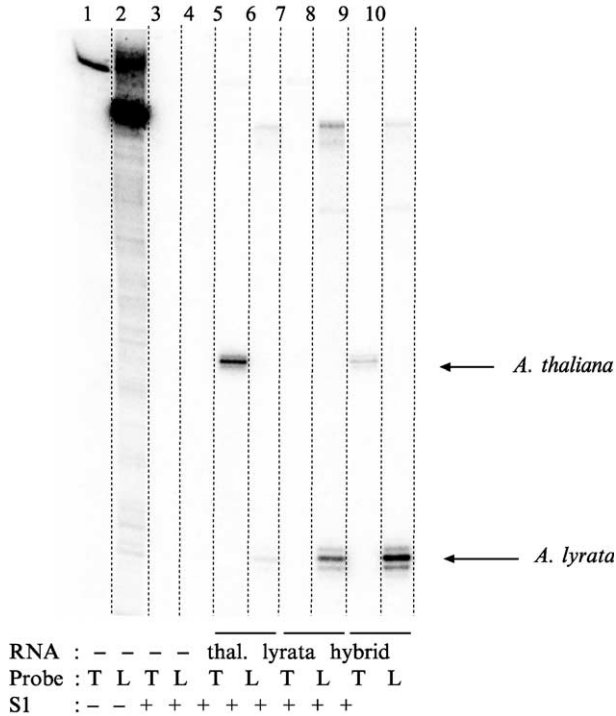


FIG. 3. Use of the S1 nuclease protection assay to reveal nucleolar dominance in F1 hybrids of *Arabidopsis thaliana* × *Arabidopsis lyrata*. Twenty micrograms of *A. thaliana* (T), *A. lyrata* (L), or F1 hybrid total RNA was hybridized with S1 probes specific for *A. thaliana* or *A. lyrata* ribosomal RNA (rRNA) transcripts. After S1 nuclease digestion and electrophoresis through a sequencing gel, the gel was dried and exposed to a PhosphorImager screen to produce the image shown. Controls include aliquots of the probes, to help recognize bands corresponding to undigested probe in the experimental lanes (lanes 1 and 2); mock reactions that include probe but not RNA, to control for possible S1 digestion products not derived from DNA–RNA hybrids (lanes 3 and 4); and tests of the two probes with RNA from the two parents, to demonstrate the parent specificity of the probes and to determine whether their specific activities are similar (lanes 5–8). The two experimentally meaningful lanes (lanes 9 and 10) test RNA purified from an *A. thaliana* × *A. lyrata* F1 hybrid. Comparison of these two lanes reveals low levels of *A. thaliana* rRNA and 10-fold higher levels of *A. lyrata* rRNA transcripts in the hybrid.

express the *A. lyrata* transcripts at about 10-fold higher levels than *A. thaliana* transcripts (Fig. 3, compare lanes 9 and 10), providing another example of nucleolar dominance.

Solutions Needed

- A. Probe-RNA hybridization buffer: 40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA, 80% deionized formamide
- B. S1 digestion buffer: 5% glycerol, 1 mM zinc sulfate, 30 mM sodium acetate (pH 4.5), 50 mM NaCl
- C. Formamide-based gel loading buffer (see the section “[Primer Extension Assay for Detection of Allele-Specific Transcripts](#),” earlier in this chapter).

S1 Probe Preparation

The S1 nuclease protection assay uses radioactive probes, so take the appropriate precautions for the safe handling of radioisotopes, some of which were mentioned previously in the section “[Primer Extension Assay for Detection of Allele-Specific Transcripts](#).”

1. Cut about 2 μg of plasmid DNA containing the cloned promoter region of interest, using 5–10 units of a restriction endonuclease that cleaves downstream from the transcription start site.

2. Dephosphorylate the 5' ends by addition of 5–10 units of shrimp alkaline phosphatase and incubation at 37° for 60 min. Heat-inactivate the shrimp alkaline phosphatase at 65° for 20 min.

