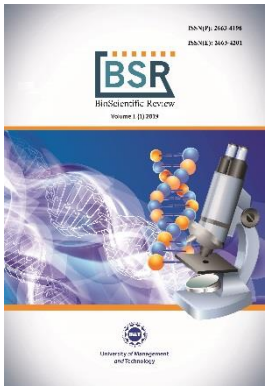

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Research Article

A Modified Method for Rapid Genomic DNA Extraction from Tuberose

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Abstract

The isolation of high yield and quality genomic DNA is crucial for studying the molecular genetics of plants. However, high contents of secondary metabolites, especially in medicinal and aromatic plants, interfere with the extraction of clean DNA, thereby rendering it useless for downstream analyses such as DNA amplification, restriction, sequencing and cloning. The chemotypic heterogeneity among plant species may not permit high quality DNA isolation with a single protocol, thus a species-specific extraction method is required for quality extractions. Here, we present a modified cetyltrimethylammonium bromide (CTAB) protocol for good quality DNA extraction from tuberose, which is an important plant for perfume and pharmaceutical industry due to its pleasant fragrance and essential oil content. In contrast to other CTAB methods, the modified procedure is rapid, omits the use of liquid nitrogen and phenol, uses inexpensive and less hazardous reagents, and requires only ordinary laboratory equipment. The procedure employed the high concentration of NaCl and use of PVP-10 to get rid of problems associated with polysaccharides and polyphenols, respectively. The yield and quality of extracted DNA were fairly good and amenable for downstream analyses. Moreover, to our knowledge, the described method is the first report of a modified DNA extraction protocol for tuberose.

Keywords: DNA extraction, CTAB, Tuberose, Polysaccharides, Polyphenols

1. Introduction

Tuberose (*Polianthes tuberosa* L.) is an important perennial flowering plant cultivated for its high potential in perfume, cut flower and pharmaceutical industry due to its lingering pleasant fragrance and essential oil content, respectively (1, 2). It is crucial to study the molecular genetics of a tuberose plant for its genetic improvement which requires an economical and clean DNA isolation. Besides genetic improvement, clean DNA extraction is also useful in phylogenetic, phylogeographic, population, and molecular taxonomic studies of plants.

In 1980, the development of CTAB protocol came as a breakthrough in DNA extraction in plants (3). CTAB is a cationic detergent that is compatible with high concentrations of salt, mostly NaCl, and is often used to separate molecular grade DNA from chromosomal proteins during extraction. Plant DNA extracted through CTAB protocol is now routinely used in several DNA based molecular biology applications, such as cloning, mapping and genotyping, which have contributed to understand the plant genes function (Allen, Flores-Vergara et al. 2006). However, high contents of secondary metabolites, such as polysaccharides, polyphenols, alkaloids, flavonoids, quinones and terpenes in medicinal and aromatic plants like tuberose hinder the extraction of good-quality DNA (4). Major problems associated with secondary metabolites are co-precipitation of the viscous polysaccharides and oxidizing compounds like polyphenols that interfere directly or indirectly with enzymatic reactions (5, 6). Thus, the presence of these secondary metabolites may compromise the purity and reduce the yield of extracted DNA, thereby rendering it useless for downstream analyses, such as DNA amplification, restriction digestion, sequencing and cloning.

DNA based molecular studies, such as restriction digestion, Random Fragment Length Polymorphism (RFLP), Single Sequence Repeats (SSR), Single Nucleotide Polymorphism (SNP), Amplified Fragment Length Polymorphism (AFLP), and Polymerase Chain Reaction (PCR) in tuberose have been obstructed by the absence of an efficient DNA extraction method. CTAB is one of the most used methods for the extraction of genomic DNA from plant (3), fungi (7), bacteria (8) and animal tissues (9). Over the years, numerous modifications (5, 10-13) have been made in CTAB protocol to remove these secondary metabolites efficiently to increase the yield and quality of the extracted DNA. However, not a single modification has been found to be applicable to all plant species universally due to their chemotypic heterogeneity, thus a species-specific extraction method is required for quality extractions (14). These problems necessitate the development of an efficient protocol for DNA extraction from tuberose.

