Extracting DNA from Cambium Tissue for Analysis of Grape Rootstocks

Hong Lin and M. Andrew Walker

Department of Viticulture and Enology, University of California, Davis, CA 95616-8749

Additional index words. RAPD, RFLP, SSR, microsatellite, *Vitis* spp., *Muscadinia rotundifolia*, leaf tissue

Abstract. The DNA extracted from cambium tissues of grape (*Vitis* spp., *Muscadinia rotundifolia* Small) rootstocks was found to be suitable for molecular analysis. Its quality was equivalent to that of DNA extracted from leaf tissues, although the yield was higher from leaves. The use of cambium tissue allows DNA extractions during dormancy or from grafted rootstocks where leaves are not available. The DNA extracted was suitable for restriction enzyme digestion and for analysis by restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD), and simple sequence repeats.

Identification of species, varieties, cultivars, and hybrids of grape has traditionally depended upon leaf and fruit characteristics. Utilizing these characters for identification is a practiced skill and is made more difficult by environmental changes and the developmental stage of the plant. There are relatively few commercially used grape rootstocks, but many of them originate from the same or very similar hybrid parentage and can be difficult to accurately identify using leaf characters. When grapes are properly propagated and grafted, foliage from the rootstock portion of a grafted plant is rarely present, and growers must rely on nursery records to ascertain the identity of their rootstocks. Rootstocks with the same or similar parentage may have very different viticultural characteristics, and the proper choice of rootstock may be essential for optimal growth and fruit quality or even vine survival. Thus, it is important to correctly identify rootstocks.

Isozyme analysis has been used to distinguish rootstocks and fruiting cultivars (Walker and Liu, 1995; Weeden et al., 1988; Wolf, 1976), but this technique is often restricted by limited numbers of detectable loci, limited polymorphisms, and seasonal or developmental expression of certain gene products. The use of DNA-based molecular markers for identification overcomes these problems, and several have been used, including RFLP (Bourquin et al., 1993), RAPD (Wu and Lin, 1994), and simple sequence repeats (SSR) (Bowers et al., 1996; Rongwen et al., 1995; Thomas and Scott, 1993).

Received for publication 31 Oct. 1996. Accepted for publication 3 Apr. 1997. Financial support for this project from the American Vineyard Foundation, the California Table Grape Commission, and the California Grape Rootstock Improvement Commission is gratefully acknowledged. We thank Carole Meredith for supplying the probe and primers used in RFLP and SSR analysis and Doug Adams and Sekar Arulsekar for critiquing this manuscript. The cost of publishing this paper was defrayed in part by the payment of page charges. Under postal regulations, this paper therefore must be hereby marked *advertisement* solely to indicate this fact.

Most DNA extraction techniques rely on leaves to produce high quality DNA in sufficient quantities for analysis. However, extraction of high-quality DNA from grape leaves is often difficult because of secondary products such as polysaccharides and polyphenolic compounds (Couch and Fritz, 1990; Lodhi et al., 1994). Since grafted rootstocks have no rootstock leaves except in the rare case of suckering from below the union, a reliable technique is needed to extract DNA from bark. This technique should allow extraction of high quality and sufficient quantities of DNA to allow development of markers for identification and other purposes. We present here a method of DNA extraction from cambium tissues of grape canes and trunks, which produces restriction enzyme digestible DNA suitable for RFLP, RAPD, and SSR analyses.

Materials and Methods

Plant materials. DNA was extracted from cambium and leaf tissues collected from eight grape rootstocks growing in the Dept. of Viticulture and Enology, Univ. of California, Davis, vineyards (Table 1). Five *Vitis* species and *Muscadinia rotundifolia* Small are included in the parentage of these rootstocks. Cambium

tissue was prepared for DNA extraction by peeling off the epidermis and bark tissue of a cane, followed by scraping the cambium layer, including cortical parenchyma cells, with a knife. The DNA was also prepared from leaves of tissue-cultured plants of the rootstocks.

