African Journal of Biotechnology Vol. 8 (17), pp. 4020-4024, 1 September, 2009
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2009 Academic Journals

Full Length Research Paper

A simple, rapid and efficient method for the extraction of genomic DNA from *Allium roseum* L. (*Alliaceae*)

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Accepted 17 July, 2007

The isolation of intact, high-molecular-mass genomic DNA is essential for many molecular biology applications including long range PCR, endonuclease restriction digestion, southern blot analysis, and genomic library construction. Many protocols are available for the extraction of DNA from plant material, but obtain it is difficult in many plants because of metabolites that interfere with DNA isolation procedures and subsequent applications. With frame of the present work, we developed the first reliable and efficient method for isolating *Allium roseum* L. genomic DNA that is free from polysaccharides and polyphenols. This protocol uses 100 mM Tris-HCl (pH 8), 20 mM EDTA, 1.4 M NaCl, 3% PVP (polyvinylpyrrolidone 40.000), 3% mercaptoethanol, and an incubation at 65°C for 1 h. The purity of isolated genomic DNA was confirmed by spectrophotometric analyses (A260/230 ratio of 1.947, A260/280 of 1.804). DNA was obtained in the amount of 189 µg per gram of leaf material, and it proved amenable to restriction digestion.

Key words: DNA isolation, DNA purification, spectrophotometric analyses, *Allium roseum* L.

INTRODUCTION

The genus *Allium* L. (*Alliaceae*) exhibits a great diversity in various morphological characters, particularly in life form (bulb or rhizome) and ecological habitat. It is of major economic importance as vegetable crops, herbal crops, and ornamental plants (Ricroch et al., 2005). This genus consists mostly of perennial and bulbous plants; and it is widely distributed over Holarctic regions from the dry subtropics to the boreal zone (Stearn, 1992). *Allium roseum* L**.** is a species with a typically Mediterranean distribution. It belongs to section *Molium* and comprises different intraspecific taxa. This species was used in traditional pharmacopoeia for its expectorant properties (Marcucci and Tornadore, 1997). Contrary to the cultivated garlic (*Allium sativum* L.) which is very well studied in terms of its morphological, agronomic and molecular aspects (Baghalian et al., 2006; Baghalian et al., 2005; Senula and Keller, 2005; Maab and Klaas, 1995), studies about rosy garlic (*A. roseum* L.) are few and limited to morpho phenologx (Jendoubi et al., 2001) and cytocaryology (Ferchichi, 1997; Marcucci and Tornadore, 1997). In the genetic improvement process, it is desirable to use molecular markers for evaluating genetic diversity of accessions, choosing of parents and selection of progeny. To achieve this process, many protocols are available for the DNA extraction from plant material. Until now, any DNA extraction protocol from *A. roseum* has been published; therefore, to determine genetic diversity in *A. roseum* a protocol for the extraction of clean DNA at a quantity that allows molecular analysis should first be devised. A good extraction procedure for the isolation of DNA should yield adequate and intact DNA of reasonable purity. However procedure should be simple, inexpen-

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Abbreviations: AP-PCR, arbitrarily primed PCR; **CTAB**, cetyltrimethylammonium bromide; **EDTA**, hexadecyltrimethylammonium bromide; **PVP 40**, polyvinylpyrrolidone 40000; **RAPD**, random amplification of polymorphic DNA; **RT**, room temperature.

Figure 1. Germaplasm collection of *Allium roseum* L. planted in experimental field in Arid Land Institute.

sive, and quick (Daneshwar and Sher-Ullah, 2004).

Molecular marker diversity has been not evaluated for *A. roseum* before. The purpose of this manuscript is to report an improved method for DNA extraction in *A. roseum* and provide some initial measure of molecular diversity in this species.

MATERIALS AND METHODS

Plant materials for DNA isolation

From a phenotypically and geographically diverse collection of *A. roseum* maintained in Arid Land Institute (Figure 1), leaves, flower petals, and bulbs were used to determine the ease of extracting enough amount of clean DNA. For routine analysis, young fresh leaves were chosen as plant material. The availability of leaves was also important for our choice of this material; indeed cutting some leaves of *A. roseum* does not damage the plant and permits therefore to maintain the established field.