In this regard, the protocol described by Khanuja et al. (1999) was modified for genomic DNA isolation from tuberose leaves. The protocol employs inexpensive reagents, ordinary laboratory equipment and omits the use of liquid nitrogen and toxic phenol. The yield of the extracted DNA remains fairly good and amenable to restriction digestion and Random Amplified Polymorphic DNA (RAPD) PCR. An individual can routinely perform 24-48 extractions in about 3 hours. Moreover, this protocol may be used for other plants that are rich in secondary metabolites.

2. Material and Methods

2.1. Reagents and Consumables

Polianthes tuberosa L. leaves were used to evaluate the efficiency of this procedure. All leaf samples were collected in polyethene bags and placed on ice.

CTAB (Calbiochem, cat. no. 219374); Tris (hydroxymethyl) aminomethane hydrochloride (Tris-HCl; Merck-Millipore, cat. no. 108219); Ethylene diamine tetraacetic acid (EDTA; Calbiochem, cat. no. 324503); Polyvinylpyrrolidone (PVP; Calbiochem, cat. no. 5295); Beta-Mercaptoethanol (βME; Merck-Millipore, cat. no. 805740); Ethanol absolute (Merck-Millipore, cat. no. 107017); Ribonuclease A (RNase A; Fermentas. USA), 2-propanol (Merck-Millipore, cat. no. 109634); 7.5 M Ammonium Acetate; 1.5 ml microfuge tubes and nuclease-free tips.

2X CTAB extraction buffer modified from Khanuja et al. (1999): 2% (w/v) CTAB; 1.5% (w/v) PVP; 0.3% (v/v) βME (add before use); 200 mM Tris-HCl (pH 8.0); 3 M NaCl and 30 mM EDTA (pH 8.0), chloroform and isoamyl alcohol (CIA): (24:1), 1X TE buffer: 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA (pH 8.0)

TissueLyser (QIAGEN) or mortar and pestle; Water bath; Heat block; Centrifuge and Micropipettes (P-200 and P-1000)

2.2. Extraction of DNA

2X CTAB extraction buffer was preheated at 65 ^{o}C in a water bath. 50 mg of leaf tissue was ground in 800 µl of 2X CTAB extraction buffer by using mortar and pestle. The mixture was transferred into a new 1.5 ml nuclease-free eppendrof and vortexed for 5 s. TissueLyzer was employed to prevent cross-contamination (Optional). Tubes were incubated at 65 ^{o}C for 30 minutes in a water bath and inverted after every 10 minutes to homogenize. Then the tubes were allowed to cool down at room temperature (RT), RNase A (25 µg/ ml) was added and inverted for 5-6 times. About 500 µl of CIA was added and vortexed for 5-10 s. Organic and aqueous phases were separated by centrifuging at 13,000 rpm for 10 minutes. The upper layer (aqueous) was carefully transferred into a new 1.5 ml eppendrof and the eppendrof containing the organic phase was discarded. To prevent mechanical damage to DNA, wide-bore tips were used (Optional step). DNA was precipitated by adding an equal volume of chilled (-20 ^{o}C) 2-propanol and 50 µl of ammonium acetate. Tubes were gently inverted for 3-4 times and incubated at -20 ^{o}C for 20 minutes. Tubes may incubate for a few hours to overnight

incubation in order to increase the DNA precipitation (Pause Step). Centrifugation was done at 13,000 rpm to pellet the DNA. The supernatant was discarded without disturbing the DNA pellet and 400 µl of 70% (v/v) chilled (-20 ^{o}C) ethanol was added. The pellet was dislodged by flicking with a finger. Tubes were centrifuged at 13,000 rpm for 5 min and the supernatant was discarded. The pellet was dried using a heat block at 40 ^{o}C. Do not over-dry the pellet because it will make the DNA pellet difficult to re-suspend. Finally, DNA pellet was re-suspended in 40 µl of TE buffer or nuclease-free water. The entire protocol is illustrated in a flow diagram in Figure 1.

