Article

A modified CTAB protocol for DNA extraction from young flower petals of some medicinal plant species

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ABSTRACT

Isolation of semi-intact, high quality genomic DNA is a crucial pre-requsite step for molecular biology applications such as polymerase chain reaction (PCR), rapid amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), PCR-RFLP, Southern blotting, and library construction of crop, forestry, desert and medicinal plants. Plant leaves of crop, tree and medicinal plants are rich in secondary metabolites, polysaccharides and polyphenolics that are problematic during isolation of genomic DNA. Besides, succulent plants freezed under liquied nitrogen turn to stone-like tiusses that are difficult to grind. Flowers were not considered as a source of genomic DNA as the case of young leaves, this might be due to the seasonal nature and short half-life feature. A cetyltrimethylammonium bromide (CTAB) protocol has been adopted for isolation of high-quality genomic DNA from young flower petals of a succulent plant (Gasteria croucheri) a shrub (Erythrina humeana) and a tree (Calpurnia aurea). Quantity of isolated genomic DNA showed a proportional relationship with petal tissues. Spectral ratio (A260/A280) measurements of isolated genomic DNA were in the range of 1.70-1.81 in average as an indication of low polysaccharides and high qualities. Isolated DNA was efficiently digestible by restriction endonucleases, and suitable for PCR amplification of genes from both nuclear and plastid genomes as an indication of high quality and co-extraction of nuclear and organelles DNA. Therefore, this isolated genomic DNA can be used for downstream molecular studies.

Keywords: Flower, petals, DNA.

Introduction

Pulverizing plant tissues under liquid nitrogen is an ideal condition for DNA extraction. Succulent tissues and leaves of some plants dipped in liquid nitrogen turn to stone-like tissues that are very difficult to crush and grind into fine powder. Avoidance of freeze-grinding tissues before addition of extraction buffer was recommended, especially for tissues with high contents of water (Križman et al., 2006). In highly differentiated plant leaves the proportion of DNA to other contents in a cell is very low (Pašakinskiené and Paplauskiené, 1999). Medicinal plants, crop plants, fruit trees, ornamental plants and desert shrubs are known for high contents of secondary metabolites, polysaccharides and polyphenolics (Khanuja et al., 1999; Aljanabi et al., 1999; Pirttilä et al., 2001; Cheng et al., 2003; Horne et al., 2004; Hameed et al., 2004; Jabbarzadeh et al., 2009) . High contents of polyphenolics and polysaccharides in plant leaves are problematic during the isolation of high-quality/-quantity intact genomic nucleic acids (Michiels et al., 2003; Puchooa et al., 2004; Karaca et al., 2005; Angeles et al., 2005). Besides complicating genomic nucleic acids extraction, polyphenolics and polysaccharides interfere in enzymatic applications as the case of restriction digestion and PCR (Porebski et al., 1997). However, there are many successful protocols to isolate nucleic acids from plant tissues with high contents of secondary metabolites, polyphenolics and polysaccharides. Some protocols adopted the use of reducing agents such as dithiothreitol and sodium metabisulfite while others successfully used the common CTAB associated with high salt as a reducing agent and selective precipitant of nucleic acids and polysaccharides (Saha et al., 1997; Sarwat et al., 2006; Križman, et al., 2006; Dehestani and Tabar, 2007; Sharma et al., 2008; Dhakshanamoorthy and Selvaraj, 2009; Hu et al., 2009;).

Due to rapid cell division and extensive DNA synthesis, Pašakinskiené and Paplauskiené (1999) exploited the floral meristems of two grasses; Lolium and Festuca to enhance the yield of extracted DNA 5.3- to 18.7-fold compared to leaves.

