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RESEARCH ARTICLE

A rapid and cost effective protocol for plant genomic DNA isolation using regenerated silica columns in combination with CTAB extraction

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Abstract

Isolation of high quality DNA from multiple samples can be both time consuming and expensive. We have developed a combined protocol to reduce the time component of the hexadecyltrimethylammonium bromide (CTAB) extraction method and reduced costs by regenerating the silica columns used to purify genomic DNA. We present data that shows, by increasing the temperature used during the CTAB method, the time required to extract crude genomic DNA can be reduced. We show that silica columns can be regenerated using HCl and still maintain their DNA-binding capacity. Furthermore, we show both spectrophotometrically, and by restriction enzyme cutting, that the quality of the eluted DNA is high. Critically, using both genomic DNA from pea and perennial ryegrass we demonstrate, using species-specific PCR primers, that there is no carry-over of DNA from repeated use of a single column. The main advantages of the method are high yield, high quality, cost effectiveness and timesaving. This method could satisfy demand when large numbers of plant genomic DNA samples are required, for example from targeting induced local lesions in genomes (TILLING) populations.

Keywords: CTAB, DNA isolation, silica columns

1. Introduction

Isolation of high quality genomic DNA is necessary for many molecular biology applications. For plant genomic DNA isolation the popular method using CTAB (hexadecyltrimethylammonium bromide) is cost effective with high DNA yield and acceptable DNA quality, but this protocol is time consuming (Doyle and Doyle 1987; Allen *et al*. 2006). On the other hand, high quality DNA can be isolated rapidly using commercial DNA extraction kits with easy protocols and then the used columns are discarded (Deavours and Dixon 2005; Tesniere *et al*. 2006). However, the application of either the CTAB method or the use of commercial kits can be limited both in terms of time and expense when large numbers of DNA samples are required. As silica matrices are extraordinarily stable over extended time periods under mild acid conditions, it has been suggested that silica columns may be reused after acid treatment to remove any DNA carried over on the binding matrix (Siddappa *et al*. 2007). The authors reported on the successful use of regenerated columns to purify plasmids. However, there are few attempts to isolate plant genomic DNA using regenerated silica columns. Lemke *et al*. (2011) reported on the regeneration and reuse of Qiagen 'DNA Easy 96 column plates' and columns, but these were regenerated using a commercial regeneration kit.

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We are interested in detecting cytokinin oxidase/dehydrogenase mutants in perennial ryegrass for yield enhancement (Jameson and Song 2016), but this requires analysing leaf material from multiple single seed lines from numerous cultivars using EcoTILLING (Comai *et al*. 2004; Till *et al*. 2006; Song *et al*. 2015). In this paper, we report a rapid and cost-effective plant genomic DNA isolation protocol to meet the requirements for preparation of large numbers of genomic DNA samples for such applications as genotyping and large-scale mutation screening. In this method, regeneration of commercial silica columns (Qiagen DNeasy Plant Mini Kit, Cat. No. 69104, Qiagen, Hilden, Germany) was assessed. To test their reliability, silica columns regenerated over 10 cycles were used for plant genomic DNA isolation in combination with a modified CTAB method. We assessed retention capacity and quality of eluted DNA, as well as using PCR to test for cross contamination when isolating genomic DNA from two different species.