3. Precipitate the dephosphorylated DNA by addition of 1/10 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of cold (–20°) absolute ethanol. Mix by vortexing, store on ice 5 min, and centrifuge at 14,000g for 15 min. Wash the DNA pellet with 70% ethanol, dry the pellet, and resuspend in 6 μl of sterile water or TE, pH 8.0. (Note that steps 1–3 can be scaled up 10-fold to yield enough dephosphorylated DNA for 10 or more experiments.)

4. End-label the DNA using T4 polynucleotide kinase and γ -³²P-ATP.
 - 6 μl dephosphorylated DNA (2 μg)
 - 2 μl 660 mM Tris-HCl pH 7.5
 - 2 μl 100 mM DTT
 - 2 μl 100 mM MgCl₂
 - 2 μl 10 mM spermidine
 - 1 μl T4 polynucleotide kinase (~5 units)
 - 5 μl gamma-labeled ³²P-ATP (10 $\mu\text{Ci}/\mu\text{l}$; 6000 Ci/mmol)

Incubate the labeling reaction at 37° for 60 min. Heat the reaction at 65° for 15 min to inactivate the kinase. Precipitate the labeled DNA by addition of 1/10 volume 3 M sodium acetate (pH 5.2) and 2 volumes of cold (–20°) absolute ethanol. Mix by vortexing, store on ice 5 min, and centrifuge at 14,000g for 15 min. Wash the DNA pellet with 70% ethanol, dry the pellet,

and resuspend in 20 μl of $1\times$ restriction endonuclease buffer (the buffer depends on the enzyme that will be used to cut the plasmid upstream of the transcription start site). Add 5–10 units of the restriction endonuclease and incubate at the appropriate temperature for 60 min.

5. Gel purify the probe DNA fragment by electrophoresis through a 5% native polyacrylamide gel. Pry apart the glass plates, cover the gel with plastic wrap, and expose the gel to X-ray film for 5–10 min, being sure to align one corner of the film with one corner of the gel plate inside the film holder. Lay the exposed film back down onto the gel, aligning the film as during the exposure step, and use a razor blade to trace the outline of the desired probe fragment, slicing through the film and the gel. Using forceps, transfer the radioactive gel slice to a 1.7-ml microcentrifuge tube and add 500 μl TE, pH 8.0. Incubate at 65° for 4 h, or overnight at 37°, to allow the labeled DNA to diffuse out of the gel slice.

6. Vortex the tube containing the gel slice in TE. Remove about 1/10 of the TE for each S1-protection reaction to be performed and pipette this into a fresh tube. Add 20–50 μg total RNA. Precipitate the RNA and probe together by addition of 1/10 volume 3 M sodium acetate (pH 5.2) and 2.5 volumes of cold (–20°) absolute ethanol. Vortex, store on ice 5–10 min, and centrifuge at 14,000g for 15–20 min. Wash the DNA pellet with 70% ethanol and then dry the pellet.

7. Resuspend the RNA and probe thoroughly in 30 μl of probe–RNA hybridization buffer. Add 50 μl of light mineral oil to prevent the reaction from evaporating during hybridization. Incubate the hybridization reaction at 90° in dry-bath incubator (or in a boiling waterbath after turning off the heat) for 15 min to denature the probe and RNA.

8. Carry the hot metal block of the dry-bath incubator to a waterbath set at the proper hybridizing temperature (generally 37–55°, depending on GC content and the length of hybrid that can form; usually, 37° works fine). Quickly move the tube(s) from the hot block to the waterbath.

9. Allow hybridization to proceed 2 h to overnight.

10. Open the tubes without removing them from the waterbath and add 270 μl of S1 digestion buffer containing about 200 units S1 nuclease/ml (S1 is added just before use). The amount of S1 nuclease can be increased in future experiments if necessary to achieve complete digestion.

11. Spin 5 s in a Microfuge tube to get the S1 digestion mix below the layer of mineral oil. Vortex briefly to mix and repeat the spin to get the thin layer of oil at the top again.

