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A simple, inexpensive and environmentally friendly method for high throughput DNA extraction from grapevine (*Vitis* spp.)

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Summary

The extraction of DNA is a basic tool for molecular analyses, which is difficult for grapevine due to its content of secondary metabolites. In terms of purity and yield, DNA extraction kits are superior; however, they are also expensive. Here we describe an efficient protocol to re-use the DNeasy 96 Plant Kit from Qiagen GmbH (Hilden, Germany) by preparing homemade buffers and regenerating the commercial 96 column plates. Leaf tissue extractions of different grapevine samples resulted in genomic DNA with a high molecular weight and sufficient purity suitable for molecular studies like restriction assays or various amplification analyses. This high throughput method is especially economical and does not require the use of laboratory robotics for pipetting steps.

K e y w o r d s : DNA extraction, leaf, grapevine.

Introduction

High-quality genomic DNA is required for most molecular analyses. Numerous DNA extraction protocols are already available for different plant species. Most of these, however, are not applicable to grapevine since this plant is rich in secondary metabolites, such as polysaccharides and phenolic compounds. These metabolites affect the extraction procedure, can interact irreversibly with nucleic acids, and interfere with the function of enzymes in subsequent analyses (Demeke and Adams 1992, Loomis 1974, Wilson 1997). Although a few protocols for DNA extraction from plants with high phenolic compounds are already established, their adaptation to high throughput methods has been found to be rather difficult due to the use of toxic organic solvents (Doyle and Doyle 1990, Lohdi *et al.* 1994). Alternatively, a number of commercial kits are available for extraction of high-quality DNA. Their application can become very expensive if large numbers of DNA samples are to be analyzed.

Here we describe a modified method optimized for isolation of DNA from *Vitis* species using homemade buffers and regenerated or re-loaded commercially available 96 column plates from the DNeasy 96 Plant Kit (Qiagen GmbH, Hilden, Germany). This protocol ensures the extraction of high quality DNA by use of silica membranes and offers many advantages: (1) easy handling and high efficiency, (2) exclusion of hazardous organic solvents, (3) re-use of column plates and other plastic components, (4) cost-effectiveness, and (5) the possibility for single sample extractions in a mini column format. The protocol is especially useful for breeders or small labs with a low budget and no access to laboratory robots for pipetting or DNA extraction.

Material and Methods

P l a n t m a t e r i a l : Plant material was taken from different accessions and cultivars of field grown *Vitis*-species of the grapevine repository at Geilweilerhof. Approximatly 80-100 mg of plant tissue was collected from 5 cm averaged young leaves, transferred to a 96 deepwell collecting plate (Fisher Scientific GmbH, Schwerte, Germany), frozen at -70 °C, and lyophilized. Dry plant material was stored at 5 °C until use. For single sample extraction, 2 mL safe lock tubes (e. g. Eppendorf AG, Hamburg, Germany) were used.

C o l u m n p r e p a r a t i o n : For DNA extraction, the commercially available "DNeasy 96 column plates" for nucleic acid extraction were used. (For single extractions, the DNeasy Mini Spin Column (Qiagen GmbH) was used). Plates and columns were regenerated by the application of MaxXBond regeneration kit "MB007" (AppliChem GmbH, Darmstadt, Germany) after every extraction procedure as follows: plates were first rinsed with 800 µL sterile water per well, incubated over night with 800 µL RG1 and centrifuged. Afterwards 800 µL RG2 buffer was applied and the plates centrifuged again. The flow-through was discarded, and the plates re-used. The columns were recharged after recurred regeneration (up to eight times) when the silica membranes were clogged or when their colour had turned to greenish or brown. The gasket rings were pulled out of the columns by a hook and the used membranes were removed with a pair of forceps. Plastic parts were rinsed with 1 % detergent (any commercial dishwashing liquid) and water to remove cell debris, incubated in 0.5 M HCl for 30 min to eliminate remaining DNA and rinsed again with 1 % detergent followed by sterile water. Cleaned plates and plastic components were autoclaved prior to further use. Fresh silica membranes were cut from GF/F borosilicate glass fiber paper (Whatman GmbH, Dassel, Germany) using a 8 mm paper punch (or for single columns a 7 mm

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paper punch was used). Two layers of silica membranes were placed in each column and fixed in place with the gasket ring. If larger yields of DNA were required, a third membrane was added and fixed.