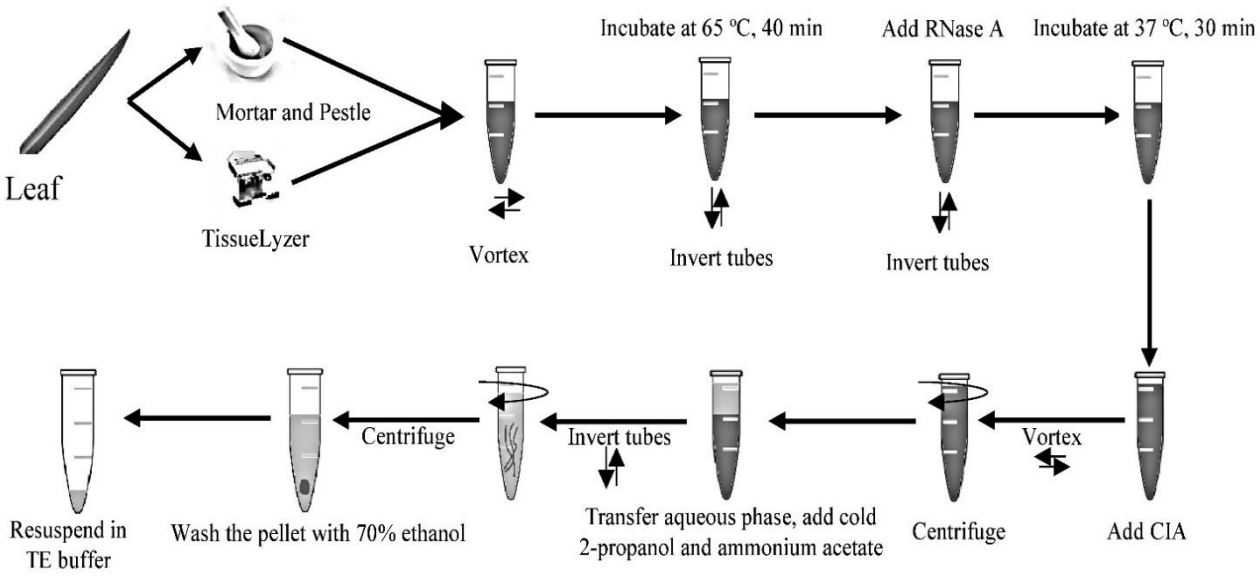

Figure 1. Flow Diagram to illustrate the major steps of the modified procedure

2.3. Yield and Quality Assessment of the Extracted DNA

The purity and yield of the extracted DNA were assessed through spectrophotometric analysis. Nano-Drop ND-2000 (Thermo-scientific, USA) was used to record $A_{260/280}$ and $A_{260/230}$ UV absorption ratios (15). Agarose gel electrophoresis was performed to determine DNA degradation and RNA contamination (16).

2.4. Restriction Digestion and RAPD-PCR Analyses

The extracted DNA was digested with *Hind III* following the manufacturer's instructions (Fermantas. USA). RAPD-PCR was performed in a 25 µl reaction volume, containing the 100 nM of RAPD primers, 1.5 units *Taq* DNA polymerase, PCR buffer (10 mM Tris-HCl and 50 mM KCl), 1.5 mM $MgCl_2$ and 0.1 mM of deoxyribonucleotide triphosphates (dNTPs). PCR product was separated on 1.5% agarose gel and analyzed on gel Doc. (BIO-RAD, USA) (17).

3. Results and Discussion

Aromatic and medicinal plants are an important source of many invaluable compounds, therefore, it is crucial to study the molecular genetics of these plants for their genetic improvement (4). Tuberose is one of the most important aromatic and medicinal plants due to its pleasant fragrance and essential oil content (2). It has high contents of secondary metabolites that hamper the molecular studies by interfering with clean DNA extraction. There are many protocols in literature, however, not a single protocol is universally applicable for every plant specie due to their chemical heterogeneity. Even different tissues of the very same plant can exhibit an enormous chemotypic heterogeneity that requires a separate protocol for each specific tissue. However, most of the procedures (18-20) employ similar extraction steps, namely cell disruption and CIA extraction, followed by ethanol/2-propanol precipitation.

We extracted DNA from tuberose leaves according to the previously reported protocol by Khanuja et al. (1999). However, the extracted DNA was viscous and the pellet was dark brown that indicates co-precipitation of the polysaccharides and oxidation of DNA by polyphenols, respectively. Thus, the extracted DNA could not be used for downstream analyses. Moreover, the DNA yield was also very low. The mean concentration and purity of the DNA extracted via Khanuja et al. (1999) and the described method are presented in Table 1. With our modified method, DNA yield was increased twofold with a consistently high purity based on both qualitative (colour and viscosity) and quantitative ($A_{260/A230}$ and $A_{260/280}$ ratios) parameters. On the other hand, the yield and quality of the DNA extracted through the method of Khanuja et al. (1999) was poor and inconsistent (Table 1).