DNA extraction. The extraction procedure was modified from the method described by Richards (1993) by the inclusion of PVP (polyvinylpyrrolidone), CTAB (hexadecyltrimethylammonium bromide), and β -mercaptoethanol. PVP and CTAB help to complex polyphenolic and polysaccharide compounds. The addition of β -mercaptoethanol ensures a reducing condition preventing phenolic compounds from being oxidized during extraction. The following solutions were used during the extraction process. The extraction buffer contained 20 mM EDTA (ethylenediaminetetraacetic acid), 350 mM sorbitol in 100 mM Tris[hydroxymethyl] aminomethane-HCl, pH 7.5. Soluble PVP [2% (m/v)] and β -mercaptoethanol [0.2% (v/v)] were added just before use. The lysis buffer contained 50 mM EDTA, 2 M NaCl, 2% (w/v) CTAB in 200 mM Tris-HCl, pH 7.5. Other solutions utilized were 5% sarcosyl (N-lauroylsarcosine); 24 chloroform : 1 octanol (v/v); isopropanol; RNase A (5.3 mg·mL⁻¹); and TE (Tris-EDTA) buffer consisting of 10 mM Tris-HCl and 1 mM EDTA, pH 8.0.

The plant tissue (0.5 g) was ground to a powder in a mortar containing liquid nitrogen. Three mL of the DNA extraction buffer was added and the suspension was briefly homogenized with a mortar and pestle for 5 to 10 s. The suspension was centrifuged in a benchtop centrifuge at 8000 *g*_n for 5 min and the supernatant was discarded. The pellet was resuspended in 0.6 mL of DNA extraction buffer minus the PVP. An 0.8-mL volume of DNA lysis buffer and 0.6 mL of 5% sarcosyl were added and mixed well. The extract was incubated at 65 °C for 45 min. A 2-mL volume of chloroform : octanol was added and mixed, then the suspension was centrifuged at 5000 *g*_n for 10 min. The upper aqueous phase was carefully transferred into a new tube. The chloroform : octanol extraction was repeated once.

An equal volume of isopropanol was added to the extract, which was then chilled at -20 °C for 30 min, and centrifuged at 12,000 *g*_n for 10

Table 1. Absorbance ratios and yields of DNA from tissues of several grape rootstocks.

Rootstock	Parentage	Tissue	Absorbance ratio (260/280)	DNA yield (µg DNA/g fresh mass)
AXR#1	<i>Vitis vinifera</i> x <i>V. rupestris</i>	Leaves (tissue cultured plants)	1.85	120
		Cambium—fresh canes ²	1.78	21
		Cambium—trunk sample	1.81	29
O39-16	<i>V. vinifera</i> x <i>Muscadinia rotundifolia</i>	Leaves—tissue-cultured plants	1.83	165
1103P	<i>V. berlandieri</i> x <i>V. rupestris</i>	Cambium—stored canes ³	1.80	29
		Cambium—fresh canes	1.82	47
		Cambium—stored canes	1.76	38
110R	<i>V. berlandieri</i> x <i>V. rupestris</i>	Cambium—trunk sample	1.74	42
5C	<i>V. berlandieri</i> x <i>V. riparia</i>	Cambium—trunk sample	1.80	32
St. George	<i>V. rupestris</i>	Cambium—trunk sample	1.84	28
3309C	<i>V. riparia</i> x <i>V. rupestris</i>	Cambium—trunk sample	1.85	36
Ramsey	<i>V. champinii</i>	Cambium—trunk sample	1.69	37

²Lignified canes taken from the vineyard in late winter.

³Canes stored in plastic bags at 1 °C for 1 year.

min. The supernatant was decanted and the tubes were washed twice with 0.5 mL of 75% ethanol. The tube was air dried for 20 min. The DNA was redissolved in 200 μ L of TE buffer, 2 μ L of RNase A added, and the solution was incubated at 37 °C for 20 min. The DNA was re-extracted once with an equal amount of chloroform : octanol, the extract was mixed with 40 μ L of 5M NaCl and 200 μ L of isopropanol, and the DNA was precipitated as described above. The DNA pellet was then redissolved in 200 μ L TE buffer.

DNA assessment. DNA quality and quantity were determined by restriction enzyme digestion, gel electrophoresis, spectrophotometric measurement, RFLP, RAPD, and SSR analysis.

DNA samples were digested with restriction enzymes (*Eco*RI, *Hind*III, *Pst*I), and then cut and uncut DNA were subjected to electrophoresis.

For RFLP, about 5 μ g of DNA was subjected to *Hind*III digestion. Digested DNA samples (5 μ g DNA/well) were separated in 0.8% agarose (Seakem, FMC Bio Products, Rockland, Maine) gel at 35 V for 36 h. Southern blotting was performed as described by Brown (1993). Probe 6E8 (provided by C.P. Meredith, Dept. of Viticulture and Enology, Univ. of California, Davis) was labeled with 32P-dCTP.