2. Materials and methods

2.1. Column regeneration

New Qiagen DNA extraction columns (Qiagen DNeasy Plant Mini Kit, Cat. No. 69104) were used to isolate genomic DNA from *Lolium perenne* (perennial ryegrass) following the manufacturer's protocol. The used columns were then washed thoroughly with water to remove any cell debris, and soaked in either 0.5 or 1 mol L^{-1} HCl for either 1, 4, 24 or 48 h. The columns were then rinsed thoroughly with sterile distilled water 3–5 times, and 500 µL equilibration buffer (QBT buffer)were added. The QBT buffer was prepared in-house based on the recipe supplied in the Qiagen Plasmid Purification Handbook and comprised 750 mmol L^{-1} NaCl, 50 mmol L^{-1} MOPS (3-(N-Morpholino) propanesulfonic acid) (pH 7.0), 15% (v/v) isopropanol, and 0.15% (v/v) Triton X-100. The columns were spun at 13 000×g for 1 min. The columns were ready for a fresh application and then used for up to 11 rounds of regeneration following the above steps. Two sets of controls were used. These were new columns used to isolate perennial ryegrass genomic DNA, but the columns were either not soaked or soaked in H_2O for 1, 4, 24, or 48 h. Elution buffer was applied to the columns and the eluate collected and treated as if it contained DNA. Eluted DNA was assessed using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and by gel electrophoresis on 1% (w/v) agarose gel prepared in 35 mL 1× TAE buffer (40 mmol L^{-1} Tris-acetate, 1 mmol L^{-1} EDTA (ethylenediaminetetraacetic acid)) and 2 µL SYBR™ Safe DNA Gel Stain (Invitrogen, USA).

2.2. DNA extraction procedure

Lysis buffer (2% CTAB buffer with 2% PVP40 (polyvinylpyrrolidone-40) (w/v); 2% β-mercaptoethanol (v/v) was added just before use) was pre-heated to 85°C. Up to 100 mg fresh weight (FW) of plant material was ground to a fine powder under liquid nitrogen using a pre-chilled mortar and pestle. Alternatively, when an Omni International Bead Ruptor 24 (Omni International, Kennesaw, Georgia, USA) was used, 100 mg tissue and 3 ceramic beads (2.8 mm Ceramic Bead Media 19-646-3; Omni International) were placed into a 2-mL freestanding microtube and stored in liquid nitrogen. Samples were disrupted using the bead ruptor with speed set at 3.9 m s^{-1} for 20 s. The powdered tissue was then scraped into a dry Axygen 1.7 mL microtube and 400 µL preheated lysis buffer was added, and the mixture incubated at 85°C for 10 min (this is a modification of the standard CTAB procedure). After 5 min incubation, the tubes were gently shaken for 5 s to disperse the material, and the incubation continued. The sample was spun at 20 000×g for 5 min at room temperature; 300 µL of the supernatant was transferred into a new 1.5 mL tube and mixed with 500 µL of binding buffer (2 mol L^{-1}) guanidine hydrochloride, 75 % (v/v) ethanol) and then 700 µL of this mixture was transferred onto new or regenerated columns. The columns were spun at 13 000×g for 1 min at room temperature. The flow-through was discarded. 700 µL of washing buffer I (10 mmol L^{-1} NaCl, 10 mmol L^{-1} Tris-HCl pH 6.5, 80 % (v/v) ethanol) was applied to each column, and the column centrifuged again at 13 000×g for 1 min. This step was repeated once. The columns were then washed with 700 µL of washing buffer II (96% v/v ethanol) and spun at 13 000×g for 1 min. The flow-through was discarded and the column centrifuged again at 13 000×g for 2 min. 50 µL elution buffer (comprising 10 mmol L^{-1} Tris-HCl pH 8.5, with RNase A (Qiagen Cat. No. 145012547) was added to a final concentration of 10 µg mL^{-1}), was preheated to 65°C, and applied to the column. The columns were spun at 13 000×g for 1 min to elute DNA. This step was repeated once using the elution buffer from the collection tube to obtain a greater DNA concentration. Alternatively, addition of a further 50 µL of elution buffer could be added and the column centrifuged again which would potentially increase yield, but reduce the DNA concentration. The eluted DNA was incubated at 37°C for 1 h to remove any remnants of RNA, and the DNA stored at –20°C.