12. Incubate the S1 digestion reaction 30–45 min at 37°.

13. Stop the reactions by removing 280 μl from the bottom of each reaction tube (i.e., avoid paraffin oil) to a fresh tube containing 10 μl of 10%

sodium dodecyl sulfate (SDS) and 5 μl of 0.5 M ethylenediaminetetraacetic acid (EDTA) (the EDTA chelates the zinc required for S1 nuclease activity). Vortex briefly to mix.

14. Add 30 μl of 7.5 M ammonium acetate and vortex to mix. Add 1 ml cold (-20°) absolute ethanol to precipitate the DNA–RNA hybrid and vortex. Store on ice 5–10 min and then centrifuge at 14,000g for 15–20 min.

15. Carefully remove supernatant (pellets may not be visible) and discard in radioactive waste container. Wash pellet with 70% ethanol and dry pellets under vacuum (or in 65° waterbath with tube caps open).

16. Resuspend the pellets in 6–8 μl of formamide-based sequencing gel-loading buffer and conduct denaturing gel electrophoresis and autoradiography as described for primer extension.

RT-CAPS Assay of Allele-Specific Transcripts

Reverse transcription of RNA, followed by CAPS analysis, exploits polymorphic restriction sites within the transcribed portions of different alleles to discriminate their transcripts. Because the only difference needed to differentiate sequences is a single nucleotide polymorphism, this method can sometimes be used when there are insufficient sequence differences for S1 nuclease protection or primer extension assays to be employed. RT-CAPS requires smaller quantities of RNA than S1 nuclease protection or primer extension and is generally less time consuming, especially when the number of samples to be assayed is large.

An example of the use of RT-CAPS is the analysis of nucleolar dominance in *A. suecica*, the allotetraploid hybrid of *A. thaliana* and *A. arenosa*. A single nucleotide polymorphism in internal transcribed spacer sequence 1 (ITS1) results in a polymorphic *Hha* I restriction endonuclease site (Fig. 4) that is present in *A. arenosa* rRNA genes but missing in *A. thaliana* rRNA genes. If one performs simple CAPS analysis using genomic DNA as the template for PCR, the *Hha* I restriction fragment patterns for the two parental species are different (Fig. 4, lanes 1 and 2). The *Hha* I fragment pattern for the hybrid is the sum of the parental patterns, indicating that both parental sets of rRNA genes are present in *A. suecica* (lane 3). If RNA from the parental species is converted into cDNA using RT and then subjected to CAPS, the same fragment patterns seen using genomic DNA are observed (lanes 4 and 5), as expected. However, RT-CAPS performed using RNA of the allotetraploid hybrid, *A. suecica* reveals the banding pattern expected for *A. arenosa* rRNA transcripts but only trace amounts of the *A. thaliana* specific band is detected (lane 6), indicating