For RAPD analysis, DNA was tested using Operon primers (OPA-01, 5' CAGGCCCTTC 3'; OPA-03, 5' AGTCAGCCAC 3'; OPA-04, 5' AATCGGGCTG 3'; and OPA-10, 5' GTGATCGCAG 3') according to the protocol described by Williams et al. (1990). The reaction tubes were placed in a PTC-100 thermal controller (MJ Research, Watertown, Mass.) that was preheated to 94 °C and subjected to the following cycling program: 4 cycles of 1 min at 94 °C, 1.5 min at 37 °C, and 2 min at 72 °C, followed by 30 cycles of 0.5 min at 94 °C, 0.5 min at 37 °C, and 1 min at 72 °C. Amplified products were separated on a 1.8% agarose gel and viewed under UV light after ethidium bromide staining.

For SSR, DNA was analyzed with the grape primers VVMD-5, VVMD-6, VVMD7, and VVMD31 (Bowers et al., 1996). About 20 ng of DNA was mixed in a 20 μ L reaction mixture containing 2 μ L of 10x buffer, 0.6 U *Taq* DNA polymerase (Perkin Elmer, Foster, Calif.), 10 pmole of the forward and reverse primers with 1 mM MgCl₂, and 0.2 mM dNTPs. The PCR amplifications were the same as for RAPD analysis except that the annealing temperature was 52 or 56 °C, depending on the primer being used (Bowers et al., 1996). Amplified DNA was separated on an 6% polyacrylamide gel and visualized by silver staining (Promega, Madison, Wis.).

To further test the practical application of DNA analysis from cambium tissue in vineyard situations, DNA was extracted from a 1.5-cm disk of tissue collected at the base of six different 20-year-old rootstock trunks with a cork borer (Table 1, Fig. 4). This disk of tissue was placed between moist paper towels and transported to the lab in plastic bags. DNA was extracted within 1 d.

Results and Discussion

Table 1 lists the results of spectrophotometer readings from some of the rootstock tissues used for DNA analysis. DNA was successfully extracted from the cambium tissue of grape, but yields were 5 to 8 times lower when compared with extractions made from leaves (Table 1). In all cases the A260/A280 values were $\approx$ 1.8, indicating that cambium DNA was relatively free of proteins and other secondary products. Agarose (0.8%) gel electrophoresis revealed that the size of genomic DNA from cambium tissue was comparable with that extracted from leaves (data not shown). DNA quality and quantity was very similar when extractions from cambium of fresh and stored (in plastic bags at 1 °C for 1 year) canes were compared (see data for rootstock 1103P in Table 1).

The DNA extracted from cambium was readily digestible with restriction endonucleases (*Eco*RI, *Hind*III, *Pst*I). RFLP analysis with AXR#1 revealed identical banding patterns for leaf and cambium DNA (Fig. 1). No differences were found between the DNA extracted from cambium vs. leaf tissues of AXR#1 and O39-16 with RAPD (Fig. 2) or from 110R, 5C, AXR#1 and O39-16 with SSR analyses using VVMD6 (Fig. 3). We have used DNA from leaf and cambium sources to produce fingerprints for more than 30 different rootstocks; no differences were observed due to DNA source (data not shown). These tests confirm that the quality of DNA extracted from cambium is equivalent to that extracted

Fig. 1. RFLP patterns comparing DNA extracted from cambium (C) and leaf (L) tissues of the grape rootstock AXR#1 with probe 6E8.

Fig. 2. RAPD comparison of DNA extracted from cambium (C) and leaf (L) tissues of grape rootstocks AXR#1 and O39-16. Separate DNA extractions are designated by 1 and 2. The set of comparisons on the left was produced with primer OPA-01 and the set on the right with primer OPA-03. The far left lane in each comparison is a 123-bp DNA ladder.

from leaves. DNA was also extracted from the cambium at the base of six 20-year-old rootstocks. After RAPD analysis, this DNA yielded consistent and reproducible amplification banding patterns (see results for three rootstocks in Fig. 4).

Results from RFLP, RAPD, and SSR analysis demonstrate that DNA extracted from cambium tissue can be used for molecular analysis. This method is particularly useful in situations where young leaves are unavailable. In addition, DNA extracted from cambium tissue may also reduce surface contamination, which might be a potential problem when DNA is extracted from leaves.