Gasteria croucheri (Hook.f.) Baker is a perennial, monocotyledonous, succulent, drought resistant, native South African plant belongs to Asphodelaceae (Zonneveld and van Jaarsveld, 2005). G. croucheri is a medicinal and ornamental plant that is over harvested by the locals in the coastal and eastern areas of South Africa, where they use it for medicinal and magical treatments (Bayley and van Staden, 1986). G. croucheri was reported as one of the top 10 traded medicinal plants in Cape Peninsula (Loundou, 2008) that lead the South African National Biodiversity Institute to rate it as vulnerable (2009). Extracts of G. croucheri have an inhibitory activity of MAO-B and these MAO-B inhibitors are used to treat neurodegenerative diseases such Parkinson's and Alzheimer's disease as well as neuroprotectants (Stafford et al., 2007, 2008). Erythrina humeana Spreng is a perennial, dicotyledonous, deciduous, shrub that belongs to Fabaceae and is widely spread in subtropics and tropics (Konozy et al.,

2002). As an ornamental and medicinal plant, the bark and leaf extracts of E. humeana have anti-bacterial activity (Pillay et al., 2001). Calpurnia aurea (Aiton) Benth is a perennial, dicotyledonous, small medicinal tree belongs to Fabaceae. Leaf powder of C. aurea mixed with water or stem honey paste are used in Ethiopia to treat internal diseases, intestinal worms, unidentified swellings/cancer neck, severe dysentery with blood, mental disorders, excessive menstruation, fungal diseases on skin and external injuries (Teklehaymanot et al., 2007; Wondimu et al., 2007; Teklehaymanot, 2009). Hydroalcoholic extracts of C. aurea contain antimicrobial activity against skin infections (Tadeg et al., 2005). Fresh leaves of C. aurea mixed with other leaves and fruits are used to treat horses (Yineger et al., 2007). Organic solvent extracts of C. aurea exhibited tick toxicity (Zorloni, 2007; Zorloni et al., 2010). Leaf and stem extracts of C. aurea possess antioxidant properties and anti-bacterial activity (Adedapo et al., 2008). Fresh leaves of C. aurea are used against fleas and lice (Waka et al., 2004). This plant species is used as forage for dromedary camels in Ethiopia during the dry and wet seasons (Dereji and Udén, 2005).

High purity genomic DNA is a basic step for further downstream molecular biology studies of G. croucheri, E. humeana, and C. aurea as important medicinal plants. The primary goal of this work was to isolate high-purity genomic DNA from young flower petals of G. croucheri, E. humeana, and C. aurea. Here, a detailed CTAB based protocol was described for high-quality/-quantity genomic DNA extraction from young flower petals of these widely different medicinal plants including a succulent.

Material and Methods

Plant Material

Fresh young flower petals of G. croucheri, E. humeana and C. aurea were collected from the Botanical Garden at School of Biological and Conservation Sciences, University of KwaZulu-Natal, Pietermaritzburg, South Africa (Photo.1).