Solutions

An extraction buffer consisting of 3% CTAB (w/v), 100 mM Tris-HCl (pH 8), 20 mM EDTA (pH 7.5), 1.4 M NaCl, 3% PVP (K40, 40,000), 3% β-mercaptoethanol, was prepared. Chloroform isoamylalcohol (24:1), 75 and 95% ethanol, 5 M NaCl, and a TE buffer consisting of 10 mM Tris-HCl (pH 8) and 1 mM EDTA (pH 8) were also needed.

DNA isolation and purification

One gram of fresh leaf material was weighed and placed on a pre-cooled mortar. Liquid nitrogen was poured onto the sample and allowed to evaporate completely. The leaf sample was macerated into small pieces with a pestle and added to 6 ml of extraction buffer. The mixture was incubated for 1 h at 65°C, with constant shaking at separate intervals of 5 min. An equal volume of chloroform-isoamylalcohol was added to the mixture. The tubes were mixed gently at room temperature (RT) for 5 min to produce a uniform emulsion. The emulsion was centrifuged at 8000 g for 10 min at RT. A micropipette was used to transfer the supernatant to a new Corning tube.

A second chloroform-isoamylalcohol extraction was performed. The supernatant was carefully decanted and transferred to a new tube and then precipitated with 1/2 vol NaCl (5 M) and 2 vol ethanol 95°C. The precipitated nucleic acids were collected and washed twice with 75% ethanol. The pellets were dried, and resuspended in 100 µL of TE buffer to dissolve genomic DNA, and RNase treatment followed.

Measurement of amount and purity of DNA

DNA yield per gram of leaf tissue was measured with a Jenway 6405 UV/VIS spectrophotometer at 260 nm. DNA purity was determined by calculating the absorbance ratio A260/280. Pure DNA has a ratio of 1.8 ± 0.1 (Clark, 1997). Polysaccharide contamination was assessed by calculating the absorbance ratio, A260/230 (Wilson, 1997).

DNA samples from the leaf tissues were digested with *BamHI* and *EcoRI* and electrophoresed on a 0.8% agarose gel.

RAPD analysis

Amplification of RAPD fragments from genomic DNA was carried out in a total reaction volume of 25 µL containing: 50 ng of genomic DNA, 100 µM dNTPs, 1 mM $MgCl_2$, 2.5 µL 10X PCR buffer (10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, pH 8.3), 0.2 µM of primer pair and 1.5 U *Taq* DNA polymerase (Fermentas); distilled water was added to complete the final volume of the reaction. Cycling conditions were: initial denaturation step at 94°C for 2 min, followed by 45 cycles, each consisting of 94°C for 30 s, annealing temperature 36°C for 1 min, and 72°C for 2 min, with a final extension at 72°C for 10 min.

RESULTS AND DISCUSSION

We performed several experiments before finding one that yielded a reasonable amount of clean DNA. The initial protocol was based on Lodhi et al. (1994). A number of experiments were conduced aiming at:

(i) Determining the plant material to be used for extraction purpose fresh leaves, flower petals, or bulbs

(ii) Eliminating polysaccharide contamination of the DNA by using sodium chloride at different concentrations (3 - 5 M)

(iii) Eliminating contamination of the DNA by polyphenols use of β-mercaptoethanol at different concentrations (1 - 3%); use of PVP (K40) at different concentrations (1 - 3 %).

An optimised protocol was devised on the basis of results obtained from the above experiments.

The CTAB method of extraction without modification gave a DNA yield of about 85 µg per gram of leaf material. However, heavy contamination, likely with polysaccharides and phenolics was revealed by spectrophotometric readings, which gave an A260/230 ratio of 1.767 ± 0.145 and an A260/280 ratio of 1.677 ± 0.137 (Table1). The sample was very viscous and pinkish in color. Upon electrophoresis, smeared bands (Figure 2, Lane 2) suggesting uneven migration were obtained, confirming the

Table 1. DNA yield (µg) and its purity for *Allium roseum* L. samples, comparing 2 different DNA extraction protocols.