Extracts were prepared from fresh canes harvested in late winter, or from canes stored in plastic bags at 1 °C for 1 year. Preparation of DNA restriction digests from the cambium tissue of some genotypes has been difficult during early dormancy (data not presented). The cambium tissues of these genotypes may be very high in polysaccharides at this time of year, which would reduce the quality of extracted DNA. However, PCR amplifications have worked with DNA from canes taken at any time of the year (data not presented).

The extraction method presented here provides a practical and reliable technique for obtaining DNA from the cambium tissue of grape. This technique is essential if DNA analysis is needed from dormant cuttings or vines, or from grape rootstocks in grafted combinations where foliage is not present. We have repeatedly performed RAPD and SSR analysis on cambium tissue DNA extracted from the lignified canes and trunks of rootstocks. The RAPD and SSR polymorphisms found among these rootstocks were consistent, and identical to those found when leaves were extracted. Extraction from cambium tissue provides a year-round source for grape rootstock DNA analysis and should work well for many other woody deciduous plants.

Literature Cited

- Bourquin, J.C., A. Sonko, L. Otten, and B. Walter. 1993. Restriction fragment length polymorphism and molecular taxonomy in *Vitis vinifera* L. *Theor. Appl. Genet.* 87:431–438.
- Bowers, J.E., G.S. Dangel, R. Vignani, and C.P. Meredith. 1996. Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). *Genome* 39:628–633.
- Brown, T. 1993. Preparation and analysis of DNA, p. 2.9.1–15. In: F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (eds.). *Current protocols in molecular biology*, vol. I. Greene Publishing Assoc., and Wiley, Plainview, New York.
- Couch, J. and P. Fritz. 1990. Isolation of DNA from plants high in polyphenolics. *Plant Mol. Biol. Rpt.* 8:8–12.
- Lodhi, M.A., G.N. Ye, N.F. Weeden, and B. Reisch. 1994. A simple and efficient method for DNA extraction from grapevine cultivars and *Vitis* species. *Plant Mol. Biol. Rpt.* 12:613.
- Richards, E. 1993. Preparation of genomic DNA from plant tissue, p. 2.3.1–3. In: F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, and K. Struhl (eds.). *Current protocols in molecular biology*, Vol. I. Greene Publishing Assoc., and Wiley, Plainview, New York.
- Rongwen, J., M.S. Akkaya, A. A. Bhagwat, U. Lavi, and P.B. Cregan. 1995. The use of microsatellite DNA markers for soybean genotype identification. *Theor. Appl. Genet.* 90:43–48.
- Thomas, M.S. and N.S. Scott. 1993. Microsatellite repeats in grapevine reveal DNA polymorphism when analyzed as sequence-tagged sites (STSs). *Theor. Appl. Genet.* 86:985–990.
- Walker, M.A. and L. Liu. 1995. The use of isozymes to identify 60 grape rootstocks (*Vitis* spp.). *Amer. J. Enol. Viticult.* 46:299–305.
- Weeden, N.F., B.I. Reisch, and M.E. Martens. 1988. Genetic analysis of isozyme polymorphism in grape. *J. Amer. Soc. Hort. Sci.* 113:765–769.
- Williams, J.G.K., A.R. Kuneth, K.J. Livak, J.A. Rafalski, and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Res.* 18:6531–6535.
- Wolf, W.H. 1976. Identification of grape varieties by isozyme banding patterns. *Amer. J. Enol. Viticult.* 27:68–73.
- Wu, L. and H. Lin. 1994. Identifying buffalograss [*Buchloe dactyloides* (Nutt) Engelm.] cultivar breeding lines using random amplified polymorphic DNA (RAPD) markers. *J. Amer. Soc. Hort. Sci.* 119:126–130.

Fig. 3. Polymorphic alleles from SSR analysis at the VVMD6 locus in cambium (C) and leaf (L) tissue DNA from four grape rootstocks (110R, 5C, AXR#1, and O39-16).

Fig. 4. RAPD polymorphisms from cambium tissue DNA of six grape rootstocks (5C, St. George, 110R, 3309C, Ramsey and AXR#1) amplified by PCR using Operon primers OPA-01, OPA-04, and OPA-10. The far left lane is a 123 bp DNA ladder.