2.3. Yield and quality determination

In order to determine the yield and quality of DNA obtained from regenerated columns, a single, pre-treated sample from 1 g FW perennial ryegrass leaves was used for the whole experiment. This was done so that identical amounts

of initial DNA could be applied to each test column. The leaves were ground to a fine powder under liquid nitrogen, the powder scraped into a dry 15 mL tube and 4 mL lysis buffer, preheated to 85°C, was added. The samples were incubated at 85°C and, because of the large volume, incubation time was extended to 18 min to lyse cells adequately. The solution was then spun at 20 000×g for 3 min at room temperature. The supernatant was transferred to a new tube. Following this, 300 µL of the mixture was transferred into new Axygen 1.7 mL microtubes and binding buffer (500 µL) was added; 700 µL of the mixture was then applied to 12 silica columns. The DNA was eluted as described above. The 12 columns included a new column and columns that had been regenerated between 1- and 11-times (regenerations 1 to 11). The experiment was repeated three times.

2.4. Assessing DNA quality using restriction enzymes

The concentration of perennial ryegrass genomic DNA extracted using new and regenerated columns was adjusted to 200 ng µL^{-1}. The DNA was cut with *BamH*I and *Hind*III (Thermo scientific, Cat. No. ER0051 and Cat. No. ER0501, respectively). Each assay contained 2 000 ng of DNA, 4 U of enzyme and 1× digestion buffer (with restriction enzyme), and was incubated for 1 h at 37°C. The reaction products were checked on 1% (w/v) agarose gels.

2.5. Quality check using amplification of genomic DNA by RT-qPCR

DNA collected from new, 1-, 5- and 10-times regenerated columns was tested. PCR primers for the elongation factor (EF) gene (LpsEFF: 5´CACCCTGGTCAGATCGGCAAC; LpsEFR: 5´CACCAACAGCAACAGTCTGCCT) were used for RT-qPCR that was carried out on a Rotor-Gene Q (Qiagen Helden, Germany). A homemade 2× SYBR Master Mix was used for the RT-qPCR (Song *et al*. 2012). Each reaction contained 100 ng of DNA. The cycler program was: 95°C for 10 min followed by 40 cycles of 95°C for 10 s, 58°C for 15 s and 72°C for 20 s. After amplification, a melting program was followed, with the temperature increasing from 72 to 95 at 1°C every 5 s. Cycle threshold (Ct) values and the melt curve analysis were generated by the Rotor-Gene Q Software (Qiagen, Hilden, Germany).

2.6. Testing for DNA cross contamination

To test whether there was carry-over of DNA in the regenerated columns, columns that had been used to isolate genomic DNA from perennial ryegrass and regenerated up to 5 and 10 times were used. Genomic DNA from *Pisum sativum* (pea) was applied to either a new column or to columns regenerated for five and 10 rounds (Re5 and Re10). The column purified DNA was then tested by PCR, using GAPDH (glyceraldehyde 3-phosphate dehydrogenase) primers specific for pea (PssGAPF: 5´TCTCTTCGGTCAGAAGCCAGTTAC; PssGAPR: 5´GCAGCTAGCATTGGAAATAATGTCAAAC) (Dhandapani *et al*. 2016; Jameson *et al*. 2016) and perennial ryegrass (LTZGAPF: 5´AGGAGGTTGCYGTSTTTGGCTG; LTZGAPR: 5´TAGCRTTRGAGACAATGKYGATGTCAGA) (Roche *et al*. 2016) to determine if there was any perennial ryegrass DNA from the regenerated columns contaminating the pea DNA.

The PCR program was: 94°C for 10 min followed by 35 cycles of 94°C for 20 s, 58°C for 30 s, 72°C for 30 s and a final amplification step at 72°C for 6 min. The PCR products were run on a 1% (w/v) agarose gel.

3. Results and discussion

When multiple DNA extractions are needed, the cost of silica columns can become prohibitive. If the silica columns can be reused, the cost can be substantially reduced. However, there is a significant risk if the DNA from previous rounds is not completely removed and is carried over in the silica column to contaminate a subsequent extraction, or if the capacity of the column to retain DNA is substantially reduced.