Procedure	DO_{260}/DO_{280}	DO_{260}/DO_{230}	DNA yield (µg/g)
on modified CTAB protocol	1.677	1.767	84.909
Modified CTAB protocol	1.804	1.947	188.89

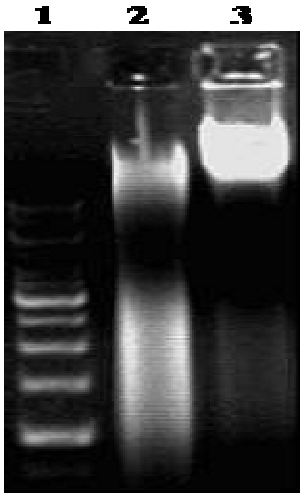

Figure 2. Electrophoresis of genomic DNA of *Allium roseum* on 0.8% agarose gel prior to treatment with RNase. Lane 1 DNA marker (1 kb DNA ladder). Lane 2 genomic DNA obtained by CTAB protocol without modification. Lane 3 genomic DNA obtained using modified CTAB method.

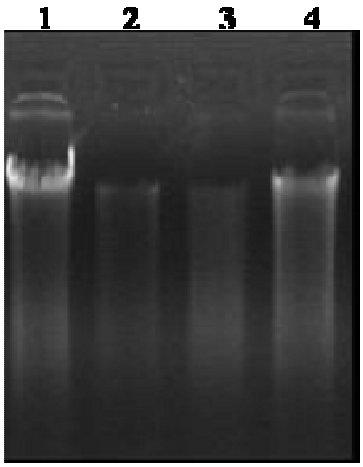

Figure 3. Restriction enzyme digestion of *Allium roseum* genomic DNA. Lane 1, undigested/incubated large scale DNA prep (LSP). Lanes 2 DNA digested with *EcoRI*. Lane 3, double digested DNA with *BamHI* and *EcoRI*. Lanes 4 DNA digested with *BamHI*.

presence of polysaccharides.

From the next set of experiments, where the NaCl concentration varied from 3 - 5 M, the quality of DNA was improved. The bands following electrophoresis were sharper (Figure 2 Lane 3). Spectrophotometric readings and electrophoresis findings indicated that the use of 5 M NaCl yielded the best result. However, upon digestion with *BamHI* and *EcoRI*, a perfect smear was obtained with the sample treated with 5 M NaCl (Figure 3 Lane 3). Several modifications were made, with one parameter tested at a time to address the problem of phenolics. Modifications included use of different concentrations of β-mercaptoethanol (1-3%), use of PVP K40 (40000) at different concentrations (1 - 3%), and use of NaCl (3 - 5 M). The use of 3% β-mercaptoethanol, 3% PVP (40000), and Nacl 5 M was found to be most appropriate.

A sufficient amount of clean genomic DNA was obtained with this modified method. The yield was about 188 µg per gram of leaf material. The A260/230 nm ratio was 1.947 ± 0.238, while the A260/280 nm ratio was 1.804 ± 0.031 (Table 1). Among the plant parts used for extraction fresh leaves reduced nucleic acid contamination from plant metabolites interfering with solubilisation of precipitated nucleic acids. DNA extraction from bulbs can cause many problems. In this experiment we speculate that secondary metabolites like organo-sulfuric compounds are particularly important in bulbs of *Allium* species (Mellouki et al., 1994).

In a set of experiments using different amounts of starting material so that more reagents is present per amount of material, we observed that the buffer-to-leaf ratio should always be at least 6:1 vol/wt to obtain a sufficient amount of clean DNA. During the addition of CTAB buffer containing β-mercaptoethanol, moving quickly at this stage was critical for obtaining good-quality DNA. Selection of 3% β mercaptoethanol instead of the 2% used by Doyle and Doyle (1990) produced nucleic acid pellets that had less color. Several authors (Stewart and Via, 1993; Porebski et al., 1997; Zhang and Stewart, 2000) recommend the use of PVP of a higher-molecular-weight PVP (i.e., 40000 at 2% [w/v]). In our experiments, adding 3% of either PVP 40000 improved the color of the nucleic acid obtained. During incubation at 65°C, no significant differences in DNA yield were found when comparing an overnight incubation with a 1 h incubation time. The latter was adopted for convenience. Once the nucleic acids were collected, they were washed in 75°C ethanol. The purity of genomic DNA was dependent on the number of washes. Increasing the number of washes improved the purity of DNA but it reduced its yield.