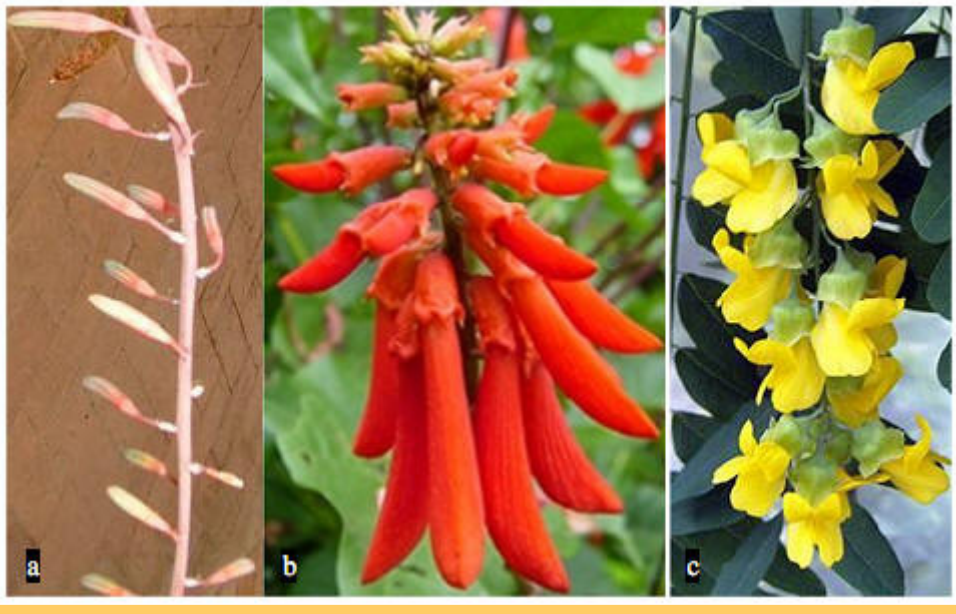

Photo 1. Plants used in this study; (a) G. croucheri, (b) E. humeana and (c) C. aurea.

Reagents and Stock buffers

Buffers used for genomic DNA isolation are listed in Table 1.

Table 1. Buffers and solutions used for DNA isolation from young flower petals and leaves.

Buffer	Contents
CTAB Extraction	2% (w/v) CTAB; 20 mM EDTA, pH 8.0; 100 mM Tris–HCl, pH 8.0; 1.4 M NaCl.
Polyvinylpolypyrrolidone (PVP)	4% (w/v) was added to CTAB extraction buffer just before extraction.
β-mercaptoethanol (βME)	3% (v/v) was added to CTAB extraction buffer just before extraction.
CTAB/NaCl	10% (w/v) CTAB; 0.7 M NaCl.
CTAB precipitation	1% (w/v) CTAB; 50 mM Tris-HCl, pH 8.0; 10 mM EDTA.
High salt TE	10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0; 1.0 M NaCl.
TE	10 mM Tris-HCl, pH 8.0; 1.0 mM EDTA, pH 8.0.
Chloroform: isoamyl alcohol	24:1, (v/v).
Iso-propanol	Absolute.
Ethanol	80% and 99.99%.
Liquid nitrogen	Used to quick freeze plant material.

DNA extraction

DNA extraction steps are outlined in Table 2.

Table 2. Steps followed for DNA extraction from young flower petals and leaves.

Step No.	Details
1	0.02 g of PVP were weighed in sterile 1.5 ml Eppendorf tube, 500 µl of CTAB extraction buffer were added and incubated in a water bath at 65 ℃. The solution was occasionally mixed by inverting the tube to dissolve PVP.
2	Young flower petals or leaf tissues were ground separately to a fine powder under liquid nitrogen with the use of pre-chilled mortar and pestle.
3	Powder of young flower petals or leaf tissues was weighed in sterile pre-chilled 1.5 ml tubes (100, 200, 300 and 400 mg) and kept in liquid nitrogen until next step.
4	15 µl of βME were added to the hot mix of CTAB extraction buffer and PVP, mixed well and the tube was returned to the water bath at 65 ℃.
5	The hot mix of CTAB extraction buffer, PVP and βME was added to the frozen powder, mixed well and incubated at 65 ℃ for 30 min with occasional mixing to avoid aggregation of homogenate.
6	One volume of chloroform:iso-amyle alcohol was added, mixed well and centrifuged at 10,000 rpm at room temperature for 5 min.
7	The upper phase was carefully transferred to new sterile 1.5 ml tube. The volume of supernatant was determined.
8	One tenth volume of CTAB/NaCl at 65 ℃ was added and mixed gently.
9	One volume of chloroform:iso-amyle alcohol was added, mixed well and centrifuged at 10,000 rpm at room temperature for 5 min.
10	The upper phase was carefully transferred to new sterile 1.5 ml tube and the volume was determined.
11	One volume of CTAB precipitation solution was added, mixed gently and stored at -20 ºC for 20 min.
12	The extracted DNA was fished out using micropipette into new tube or precipitated by centrifugation at 14,000 rpm for 5 min.