3.1. Modification of the CTAB method

Based on the traditional CTAB method, we used 2% CTAB buffer to lyse the plant cells but at 85°C, instead of at 60°C (Doyle and Doyle 1987) and successfully reduced the incubation time from 40 to 10 min. The integrity of DNA from samples incubated at 85°C for 10 min was similar to DNA from samples incubated at either 65 or 75°C for 20, 30 or

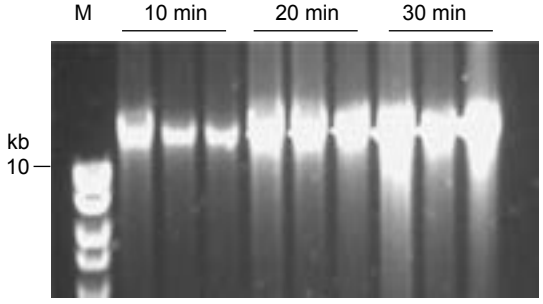

Fig. 1 Modification of hexadecyltrimethylammonium bromide (CTAB) method to reduce lysis incubation time. Ground samples, in three replicates, were incubated in lysis buffer preheated to 85°C for 10, 20 and 30 min. The DNA was purified through Qiagen DNeasy columns and the products applied to 1% (v/v) agarose gel. M, molecular weight marker (HyperLadder 1 kb, Bioline, UK). The same as below.

40 min (data not shown). The DNA product from the 85°C incubation was not degraded within 10 min and the yield and quality were still excellent (Fig. 1). However, a longer incubation at 85°C (e.g., for 20 min) led to degradation of the DNA (Fig. 1). In some cases, for example, when the DNA quality is more important than speed, an intermediate incubation temperature (60–65°C) should be tested.

3.2. Column regeneration

To determine the optimal method for cleaning the columns, HCl was used as first proposed by Siddappa *et al*. (2007). As shown in Fig. 2, following application of perennial ryegrass DNA to the columns, all DNA was removed when the columns were soaked in HCl, even in 0.5 mol L^{-1} HCl for as little as 1 h. In Siddappa *et al*. (2007), plasmid DNA cannot be visualized on the 1% agarose gel after 4 h incubation in 1 mol L^{-1} HCl and the DNA binding capacity of the columns was not reduced by prolonging soaking time in 1 mol L^{-1} HCl for as long as 30 d. It is still possible that a trace of DNA could be in the columns after 1 h, but not visible on our gels, so we propose that columns are soaked in 1 mol L^{-1} HCl overnight (16 h), with the columns then stored in 0.1 mol L^{-1} HCl. The columns must then be rinsed with dH_2O and QBT buffer before use.

3.3. Yield and quality of DNA were not compromised when regenerated columns were used

Using the above regeneration protocol, we tested whether the binding capacity of the columns declined with increasing numbers of regeneration. We found that there was some impact on binding but, even after 11 regenerations, the product concentration was still about 300 ng μL^{-1} (Fig. 3-A), which is sufficient for most experiments, including PCR and RT-qPCR. Furthermore, the integrity of the DNA was maintained. Fig. 3-B shows that the product was not degraded. In addition, when the purified DNA was assessed spectrophotometrically, acceptable values between 2 and 2.2 for ratios of 260/280 and 260/230 nm, respectively, were obtained (Appendix A).

A further test of the DNA quality included assessing the effectiveness of cutting by restriction enzymes. Restriction digestion is a commonly used molecular technique, which utilises restriction endonucleases. Two frequently used restriction enzymes, *BamH*I and *Hind*III, were used to test the quality of genomic DNA isolated by the protocol using new and regenerated columns. As shown in Fig. 4, the perennial ryegrass genomic DNA, from new, Re1, Re5 and Re10 columns, showed good integrity prior to enzyme digestion, but after 1 h of digestion, the DNA appeared as a smear on the gel, indicating that the DNA was completely cut by both *BamH*I and *Hind*III. This indicates that high quality DNA was produced even after the columns had been regenerated multiple times.