Solutions: Lysis Buffer (0.5 % (w/v) SDS, 8 % (w/v) PVP-10, 250 mM sodium chloride, 25 mM Na_2-EDTA, 200 mM Tris-HCl pH 7.5. RNase A (0.25 mg·mL^{-1}) was added before use. RNase A (100 mg·mL^{-1}, 7,000 U·mL^{-1}) (Qiagen GmbH). Precipitation Buffer (5 M potassium acetate, pH 6.5 adjusted with acetic acid). Binding Buffer (2 M Guanidinhydrochloride, 75 % (v/v) Ethanol). Washing Buffer I (10 mM NaCl, 10 mM Tris-HCl pH 6.5, 80 % (v/v) Ethanol). Washing Buffer II (96 % Ethanol). Elution Buffer (10 mM Tris-HCl pH 8.5).

Extraction procedure: Lyophilized plant tissue was disrupted in a TissueLyser (Qiagen GmbH) for 2 x 1 min at 25 Hz using one 3 mm tungsten carbide bead per well (Qiagen GmbH). Pulverized tissue was suspended in 400 µL Lysis Buffer (preheated to 65 °C) and incubated at 65 °C for 10 min. Sample plates were centrifuged at 1,500 x *g* for 10 s in a Sigma centrifuge 4K15; for single columns a Sigma centrifuge 2K15 was used (Sigma-Aldrich GmbH, Munich, Germany). All centrifugation steps were performed at room temperature. After adding 200 µL Precipitation Buffer the tubes were closed with collection microtube caps (Qiagen GmbH) and the suspension was mixed by inversion. Samples were incubated at -20 °C for 15 min and centrifuged for 15 min at 4,000 x *g* to separate the cell debris. The supernatant was transferred to a new deepwell plate and centrifuged again (4,000 *g* x 15 min). Approximately 400 µL of supernatant was transferred into a new deepwell plate and mixed with 600 µL of Binding Buffer. The DNA-containing mixture (approx. 1 mL) was applied to a 96 column plate placed on a collection rack (S-Block, Qiagen GmbH). The 96 column plate was sealed with an AirPore Tape Sheet (Qiagen GmbH) and centrifuged at 6,000 x *g* for 4 min. The flow-through was discarded and 800 µL of Washing Buffer I added to each well. After resealing with AirPore Tape, the plate was centrifuged again at 6,000 x *g* for 4 min. The washing step was performed twice with Washing Buffer I followed by one wash with Washing Buffer II. An additional centrifugation step at 6,000 x *g* for 15 min ensured the complete removal of residual ethanol. The 96 column plate was placed on a multisample rack (Fisher Scientific GmbH) and DNA eluted from the membrane with 50-100 µL of Elution Buffer preheated to 65 °C. The elution step was repeated once.

For extraction in the single sample format the DNeasy Mini Spin Column (Qiagen GmbH) and 2 mL tubes (e.g. Eppendorf AG, Hamburg, Germany) were used following the protocol described above.

DNA-quantity and quality: Final DNA concentrations were estimated electrophoretically on 1 % agarose gels in comparison to a known amount of linear λ-DNA as standard. In addition, the DNA was subjected to restriction assays, PCR amplification and microsatellite locus analysis.

Restriction assays: Restriction reactions were performed with different enzymes (e.g. *Mse* I or *Eco* RI New England Biolabs GmbH, Frankfurt/Main, Germany). One unit of enzyme was combined with 500 ng of genomic DNA and incubated for 1h at 37 °C in the supplied buffer. The reaction products were separated on 1 % agarose gels.

Amplification of DNA fragments and microsatellite analysis of genomic DNA: All PCR amplifications were performed using the Gene Amp PCR System 9700 cycler (Applied Biosystems, Darmstadt, Germany).

Primers used for amplification of the chloroplast *atp*B-*rbc*L spacer were designed by MANEN *et al.* (1994). Each assay contained 2-5 ng of DNA, 20 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$, 2.5 mM each of forward and reverse primer, 0.2 mM of each dNTP (Axon Labortechnik GmbH, Kaiserslautern, Germany), 2.5 µg BSA (bovine serum albumine) and 0.1 U of *Taq* DNA polymerase (Invitek GmbH, Berlin, Germany). The cycler program was: 94 °C at 5 min followed by 35 cycles of 94 °C 1 min, 58 °C 1 min, 72 °C 1 min and a final amplification step at 72 °C for 10 min. The PCR products were separated on 1 % agarose gels.