13	The pellet was carefully recovered and dissolved in 300 µl of high salt TE buffer.
14	200 µl of ice-cold Iso-propanol were added, mixed and followed by centrifugation at 14,000 rpm for 10 min
15	The pellet was carefully recovered by decanting solution.
16	The pellet was washed with 80% ice-cold ethanol, spun down and carefully recovered.
17	A second wash of pellet was carried out with 99.99% ice-cold ethanol, spun down and carefully recovered.
18	To dry up the DNA pellets, tubes with their lids open were inverted on sterile tissue paper for 10 min then DNA pellets were dissolved in 50 µl TE buffer for each.

DNA quantification

UV-Visible spectrophotometer (Varian, Australia) was used to measure the absorbance of isolated genomic DNA at A_{260} and A_{280} nm. While the purity of extracted DNA was determined based on the ratio of A_{260}/A_{280}, the yield was measured according to the formula (DNA (µg) = A_{260} x 50 x Dilution factor). A sample run on 0.8% agarose gel was utilized to have a visible test of quantity and quality of extracted DNA. The gel was run in 1 x TAE (Tris-base, glacial acetic acid, 0.5 M EDTA) buffer for ~45 min and stained in 0.5µg/ml ethidium bromide solution. The isolated DNA was compared with GeneRuler^{TM} DNA Ladder Mix (Fermentas, USA) as a DNA marker. The gels were visualized and photographed under a UV light source (UVItec Ltd, BTS-20 M model, Cambridge, UK).

PCR amplification

Degenerate primers for the conserved domains II (5'-RTIGTIGGITGCCICCIRT-3') and IV (5'-A-CRTCICCIRCIARCATCCA-3') of *Aux/IAA* nuclear genes and primers for the promoter (PLf; 5'GATCAGGTTGC-GCCATACATATG3' and PLr; 5'CTTTAACACAAGCTTTGAATCCAACAC3') of the chloroplast /chromoplast *rbcL* gene were employed to test PCR amplify-ability of isolated genomic DNA. Each PCR reaction mixture (25 µl in 200 µl thin-walled tubes) contained ~50 ng of template DNA, 0.625 U *Taq* DNA polymerase (Fermentas, USA), 4 mM $MgCl_2$, 0.4 mM of each dNTP, and 0.2 µM of each forward and reverse primer. In a Corbett Research PC 960 Thermal Cycler (Corbett Research, Mortlake, Sydney, Australia), PCR mixtures were subjected to the following PCR protocol: one initial denaturizing cycle at 94 ℃ for 2 min, initial annealing step at 50 ℃ for 30 s, and elongation step at 72 ℃ for 1 min (*Aux/IAA* genes) or 30 s (*rbcL* gene promoter), which was followed by 35 cycles at 94 ℃ for 1 min, 50 ℃ for 30 s, 72 ℃ for 1 min (*Aux/IAA* genes) or 30 s (*rbcL* gene promoter), then followed by a final extension step at 72 ℃ for 5 min. Amplified PCR products were analyzed using electrophoresis in agarose gel (1.5%), stained with ethidium bromide, visualized and photographed under UV.

Restriction digestion

Genomic DNA isolated from flower petals was tested for digestibility by restriction enzymes. Digestion reactions were performed at 37 ℃ overnight in a PCR machine. Each reaction of 20 µl volume contained 5 µg DNA, 2 µl 10 x recommended restriction buffer, and 5U of EcoRI (Fermentas, USA). DNA digestion was visually inspected under

UV light after agarose gel (1.5%) electrophoresis and ethidium bromide treatment.

Results and Discussion

The quantity and quality of extracted genomic DNA from different amounts of young flower petals are shown in Table 3. Gel electrophoresis, spectrophotometry, PCR amplification and restriction digestion were used to test the quantity and quality of isolated genomic DNA.