As isolated genomic DNA is frequently used for cloning, the quality of the DNA was also checked by amplification of genomic DNA using RT-qPCR. The purity of the genomic DNA is critical for PCR analysis, so the DNA from different regenerated columns was used for RT-qPCR. RT-qPCR can only be used reliably when the amplification is without error, so the quality of the template is critical to success. The fluorescence curves and the melt curve analysis (Fig. 5) show that the amplifications were performed successfully and the products were of high specificity. Based on these results, the DNA from different columns (new, Re1, Re5, Re10) is of high quality.

3.4. Sample-to-sample cross contamination did not occur

When HCl was used to clean columns, the result was

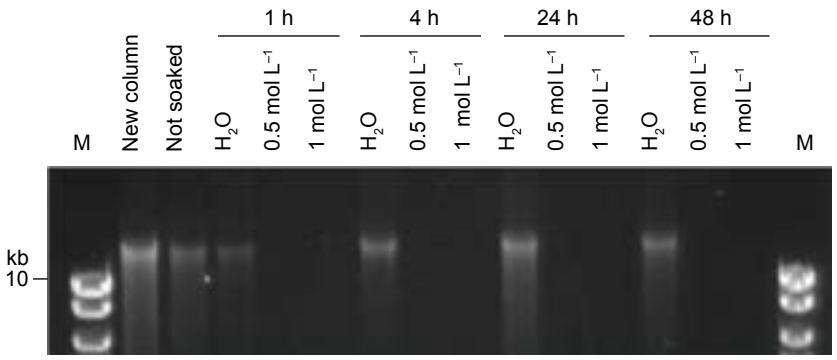

Fig. 2 Optimization of the regeneration protocol. After perennial ryegrass DNA was applied and eluted, the used columns were soaked in 0.5 mol L^{-1} HCl, 1 mol L^{-1} HCl or H_2O for 1, 4, 24, and 48 h. The soaked columns were washed with distilled water twice and QBT buffer once, and then eluted with 50 µL elution buffer. The controls included a used column without soaking and a new column to which DNA had been applied. All elution products were run on a 1% (w/v) agarose gel.