Primers for VvMD37 were adopted from BOWERS *et al.* (1999). Amplification reactions were performed with 0.5 ng of genomic DNA, 1x PCR buffer containing 50 mM KCl; 10 mM Tris-HCl (pH 8.3); 1.5 mM $MgCl_2$, 0.4 µM of each primer, 0.125 µM of each dNTP, 0.2 U *Taq* DNA Polymerase (Roche Diagnostics GmbH, Mannheim, Germany). The cycling program was as follows: 95 °C at 2 min followed by 40 cycles of 95 °C 30 s, 56 °C 30 s, 72 °C 2 min and final elongation at 72 °C for 10 min. The amplification products were analyzed with an ABI 3130xl Genetic Analyzer (Applied Biosystems).

Results and Discussion

Here we present a protocol for high throughput DNA extraction optimized for grapevine leaves. The protocol is a modification of the DNeasy 96 plant kit (Qiagen GmbH). Using this method more than 3500 DNA samples were successfully extracted in four weeks by one person without the use of robotics for pipetting. The extraction results of eight representative samples from accessions of some widely investigated *Vitis* species (*V. riparia, V. aestivalis, V. amurensis, V. rupestris, V. berlanderi, V. cinerea, V. labrusca, V. vinifera)* are shown in the Figure A. DNA extraction from 80 to 100 mg of fresh tissue resulted in the yield of 10-30 µg DNA in a total elution volume of 100 µL. The DNA is of high quality and quantity compared to samples purified with commercial kits (e.g. DNeasy 96 Plant Kit (Qiagen GmbH); data not shown; see also JANSSEN *et al.* 1997). Samples of different grapevine species showed nearly equal amounts of DNA yield. The genomic DNA was homogenous in size. Only slight traces of DNA degradation but no RNA contaminations were visible on the gel.

This method is simple and allows extraction of DNA with a high average molecular weight. In addition, it allows