Table 1. Qualitative and Quantitative Analyses of the Extracted DNA from Tuberose (± SD, N =120)

Procedure	**DNA yield (ng/µl)**	**$A_{260/280}$**	**$A_{260/230}$**	**Colour/Viscosity**
Khanuja et al., (1999)	270 ± 53	1.47 ± 0.12	1.32 ± 0.34	Dark-Brown/Viscous
Optimized protocol	450 ± 66	1.82 ± 0.06	2.13 ± 0.05	Clear/Non-viscous

In this regard, the method of Khanuja et al. (1999) was modified for clean and rapid genomic DNA isolation from tuberose. The concentration of NaCl was increased from 1.5 M to 3 M to prevent the co-precipitation of polysaccharides. PVP and βME concentrations were increased from 1% and 0.2% to 2% and 0.4%, respectively. Furthermore, to reduce cost and time of extraction, RNase A was added before chloroform extraction and the use of liquid nitrogen and phenol were omitted. The extracted DNA was further subjected to agarose gel electrophoresis, restriction digestion and RAPD-PCR to assess its applicability for downstream analyses. As a result, the DNA isolated via the described method was found to be highly susceptible to restriction enzyme (Figure 2) and allowed PCR amplification (Figure 3). Conversely, the DNA concentration extracted by Khanuja et al. (1999) was very low and partially digested with a restriction enzyme (Figure 2).

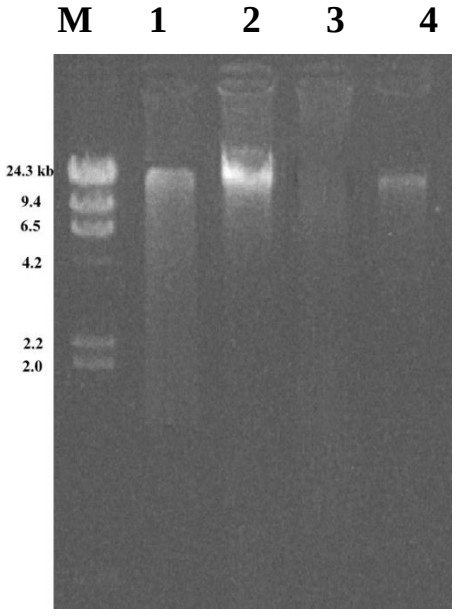

Figure 2. Electrophoretic and restriction digestion analyses of the isolated DNA from tuberose leaves. 2 µl of undigested and 5 µl of *Hin*d III digested DNA resolved on 1% agarose gel. Lane M:

λ-*Hin*d III ladder; lane 1 and 3: modified protocol and Khanuja et al., (1999) *Hin*d III digested DNA, respectively; lane 2 and 4: modified protocol and Khanuja et al., (1999) undigested DNA, respectively

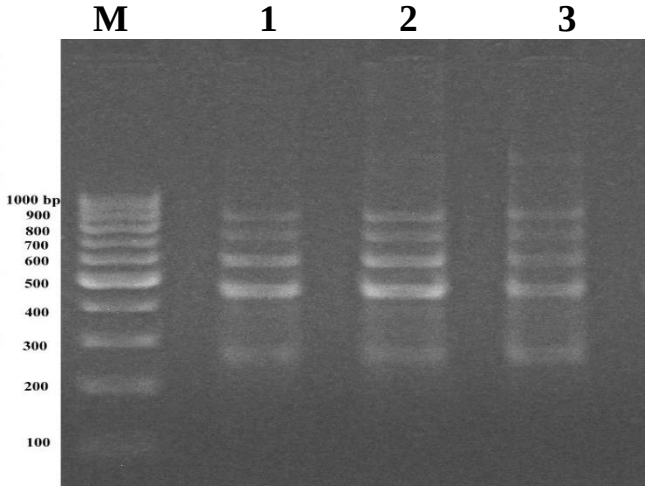

Figure 3. RAPD-PCR of the isolated DNA extracted through modified protocol separated on 1.5% agarose gel. Lane M: 100 bp ladder (Fermentas, USA); lane 1-3: amplified sequences.

4. Conclusion

To conclude, we described an efficient extraction procedure for rapid DNA isolation from tuberose and the isolated DNA was amenable to downstream analyses, such as RAPD-PCR and restriction digestion. Moreover, this method may be used for other aromatic and medicinal plants that are reluctant to other methods.

Competing interest

None

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