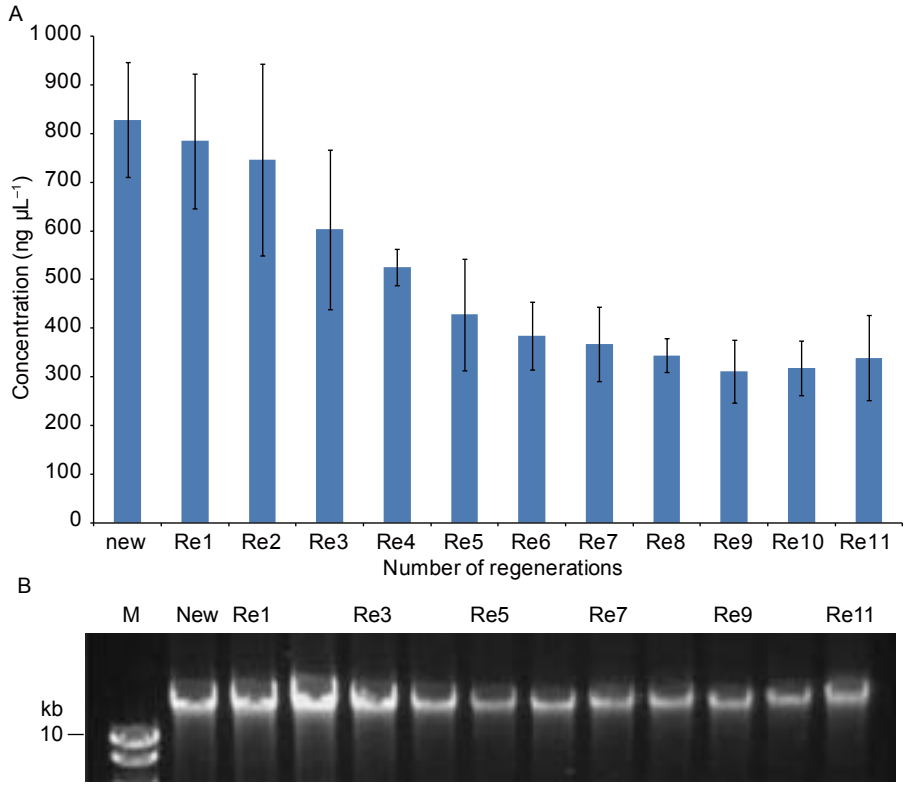

Fig. 3 Comparison of yield and integrity of DNA obtained from a new column and columns regenerated up to 11 times (Re1–11). Perennial ryegrass DNA was added to the pre-prepared, regenerated columns and new columns. DNA products were detected by NanoDropND-1000 (A) and gel electrophoresis on 1% agarose (B). The DNA yield is presented as the mean of three replicates and the error bars are ±SD.

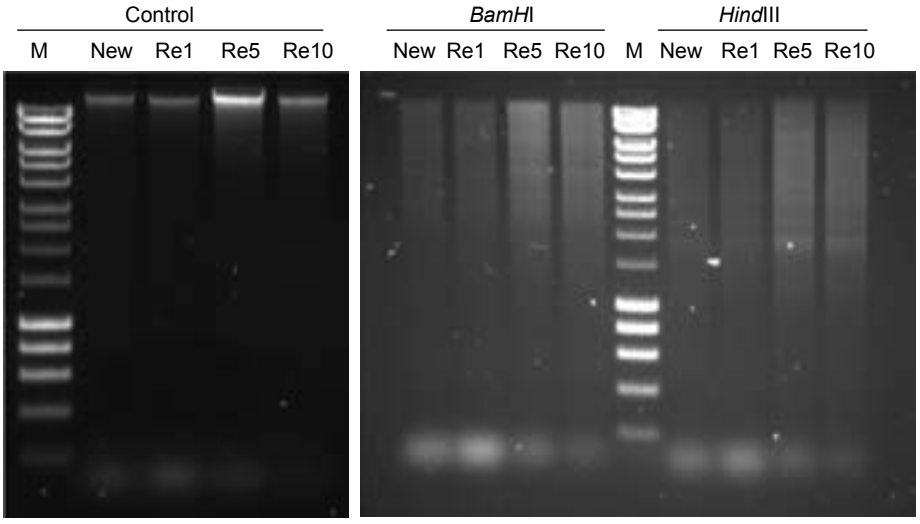

Fig. 4 Quality assessment using restriction enzyme assay of DNA eluted from a new column and columns regenerated up to 11 times (Re1–11). Perennial ryegrass DNA was applied to a new column, and columns regenerated for 1 (Re1), 5 (Re5) and 10 (Re10) times. The eluted samples were cut by *BamH*I and *Hind*III, respectively. The DNA products without digestion and the digestion products were run on a 1% agarose gel.

acceptable visually (Fig. 2). However, it was still possible that there may have been traces of DNA carried over in the regenerated columns, leading to cross contamination. To test this, columns that had previously been used to isolate DNA from perennial ryegrass were used to isolate genomic DNA from pea. As Fig. 6 shows, 5th and 10th regeneration