Because CTAB is soluble in ethanol, residual amounts are removed in the subsequent wash. In fact during etha-

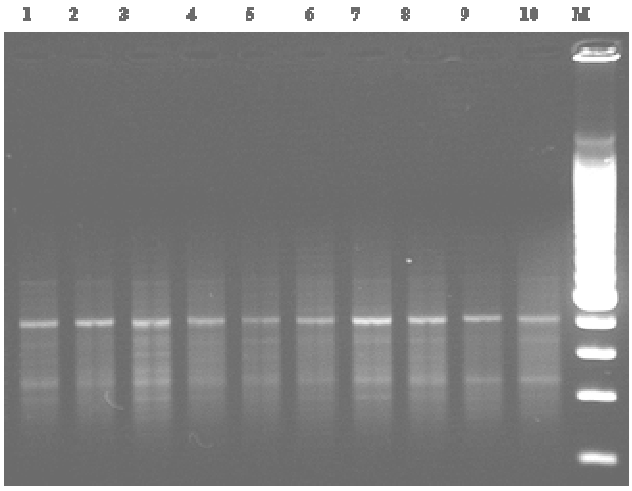

Figure 4. Amplification of purified DNA with RAPD-PCR. DNA was purified using the method described. The amplification products were separated on a 1.5% agarose gel, stained with ethidium bromide and visualized with UV light. Lane M: contains a 100 bp DNA size marker. Lanes 1 **-** 10: *Allium roseum* plants amplified using OPJ 20 primer (5'-AAGCGGCCTC- 3').

nol precipitation of nucleic acids from 1.5 M NaCl, polysaccharides remain dissolved in the ethanol (Fang et al., 1992). If resuspending the pellet obtained from the first ethanol precipitation from 5 M NaCl, washing in 75% ethanol gave better DNA because of the removal of any residual NaCl and/or CTAB.

The DNA obtained was easily digested with restriction enzymes. Figure 3 shows an ethidium stained agarose gel, following electrophoretic separation of digested /undigested DNA. It is clear from lanes 3 that the majority of DNA was digested. Undigested DNA can also be analyzed after incubation without the enzyme, but in the presence of its appropriate buffer. Nuclease contamination is another major concern during DNA isolation since it may degrade the DNA. The degraded DNA can resemble restriction enzyme digested DNA when separated electrophoretically. Thus, the absence of nucleases in the preparation was confirmed by incubation of DNA at 37°C followed by electrophoretic analysis (Figure 3, lanes 1).

The isolated DNA with this method was successfully amplified using the RAPD PCR procedure (Figure 4). In all samples, RAPD primers produced a constant and reproducible banding pattern across all samples.

The method described here for the extraction of genomic DNA will be useful for molecular, genetic diversity and transgenic studies in *A. roseum* L. This procedure may also be useful for other plants containing high levels of secondary metabolites.

Conclusion

In an attempt to isolate pure nuclear DNA from *A. roseum*, a modified CTAB protocol (Lodhi et al., 1994) was adopted. To our knowledge, it is the first reliable and efficient method for isolating *A. roseum* L. genomic DNA that is free from polysaccharides and polyphenols. Since we were interested in isolating large quantities of DNA, we chose chloroform/isoamylalcohol extractions to purify DNA rather than more expensive methods involving commercial kits. The DNA we obtained appeared to be as pure as DNA isolated with other purification techniques from many species of genus *Allium* (Friesen et al., 1997; Friesen et al., 1999; Ricroch et al., 2005). Because different plants can vary considerably in the number and types of secondary compounds they produce, it is unlikely that any one technique for isolating contaminant-free nuclear DNA will ever be developed (Loomis, 1974). However, it is likely that our *A. roseum* DNA isolation protocol can be used to isolate nuclear DNA from a variety of other plant species.

ACKNOWLEDGEMENTS

The authors are grateful to Pr Philipp W. Simon (Professor USDA, from Horticulture at the University of Wisconsin**)**, for critically reviewing the manuscript. We are thankful to Dr. Ouled Belagacem Azieze for his comments on an early version of manuscript. The authors also acknowledge the technical assistance rendered by all members of Plant Biotechnology Laboratory in National Institute of Applied Sciences and Technology Tunisia.

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