DNA production

Gel electrophoresis test showed high molecular weight of well intact isolated genomic DNA (Fig. 1). This could be attributed to the nature of flower petal tissues compared with photosynthetic leaves or succulent tissues. It was found that crushing, grinding and lyses process of young petals were much easier than leaves or succulent tissues (this study). It was also found that the quantity of extracted genomic DNA is proportional to the amount of petal tissues in both G. croucheri and E. humeana, but in C. aurea the amount of DNA remained stable or slightly lower despite increased amount of tissue from 100 to 400 mg (Table 3). It was reported that genomic DNA extracted from photosynthetic leaves decreases with the increase of leaf tissue amount per extraction buffer unit (Moyo et al., 2008). This could be the case for C. aurea, but not G. croucheri and E. humeana, at least in the range of 100-400 mg tissue.

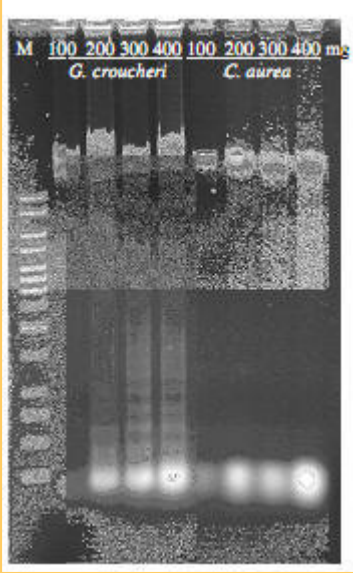

Fig. 1. Gel photo shows DNA extracted from 100, 200, 300 and 400mg of flower petal tissues from G. croucheri and C. aurea. (M) refers to 1kb GeneRulerTM DNA Ladder Mix (Fermentas, USA).

DNA purity

Spectrophotometry test relies on the fact that an absorbance ratio of A260/A280 between 1.8 and 2.0 is an indication of a highly pure genomic DNA, while lower or higher ratio indicates the presence of polysaccharides and other contaminants (Pašakinskiené and Paplauskiené (1999). The A260/A280 ratio ranged from 1.70 to

1.81 for G. croucheri, 1.70 to 1.75 for E. humeana, and 1.77 to 1.81 for C. aurea, which reflects high purity of isolated genomic DNA (Table 3).

Table 3. Young flower petals tissue amount in mg, DNA purity (A260/A280 ratio), quantity in µg and concentration in µg/µl.

Plant Species	Petals mass (mg)	A260/A280	DNA Yield (µg)a	Concentration (µg/µl)
G. croucheri	100	1.71 ± 0.38	16.75 ± 7.90	0.34 ± 0.16
	200	1.81 ± 0.11	30.45 ± 9.41	0.61 ± 0.19
	300	1.71 ± 0.05	47.82 ± 15.08	0.96 ± 0.30
	400	1.70 ± 0.04	54.82 ± 10.49	1.10 ± 0.21
E. humeana	100	1.75 ± 0.16	22.90 ± 7.62	0.36 ± 0.30
	200	1.73 ± 0.01	62.60 ± 9.61	1.13 ± 0.16
	300	1.70 ± 0.01	100.17 ± 16.79	2.00 ± 0.34
	400	1.70 ± 0.01	110.43 ± 21.16	2.21 ± 0.42
C. aurea	100	1.80 ± 0.02	41.95 ± 0.02	0.84 ± 0.01
	200	1.78 ± 0.01	42.95 ± 0.01	0.86 ± 0.03
	300	1.81 ± 0.03	29.50 ± 0.03	0.59 ± 0.04
	400	1.77 ± 0.02	35.30 ± 0.02	0.71 ± 0.14

[a] Data are means ± Standard Error (SE), n = 3 replicates.

PCR amplification

Successful PCR amplification of the chloroplast/chromoplast rbcL gene promoter and nuclear Aux/IAA genes indicates that organelles' genomic DNA was co-extracted with nuclear genomic DNA and both were of high quality and amplifiable (Fig. 2). Aux/IAA genes are nuclear auxin responsive genes (Abebie et al., 2008). From Fig. 2 Aux/IAA genes show a wide diversity of length among these different plants. G. croucheri expressed the shortest Aux/IAA genes while G. barbadense showed the longest. Despite the widely different plants from far related families as DNA sources, the promoter of plastid rbcL gene expressed similar length. This could be due to the well conserved nature of plastid genes (Shimada and Sugiura, 1991).