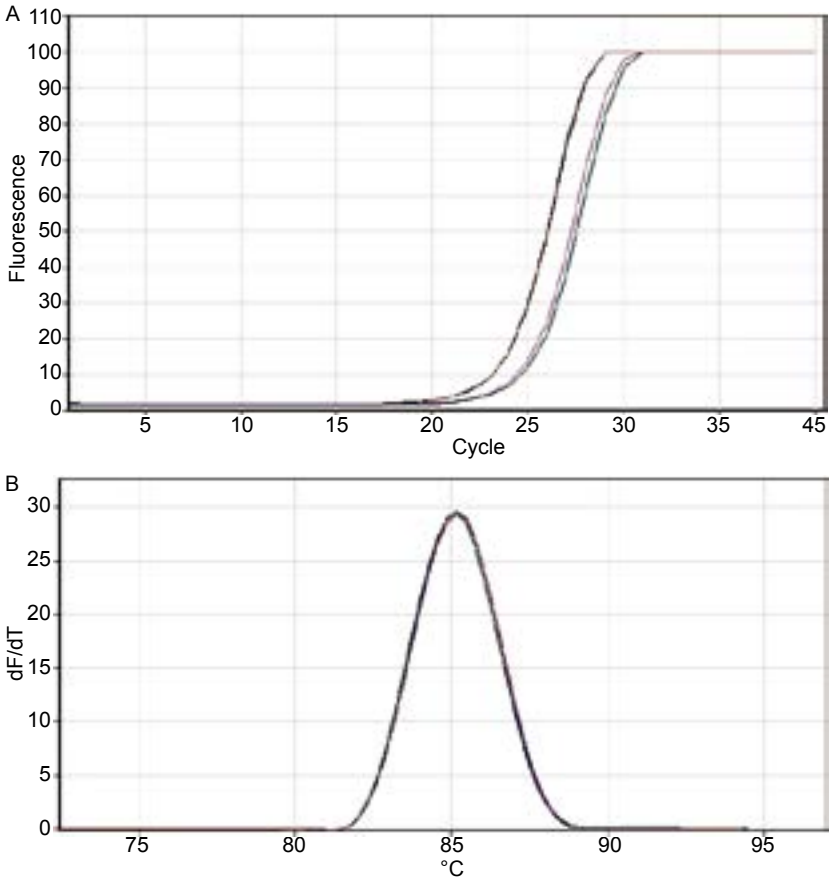

Fig. 5 Amplification of genomic DNA with RT-qPCR. The DNA are products from New, Re1, Re5, and R10 using the procedure described in Fig. 4. The fluorescence curves (A) and the melt curve (B) analysis were generated by the Rotor-Gene Q software 2.1.0.9.

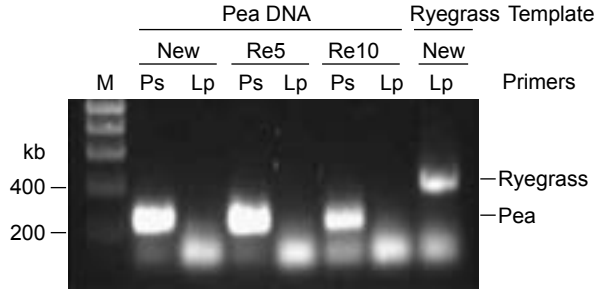

Fig. 6 Comparison of PCR products after elution of pea genomic DNA from new and regenerated columns. The columns were initially used for perennial ryegrass DNA extraction and were regenerated 5 (Re5) and 10 (Re10) times. After that, new, Re5, and Re10 columns were used to extract pea DNA. Pea DNA was amplified by standard PCR with pea primers and perennial ryegrass primers. A control is perennial ryegrass DNA with perennial ryegrass primers. The PCR products were checked on 1% agarose gel. Ps, pea primers; Lp, perennial ryegrass primers.

columns were used to isolate pea DNA having previously been used to purify perennial ryegrass DNA. A PCR was then run on the products eluted from the columns using pea and perennial ryegrass gene-specific primers. Positive controls were run for both perennial ryegrass and pea DNA using new columns. The PCR products from the regenerated columns (previously used for perennial ryegrass DNA) were the same as from the new column to which only pea extract had been applied: no PCR product was detected using the perennial ryegrass primers, indicating that there was no perennial ryegrass contamination of the pea genomic DNA.