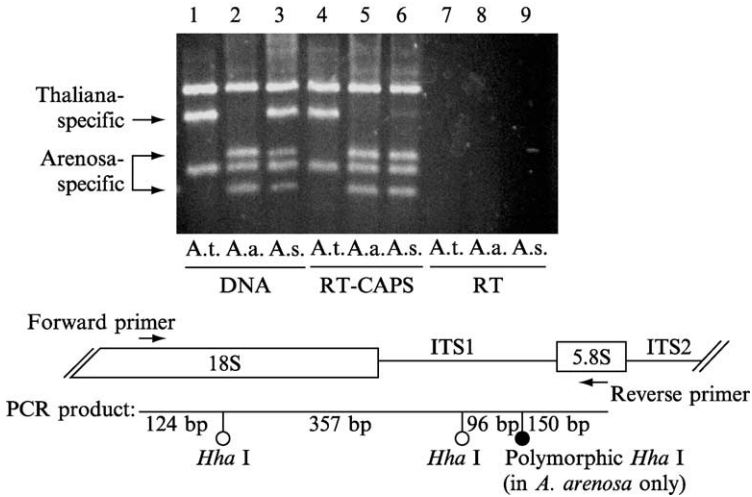


FIG. 4. Use of the reverse-transcription–cleaved amplified polymorphic sequence (RT-CAPS) assay to show that *Arabidopsis thaliana* ribosomal RNA (rRNA) genes are repressed and *Arabidopsis arenosa* rRNA genes are dominant in the allotetraploid hybrid *Arabidopsis suecica*. Genomic DNA (lanes 1–3) or reverse-transcribed (RT) total RNA (lanes 4–6) was amplified by polymerase chain reaction (PCR) using primers flanking internal transcribed spacer 1 (ITS1; see diagram) and then cleaved using the restriction endonuclease *Hha* I. An extra (polymorphic) *Hha* I site in ITS1 of *A. arenosa* rRNA genes allows *A. arenosa* (A.a.) and *A. thaliana* (A.t.) genes and their transcripts to be discriminated in *A. suecica* (A.s.). Both progenitors' rRNA genes are present in *A. suecica* (lane 3), but only *A. arenosa* rRNA gene transcripts are abundant in the hybrid (lane 6). Controls in which RNA samples were not incubated with reverse transcriptase before PCR show that the RNA samples are free of contaminating DNA (lanes 7–9). Reprinted, with permission and minor modifications, from Lewis and Pikaard (2001).

that the *A. thaliana* rRNA genes are mostly silenced. As controls, mock reactions that include RNA but no reverse transcriptase are performed to be sure that contaminating genomic DNA is not contributing to the signals (lanes 7–9).

Reagents Needed

- A. RQ1 DNase reaction buffer and stop solution (Promega).
- B. RT kit (e.g., Invitrogen Superscript kits).
- C. 10X PCR buffer (various commercial sources).
- D. Restriction endonuclease and buffer (various commercial sources).

Procedure

1. Isolate total RNA from both the hybrid (or allopolyploid) and the parental species. Treat 500 ng of total RNA from each species with RNase-free DNase (we use Promega RQ1 DNase) in a 10- μ l reaction to eliminate contaminating genomic DNA. Incubate for 30 min at 37°. Stop the reaction with 1 μ l of RQ1 stop solution. Incubate for 15 min at 65° to inactivate the enzyme.

2. Add 5 μ l of the DNase-treated RNA to a 20 μ l RT reaction using 125 ng of random hexamer oligonucleotide primers (New England BioLabs) to prime cDNA synthesis. For the RT reaction itself, we use the reagents supplied in the Superscript Reverse Transcriptase kit (Invitrogen). As a negative control, add 5 μ l of DNase-treated RNA to a 20 μ l reaction but omit the RT. Incubate the RT reaction for 50 min at 42°. Stop the reaction by heating at 85° for 5 min.

3. Perform a 50 μ l PCR containing the following:

1-2 μ l of RT reaction product (or -RT control)

5 μ l 10 \times PCR buffer

0.2 mM each dNTP (dATP, dCTP, dGTP, TTP)

2.5 mM MgCl₂

10-20 pmol each of the forward and reverse primers

2 units *Taq* polymerase

Perform 25-35 cycles of PCR using conditions appropriate for the primers chosen.

4. Digest 20 μ l of the PCR with the restriction enzyme that will reveal the polymorphism. It may be necessary to first ethanol precipitate the PCR products if the PCR buffer is incompatible with the restriction endonuclease to be used.

5. Analyze digestion products on a 1-2% agarose gel and visualize DNA bands by staining with ethidium bromide or an alternative reagent.

Concluding Remarks

Of the three techniques described in this chapter, we tend to use S1 nuclease protection most frequently. Despite being the most laborious of the three assays, the S1 nuclease protection assay consistently yields the most reliable quantitative data. Presumably, this is due to the longer, more stable probe-RNA hybrids that are formed, compared to the primer-RNA hybrids formed for the primer extension assay. Furthermore, the S1 nuclease protection assay does not require signal amplification as in the PCR-based RT-CAPS method, which can lead to skewed results. In our hands, RT-CAPS analysis typically makes differences in allele expression appear smaller than they really are. For this reason, we strongly advise that

any differences in allele expression found between two alleles using the RT-CAPS method be confirmed using a second technique.

For additional discussion of the S1 nuclease protection assay, primer extension assay, and RT-PCR, including detailed protocols that may vary slightly from ours, we highly recommend the *Molecular Cloning Laboratory Manual* (Sambrook and Russell, 2001).

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