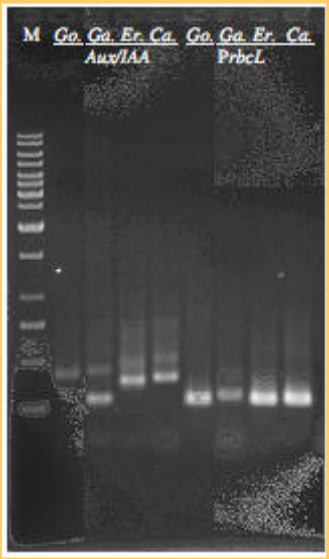

Fig. 2. PCR amplification of Aux/IAA gene(s) and promoter of rbcL plastid gene (PrbcL) from Go; G. barbadense, Ga; G. croucheri, Er; E. humeana, and Ca; C. aurea. (M) is 1kb GeneRulerTM DNA Ladder Mix (Fermentas, USA).

Restriction digestion

Endonuclease digestion test was carried out with the use of EcoRI restriction enzyme and the isolated genomic DNA. The gel profile showed complete digestion, which reflects high purity of isolated genomic DNA (Fig. 3). PVP is used to reduce Phenolics during DNA extraction due to its ability to form complex hydrogen bonds and co-precipitates with cell debris upon lyses in the presence of chloroform (Haaf et al., 1985). High salt concentrations are used to remove polysaccharides (Porebski et al., 1997). CTAB forms complexes with polysaccharides and co-precipitate during chloroform extraction (Michiels et al., 2003). Therefore, CTAB was added to reduce polysaccharides and their inhibitory effects on biological enzymes that are used for downstream applications such as polymerases, endonucleases and ligases (Porebski et al., 1997; Zhang and Stewart, 2000).

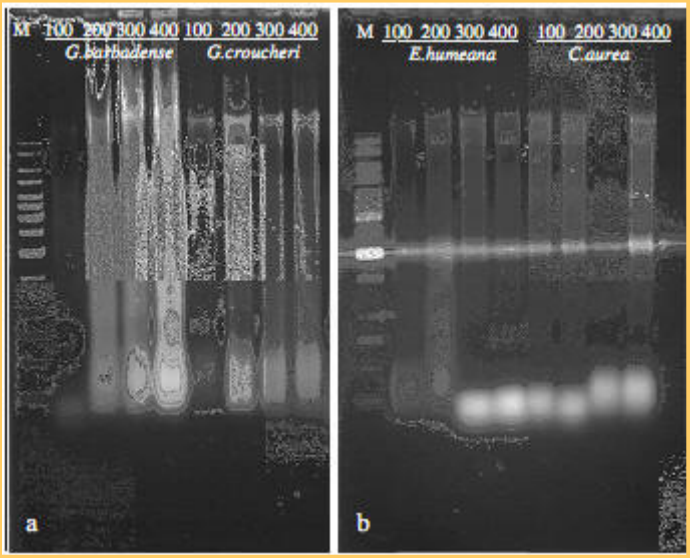

Fig. 3. Gel photos show EcoRI digestion of extracted genomic DNA from (a) G. barbadense and G. croucheri, (b) E. humeana and C. aurea. 100- 400 refer to DNA extracted from 100-400 mg of young flower petals tissue. (M) is 1kb GeneRulerTM DNA Ladder Mix (Fermentas, USA).

In conclusion, these results show that young flower petals can be an alternative source for total genomic DNA from medicinal and succulent plants that contain high quantities of secondary metabolites. Flower petals from succulent plants were easier to crush and grind under liquid nitrogen as well as lyses in buffer than succulent tissues. The isolated genomic DNA was of high molecular weight and the amount increased proportionally as the amount of petals tissue increases. The extracted DNA from tested species proved suitable for endonucleases digestion and polymerases amplification, which reflects high purity DNA. This protocol will be used in future to isolate genomic DNA from tested and other related plant species for downstream molecular biology studies.

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