4. Conclusion

A rapid and cost effective protocol for isolating plant genomic DNA is described. This protocol not only combines the traditional CTAB method with commercial silica columns, but also improves the CTAB method to reduce incubation time, and re-uses regenerated silica columns up to 11 times. The reliability of the regenerated columns was tested for yield, quality and purity. High yield and good quality genomic DNA was produced by a low-cost and facile method. In addition, our method used individual silica columns, providing greater flexibility and convenience than using 96-well column plates

(Lemke *et al*. 2011). Based on these benefits, this protocol could meet the requirements for preparation of large numbers of genomic DNA samples for such applications as genotyping and large-scale mutation screening.

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Appendix associated with this paper can be available on http://www.ChinaAgriSci.com/V2/En/appendix.htm

References

Allen G C, Flores-vergara M A, Krasnyanski S, Kumar S, Thompson W F. 2006. A modified protocol for rapid DNA isolation from plant tissues using cetyltrimethylammonium bromide. *Nature Protocols*, **1**, 2320–2325.

Comai L, Young K, Till B J, Reynolds S H, Greene E A, Codomo C A, Enns L C, Johnson J E, Burtner C, Odden A R, Henikoff S. 2004. Efficient discovery of DNA polymorphisms in natural populations by Ecotilling. *The Plant Journal*, **37**, 778–786.

Deavours B E, Dixon R A. 2005. Metabolic engineering of isoflavonoid biosynthesis in alfalfa. *Plant Physiology*, **138**, 2245–2259.

Dhandapani P, Song J, Novak O, Jameson P E. 2016. Infection by Rhodococcus fascians maintains cotyledons as a sink tissue for the pathogen. *Annals of Botany*, doi: 10.1093/aob/mcw202

Doyle J J, Doyle J L. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemical Bulletin*, **19**, 11–15.

Jameson P E, Dhandapani P, Novak O, Song J. 2016. Cytokinins and expression of SWEET, SUT, CWINV and AAP genes increase as pea seeds germinate. *International Journal of Molecular Sciences*, **17**, 2013.

Jameson P E, Song J. 2016. Cytokinin: A key driver of seed yield. *Journal of Experimental Botany*, **67**, 593–606.

Lemke L, Rex M, Zyprian E, Töpfer R. 2011. A simple, inexpensive and environmentally friendly method for high throughput DNA extraction from grapevine (*Vitis* spp.). *Vitis*, **50**, 7–10.

Roche J, Love J, Guo Q, Song J, Cao M, Fraser K, Huege J, Jones C, Novák O, Turnbull M H, Jameson P E. 2016. Metabolic changes and associated cytokinin signals in response to nitrate assimilation in roots and shoots of *Lolium perenne*. *Physiologia Plantarum*, **4**, 497–511.

Siddappa N B, Avinash A, Venkatramanan M, Ranga U. 2007. Regeneration of commercial nucleic acid extraction columns without the risk of carryover contamination. *BioTechniques*, **42**, 186.

Song J, Jiang L, Jameson P E. 2012. Co-ordinate regulation of cytokinin gene family members during flag leaf and reproductive development in wheat. *BMC Plant Biology*, **12**, 1–17.

Song J, Jiang L, Jameson P E. 2015. Expression patterns of Brassica napus genes implicate IPT, CKX, sucrose transporter, cell wall invertase and amino acid permease gene family members in leaf, flower, silique and seed development. *Journal of Experimental Botany*, **66**, 5067–5082.

Tesniere C, Torregrosa L, Pradal M, Souquet J M, Gilles C, Dos Santos K, Chatelet P, Gunata Z. 2006. Effects of genetic manipulation of alcohol dehydrogenase levels on the response to stress and the synthesis of secondary metabolites in grapevine leaves. *Journal of Experimental Botany*, **57**, 91–99.

Till B J, Zerr T, Comai L, Henikoff S. 2006. A protocol for TILLING and Ecotilling in plants and animals. *Nature Protocols*, **1**, 2465–2477.

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