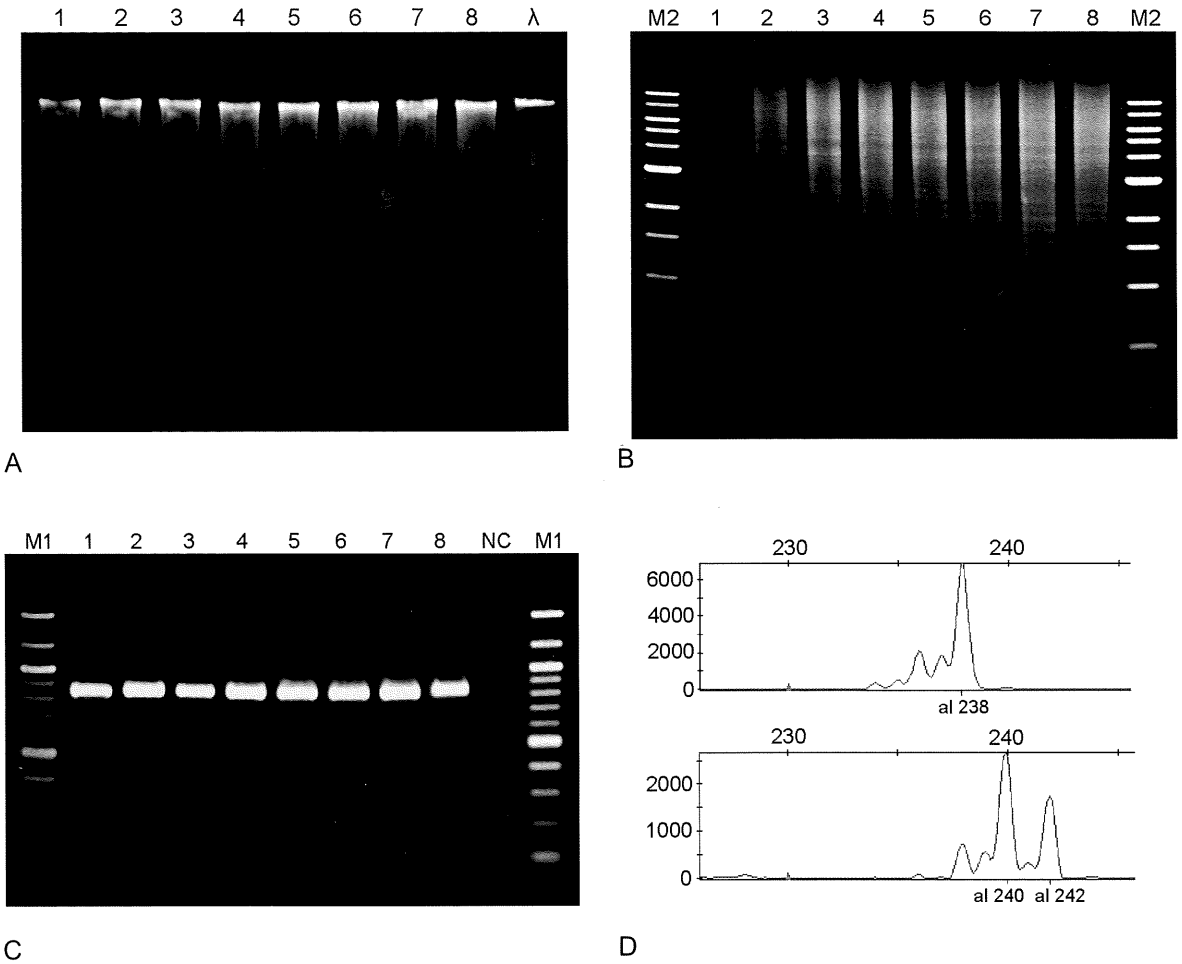

Figure: **A)** Extracted genomic DNA using the newly developed technique. **B)** *Eco*RI restriction of DNA. **C)** Amplification results using *atp*B-*rbc*L primers. - Amplification from cp DNA. **D)** Representative microsatellite analysis of sample 1 and 2 with VvMD37; data for samples 3-8 not shown. - Amplification from nuclear DNA. (**1**) *Vitis riparia,* (**2**) *Vitis aestivalis,* (**3**) *Vitis amurensis,* (**4**) *Vitis rupestris,* (**5**) *Vitis berlanderi,* (**6**) *Vitis cinerea,* (**7**) *Vitis labrusca* (**8**) *Vitis vinifera,* (λ) λ-DNA as standard 20 ng (New England Biolabs), (**M1**) 100bp DNA ladder, (**M2**) 1kb DNA ladder (New England Biolabs), (NC) Negative control.

the cheap and easy processing of large sample numbers (up to 384 samples per day) required for high throughput applications. In contrast, sampling in the field is a bottleneck due to the high time expenditure and high logistic effort.

The protocol is especially suitable for high throughput DNA extraction for downstream analysis, allowing rapid DNA extraction and simple screenings sufficient for large scale handling of samples. Optimization of the buffers reduces the inhibitory effects of polyphenolic compounds intrinsic to *Vitis* species. The extracted DNA is suitable for molecular investigations such as subsequent enzymatic treatments required in molecular analysis.

Restriction analysis of DNA is an important step for many analytical techniques. The method described here provides DNA suitable for restriction analysis with endonucleases and for amplification of nuclear and chloroplast DNA. Figure B gives an example of an *Eco*RI restriction assay (New England Biolabs) which appears as a smear on the agarose gel.

PCR analysis is another crucial technique in functional genomics which requires DNA of sufficient purity. Amplification of DNA fragments is the basic method for countless applications such as marker analysis or cloning. For standard amplification DNA extractions generated with this protocol can be used undiluted or diluted up to 50 fold.

We amplified a chloroplast *atp*B-*rbc*L spacer region which is highly conserved among higher plants (Manen *et al.* 1994). Successful PCR provided the expected fragment length of 845 bp for all analyzed samples (Figure C).

In addition a successful SSR analysis using primers flanking the region of the nuclear microsatellite VvMD37 (Bowers *et al.* 1999) could be performed. Figure D gives an example of an amplicon length determination profile of *V. riparia and V. aestivalis* with fragments at 238 bp and 240/242 bp.

Regenerated silica columns were tested for any potential contamination of genomic DNA by PCR amplification of different marker types (nuclear or cpDNA markers). No contamination could be detected (data not shown).

The preparation of homemade buffers and the regeneration or exchange of columns is slightly more time consuming than the application of the original kit. On the other hand, the costs of DNA extraction could be reduced up to

70 %. The use of the MaxXBond regeneration system and re-loading of silica columns prevent DNA contamination of the samples and allows re-using the multicolumn plates several times, respectively.

This newly established DNA extraction protocol avoids the use of organic solvents, produces no hazardous waste and generates much less plastic waste, thus contributing to environmental protection.

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